MUTAGENESIS OF HUMAN LYMPHOBLAST CELLS

by

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B. S., University of Illinois, 1973

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1976

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Previous work with mutagenesis of human lymphoblasts used 6-thioguanine (6-TG) as the selective agent and either ethylmethane sulfonate (EMS) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as mutagens for induction of cells that are deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Extensive experiments were conducted which examined the effects of cell density, pH, serum lot, dose-response relationships, and the optimum time for expression of the 6-TG^R phenotype.

The main goals of this research were to study the frequency and characteristics of human lymphoblasts lacking HGPRT using X-rays as a mutagen and 6-TG as a selective agent and to compare the results with those already obtained using EMS. The suitability of using 8-azaguanine (8-AZG) instead of 6-TG as a selective agent was also examined. This thesis shall consist of three sections:

A. A literature review,

B. A manuscript comparing X-ray and EMS mutagenesis in three lymphoblast cell lines, and

C. A manuscript comparing 6-TG and 8-AZG selection of MGL33C19 cells that are deficient in HGPRT.

The papers are presented in a form suitable for publication in Mutation Research.

Literature Review

Studies on Genetics of Mammalian Cells

The most common approach to the study of mammalian cell mutagenesis and genetics is that of mutation of animal cells to resistance to the purine analogs 8-azaguanine (8-AZG) and/or 6-thioguanine (6-TG). Although much information is available from such drug-resistant mutants of animal cells, many unanswered questions and problems are still associated with the biochemical identification of particular mutant clones and in quantifying mutation rates for resistance to 6-TG and 8-AZG.

Resistance to 8-AZG or 6-TG is usually associated with lack of activity of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). This enzyme catalyzes the transfer of a 5-phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate to hypoxanthine and guanine to form their respective ribonucleotides[1]. HGPRT is necessary to convert 8-AZG and 6-TG into their cytotoxic nucleotides[2]. It also appears that HGPRT plays a role in the transport of purines into cells[3]. Evidence suggests that there is a saturable process that controls the rate at which hypoxanthine, guanine and related purine analogues enter a cell[4,5]. Lack of HGPRT activity might cause a build-up of non-utilized hypoxanthine which consequently slows the transport process[4].

Applications of Mammalian Cells Resistant to Purine Analogs

Cells lacking HGPRT are useful in studying a variety of problems, including the control of biochemical pathways, selection of hybrid cells, and the regulation of gene expression. HGPRT-deficient cells are used to study the mechanism controlling purine transport in mammalian cells[3,4,5]. Studies of mechanisms controlling nucleic acid biosynthesis are also possible, since cells lacking activity of various other enzymes in purine and pyrimidine

biosynthesis are available[6-10]. Studies on the regulation of gene expression in somatic cell hybrids are facilitated by the use of 8-AZG^R/6-TG^R cells since hybrids may be isolated by the HAT (hypoxanthine, amethopterin and thymidine) selection system[11]. Amethopterin (or aminopterin) inhibits the de novo biosynthesis of purines and pyrimidines and only cells which contain both purine and pyrimidine salvage enzymes, HGPRT and thymidine kinase (TK), survive in HAT medium.

Other uses of cells resistant to 8-AZG/6-TG are to study various cell population phenomena, such as metabolic cooperation and population growth dynamics. Using autoradiography, investigators demonstrated that 8-AZG^S cells can transfer the ability to incorporate substrate to 8-AZG^R cells by a process called "metabolic cooperation"[18-20].

The observation that the frequency of recovery of 8-AZG^R cells often decreases as plating densities are increased, is thought by many to be due to metabolic cooperation[15,21,26,53]. Van Zeeland, et al.[21] found that the frequency of recovery of 8-AZG^R cells did not decrease from values obtained in the absence of 8-AZG^S cells if they were separated from 8-AZG^S cells by a fibrin layer. Their result indicated that cell-cell contact is necessary for metabolic cooperation to occur[21]. Morrow[22] investigated the population dynamics of mixed cultures of drug resistant and drug sensitive cells by plating 8-AZG^R or BUdR^R cells together with cells sensitive to each respective analog. He observed that drug resistant cells are at a selective disadvantage in regular medium and their frequency was reduced to that normally obtained for wild type populations[22].

In humans, the gene coding for HGPRT is linked to the X chromosome[24]. According to the Lyon hypothesis[13] the gene is present in only one functional dose in both female as well as male diploid cells. This selective inactivation permits the selection of HGPRT deficient cells in a single

mutagenic step[2] and eliminates the need to first select for heterozygotes as is necessary when one selects for recessive traits on autosomes.

The convenience of single step selection, the ability to select for revertants with HAT medium and the relative ease of working with drug resistant markers has led to widespread use of the 8-AZG/6-TG selection systems in quantitative mutagenesis studies. These systems have been used to estimate spontaneous and induced mutation rates[25-29], in setting up systems for the assessment of the hazards of environmental mutagens[30] and in attempts to establish correlations between mutagenic and carcinogenic compounds[31-33].

Inconsistencies in the Data on Quantitative Mutagenesis at the HGPRT Locus

Unfortunately, data on quantitative mutagenesis at the HGPRT locus are presently confused by inconsistent results. Estimates of mutation rates vary widely, especially for systems in which 8-AZG is used as the selective agent. Even within the same cell line, mutation rates often differ by as much as one-hundred fold[37]. Such inconsistencies in mutation frequencies may be due to various factors, such as: 1) Differences in mechanisms of toxicity and in metabolism of 8-AZG and 6-TG that may result in non-cross-resistance to the two drugs, 2) Differences in the kinds of alterations that may result in resistance of cells to 8-AZG and/or 6-TG, 3) Various experimental errors affecting the quantification of mutation frequencies that are not uniformly corrected for in experiments from separate laboratories.

Evidence of Non-cross-resistance to 6-TG and 8-AZG

Although selection with 8-AZG and 6-TG can result in production of drug-resistant cells with apparently the same genotype, $HGPRT^-$, evidence indicates that the phenotypes produced by these two selective agents are quite distinct in some cases. $8\text{-}AZG^R$ cells, for example, may not be cross-resistant to 6-TG[35], or vice versa[36]. 6-TG binds to HGPRT much more strongly

than does 8-AZG[17], which may possible account for the selection of cells that are resistant to 8-AZG, but not to 6-TG. Differences in the metabolism of the two drugs may also account for non-cross-resistance. 8-AZG^R tumor cells have been found that contain an enzyme capable of deaminating 8-AZG, but not 6-TG, to a non-toxic derivative (7-azaxanthine). Three out of four 8-AZG^S tumor cell lines that were examined contained relatively low levels of the deaminating enzyme[65]. In view of the differences in affinity for HGPRT and in metabolism, mutation data obtained with 8-AZG might not necessarily be similar to mutation frequencies obtained with 6-TG as a selective agent.

Mechanisms of Resistance to Purine Analogs

A number of different changes may occur to produce resistance to purine analogs: 1) mutations affecting the structural gene for HGPRT, 2) mutations affecting other gene loci, and 3) stable changes, other than mutations, which result in resistance to purine analogs.

Cells resistant to purine analogs generally have decreased activity of HGPRT. Several investigators have isolated 8-AZG^R or 6-TG^R cells that lack HGPRT activity and contain a protein (CRM) that cross-reacts with antibody specific for HGPRT[41,42]. This result indicates that a nonsense mutation may occur in some cases at the structural gene which results in production of an inactive gene product[41,42]. Some 8-AZG^R clones have been found to have residual HGPRT activity, but possess an altered HGPRT enzyme having a higher K_m for its substrate or a different thermosensitivity than the normal HGPRT enzyme[39,42-45]. This evidence suggests that missense mutations may cause the drug-resistant phenotype. Other mutants which lack HGPRT activity appear to be non-revertable[14]. This result suggests that such mutants possess a deletion at the structural gene coding for HGPRT.

Mutations do not always need to occur at the HGPRT locus to produce 8-AZG^R cells. It is possible, for example, to isolate 8-AZG^R cells that

have full HGPRT activity[35]. Harris[48] isolated an 8-AZG^R mutant from Chinese hamster ovary cells (CHO) that exhibited full HGPRT activity but had a temperature dependent defect in purine transport[48]. Mutations of regulatory gene loci also appear to be possible. Some HGPRT$^-$ cell lines regain HGPRT activity specific for their species at a rate much higher than the spontaneous reversion rate when fused to HGPRT$^+$ cells of a different species[46,47]. That result suggests that the gene coding for HGPRT in the deficient cells was initially repressed, but that possibly a substance produced by the HGPRT$^+$ cells removed the repression.

Harris[28] demonstrated in an earlier study that the spontaneous mutation frequency of CHO cells to the 8-AZG^R phenotype did not decrease with an increase in ploidy[28]. Since 8-AZG^R appears to behave as a recessive trait in hybridization experiments[64], Harris expected the frequency of 8-AZG^R mutants to decrease as the ploidy increased. He suggested that 8-AZG^R cells in his experiments result not from mutation, but by "stable shifts in phenotypic expression." Such shifts in expression, he suggested, form a familiar part of embryonic development, but would not be expected to occur in normal cells.

Investigators working with other cell lines and different traits also observed similar discrepancies in "mutant" frequencies which did not decrease as expected at higher ploidy levels[49-51]. Chasin[52] working with Chinese hamster cells, observed 25-fold fewer AZG^R mutants with tetraploid than with diploid cells. His study, however, differed from Harris' in that he was observing mutagen-induced, rather than spontaneous mutation frequencies. Chasin suggested that AZG^R cells might often arise spontaneously due to a shift in gene expression. The frequency of such cells would not vary as expected with changes in ploidy since they do not arise by mutations. He also suggested that treatment with a mutagen will more likely produce AZG^R cells that arise by mutations. The frequency of such cells would be expected

to be lower at higher ploidy levels, as his results indicated[52].

Due to the variety of ways that cells with the AZG^R phenotype may be obtained, it is evident why estimates of mutation frequencies have varied in separate studies. The HGPRT locus, nevertheless, is useful to study mutagenic mechanisms in mammalian cells and may help us to discern the various genetic changes which occur in eukaryotic cells.

Quantification of Mutation Frequencies at the HGPRT Locus

The phenotypic variation in AZG^R cells is probably only partially responsible for the inconsistent results obtained in quantitative studies of mammalian somatic cell mutation frequencies. There are apparently several sources of error in many of the previously reported mutation frequencies. In a review article, Simons[38] discussed the various factors that may interfere with estimation of mutation frequencies and the dose-response relationships that influence these results. Briefly, many of these factors are attributable to the heterogeneous nature of cell populations and variations in substances present in the serum component of the medium. Other experimental factors such as differences in growth rates and cloning efficiences between mutant and wild-type populations are also difficult to assess and are sources of error. Experimental variability may be controlled to a great degree by standardization of experimental conditions, determination of optimum expression times for the mutant phenotype in each cell line studied, and estimation of appropriate correction factors, such as determination of colony forming ability (plating efficiency) of mutagenized cells under optimum, non-selective conditions. Simons specifically stated that the importance of a particular type of error on influencing mutation frequencies will vary, depending upon the particular kinds of cells that one is working with[38].

Other investigators also noted that various factors may influence the frequency of mutant recovery. Cell density effects, concentration of selec-

tive agent, metabolic cooperation, population dynamics and serum effects all play a part in modifying mutation frequencies. Carver[53], working with CHO cells, studied the effects of cell density[53]. He observed a 9-fold increase in observed mutation frequency with a 2-fold increase in number of cells plated when the AZG medium was not changed frequently. However, both Carver and others[21,26,15] observed a decrease in the frequency of mutants when the number of cells plated per petri dish was above a certain optimum concentration. He suggested the effect of cell density is due to depletion of the selective agent in the medium and metabolic cooperation between mutant and "wild type" cells. It is possible that as cell density increases, the effective drug concentration in the medium decreases, which results in an apparently higher frequency of mutants because of growth of partially enzyme-deficient cells or phenocopies[55], which have full enzyme activity and later cannot be distinguished from "wild type" cells on the basis of sensitivity to the selective agent.

The appearance of phenocopies at low 8-AZG concentrations was observed by several workers[55,56,40]. When cell concentrations are high enough so that cell-cell contact is extensive, then metabolic cooperation may become a significant factor in altering mutation frequencies. A related observation in Chinese hamster cells indicates that dispersal of cells and replating prior to selection in 8-AZG increases the observed mutation frequency[55,56]. Morrow showed in reconstruction experiments of mixed populations of mutant and normal cells that resistant cells are at a selective disadvantage in regular media[22]. This result may explain why the frequency of mutants often decreases after a period of growth and why the maximum number of mutants is expressed at a particular time after mutagenesis of the cells.

Serum may also influence mutation frequencies. An uncharacterized component in serum is thought to be present which can act to degrade or in-

activate 8-AZG[34] or azahypoxanthine[34,57]. (Azahypoxanthine is another purine analogue useful for selection of HGPRT-deficient mutants.) A serum factor is also thought to interfere with the toxic effects of amethopterin[58]. Serum concentration can indirectly affect mutation frequencies by influencing the cell cycle. If the serum content of the medium in which Chinese hamster cells are grown is decreased below an optimum concentration, the doubling time of the cell population is increased which means that the proportion of cells in the G_1 phase is increased. During the G_1 phase, the cells are radiation resistant relative to the other stages in the cell cycle[16].

Mutation Studies with Human Lymphoblast Cells

Previous work with mutagenesis of human lymphoblasts used 6-TG as the selective agent and either EMS or MNNG as mutagens[59,60]. All 6-TG resistant clones isolated in those experiments were completely deficient in HGPRT[59,61]. Thus, the heterogeneity often found in mutants selected with 8-AZG was not observed in these extensive experiments which examined the effects of cell density, pH, serum lot, dose-response relationships, and the optimum time for expression of the 6-TG^R phenotype[60].

The goals of this research were to study the frequency and characteristics of human lymphoblasts lacking HGPRT using X-rays as a mutagen and 6-TG as a selective agent, and to compare the results with those already obtained using EMS as a mutagen. I also compared the purine analogs 8-AZG and 6-TG as selective agents for HGPRT deficient lymphoblasts.

Survival curves and the frequency of induced and spontaneous mutations were studied. Mutants obtained in various experiments were characterized by their growth rates in selective and non-selective media, determination of *in vitro* HGPRT activity, and quantification of *in vivo* incorporation and uptake of tritiated hypoxanthine.

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Paper: COMPARISONS OF X-RAY AND ETHYLMETHANESULPHONATE MUTAGENESIS OF CULTURED HUMAN LYMPHOBLASTS

Summary

The cytotoxicities and mutagenic efficiencies of X-rays and ethylmethanesulphonate (EMS) were compared in three lymphoblastoid cell lines--MGL33C19, MGK8E, and KS3A4. These cell lines were found to differ in their spontaneous mutation frequencies to 6-thioguanine resistance (6-TG^R). The average spontaneous mutation frequency of MGL33C19 was found to be $3.74 \pm 0.98 \times 10^{-5}$, while values found for KS3A4 and MGK8E were 7.76×10^{-6} and 2.10×10^{-6}, respectively. KS3A4 was found to be significantly more sensitive to both mutagens than the other two lines. MGK8E was similar to MGL33C19 in radiosensitivity, but was much more resistant than MGL33C19 to EMS. The optimum time necessary for expression of MGL33C19 6-TG^R mutants following both X-ray and EMS treatments appeared to be about 4-5 generations, despite obvious differences in the growth response following X-ray and EMS treatments. Some growth occurred in the first 2 days, even after high doses of EMS (500 µg/ml), while high doses of X-rays (200-400 rads) were much more inhibitory. When compared at equitoxic doses, X-rays and EMS appeared to differ by less than two-fold in effectiveness at inducing 6-TG^R mutants in MGL33C19. In contrast, EMS appeared to be greater than ten times as effective as X-rays at inducing 6-TG^R mutants in KS3A4 and MGK8E. Differences in mutability among these cell lines do not appear to be related to mutagen sensitivities, as the similarly mutable KS3A4 and MGK8E differed widely in X-ray and EMS sensitivities. A correlation was noted between mutability and plating efficiency. MGL33C19, which had an average plating efficiency greater than twice that of the other two lines, was the most easily mutated.

Introduction

Much less is known about mutagenic mechanisms in mammalian cells than in procaryotes, because methods of studying recombination and segregation are not readily available in mammalian cells[11]. Methods which can be used, however, include reversion analyses of known mutants, studies of altered structure or function of proteins in mutant cells, or comparisons of the effectiveness of various mutagens which are known to produce specific effects on DNA. Data obtained from such studies suggest that mutagens which produce specific and well characterized effects in procaryotic cells[7,11], also produce specific, although currently less well characterized alterations on mammalian DNA.

The specificity of particular mutagenic agents is supported by studies on the types of mutants produced by mutagen exposure. Ethylmethane sulfonate (EMS), for example, was shown to induce more 6-thioguanine resistant (6-TG^R) than 8-azaguanine-resistant (8-AZG^R) mutants in mouse carcinoma cells[17]. N-methyl-N'-nitrosoguanidine (MNNG), in contrast, induced more 8-AZG^R than 6-TG^R mutants in the same cells[17]. In other studies, EMS was more efficient than MNNG for inducing 6-TG^R mutants of human lymphoblasts[16], while MNNG induced more 8-AZG^R mutants than EMS for Chinese hamster V79 cells[4]. In another example, Chu[5] found that reversion of 72 Chinese hamster cell mutants resistant to 8-AZG could be induced by some mutagens but not others. Also, some of the mutants in his study could not be reverted by any mutagen tested.

Alkylating agents, such as EMS are commonly recognized to be much more efficient at inducing mutations of various kinds than physical agents such as X-rays and gamma rays[3,10,13,14,18,21]. This result suggests that X- and gamma rays produce more inactivating damage, such as chromosomal breakage, than EMS, and less mutagenic alterations, such as base-pair sub-

stitutions[11].

The goals of our study were to compare the effectiveness of EMS and X-rays as mutagens for human lymphoblastoid cells and to study the mutagen sensitivity and mutability of three lymphoblastoid cell lines which differed in their spontaneous mutation frequency to the 6-TG^R phenotype. The three lymphoblast cell lines were found to differ in both mutagen sensitivity and mutability. However, there did not appear to be a correlation between sensitivity of a cell line to a mutagen, and the ability of a cell line to be mutagenized.

Materials and Methods

Cells

The MGL33C19 lymphoblast line is a clone of the PGLC33H culture initiated by Dr. P. R. Glade from the peripheral blood of a female patient with infectious mononucleosis. MGL33C19 has been grown in culture for over 7 years and has a heteroploid chromosome distribution with predominant modes at 46 or 47 chromosomes. The 8E cell line was cloned from MGL8, which was initiated from a female patient, a possible heterozygous galactosemic, at Massachusetts General Hospital. KS3A4 is a clone of KS3, which was initiated at Kansas State University by Dr. R. S. Slesinski from the peripheral blood of a normal male. LN326 human fibroblasts, deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT), were initiated from a patient with the Lesch-Nyhan syndrome and used as "feeder" cells in all soft-agarose plating experiments. LN326, MGL33C19, and MGL8 were originally obtained from Dr. J. W. Littlefield, Johns Hopkins Medical School.

Cell Culture Maintenance and Cell Plating Procedures

Lymphoblast cells were maintained in liquid media and plates in soft agarose[16]. The routine growth medium was R.P.M.I. 1640 + 14.36% undialyzed, heat-inactivated fetal calf serum (FCS); the same medium with 20% FCS was used in all soft-agarose plating experiments. The agarose concentration used in this study was 0.28% for both the top and bottom layers.

Serum and powdered media were purchased from Grand Island Biological Co. Lymphoblasts were maintained in continuous growth and regularly passed into fresh medium at 8-12 day intervals. Only actively growing cells in the logarithmic stage of growth were used for mutagenesis experiments, growth studies, or enzyme assays. Numbers of cells were determined with a model Z Coulter counter which was standardized regularly with a precounted latex bead suspension (Clinton Laboratories).

X-Ray and EMS Mutagenesis

Cells were centrifuged and resuspended in fresh growth medium at a concentration of 2.5 X 10^6 cells/ml. Two mls. of cell suspension were added to 60 mm petri dishes and exposed to X-radiation at a rate of 126.9 roentgens per minute (determined by Victoreen), using a Pickard X-ray machine operated at 50 kV and 20 mA (1.8 mm Al filtration). The irradiated cells and controls, treated similarly but not irradiated, were then diluted with growth medium, to a concentration of 2 to 5 X 10^5/ml, and plated in soft agarose to determine the percentage of surviving cells.

Ethylmethane sulfonate (EMS) mutagenesis was performed by modifications of the methods described by Sato et al.[16]. Cells were treated with various concentrations of EMS (Eastman Organic Chemicals) at 37° in 1640 medium (pH 7.2) for a period of 4 hours. The cells were then centrifuged, resuspended in fresh medium and plated in soft-agarose to determine the percentage of surviving cells. Treated cells were counted at 2-3 day intervals and a-lowed to grow for 4 generations to allow expression of the mutant phenotype. Mutagenized cells were then plated in 6-thioguanine (6-TG)-soft agarose medium, to determine the mutant frequency.

Mutant Selection with 6-TG

Cells treated with EMS were exposed to 6-TG (5 µg/ml) following 4-5 generations of growth, the optimum growth period required to express the 6-TG^R phenotype in MGL33C19 cells[20]. An expression time of 4-5 generations was also used in our X-ray experiments, since preliminary data indicate that this period of growth is also optimal for recovery of 6-TG^R mutants produced by X-rays.

The frequency of mutants resistant to 6-TG was calculated by dividing the number of colonies which appeared in 6-TG medium by the plating efficiency of the same cells plated in standard medium. The frequency of induced mutants

was calculated by subtracting the number of spontaneous mutants from the total number of mutants obtained from mutagenized cells.

Preparation of Enzyme Extracts

Cells, growing exponentially at a density of 4-6 X 10^5 cells per ml., were harvested by centrifugation, rinsed twice with 0.9% saline solution, and resuspended in 0.01 M Tris-HCL buffer (pH 7.4) at a concentration of 10^7 cells/ml. Enzyme extracts were prepared from cells disrupted by 20-25 bursts from a Branson Sonifier. Cells were often stored frozen at -90°C prior to assay, but enzyme extracts were always prepared immediately prior to enzyme assay. Extracts were centrifuged at approximately 6000 X g for 20 min. at 4° C and the supernatant fluid was used for both protein analysis and for enzyme assay. Protein concentrations of the cell extracts were determined by the Lowry method and typically varied from 0.11 to 0.28 mg/ml.

Assay of Hypoxanthine-guanine Phosphoribosyltransferase Activity

Reactions were carried out at 37° C for 180 min. Reaction mixtures contained: 20 µl of cell extract, 20 µl of 10 mM 5-phosphorylribose-1-pyrophosphate (Sigma) and 60 µl of a mixture containing 3 volumes of (^{3}H) hypoxanthine (3.64 X 10^{-3} M, 100 µCi/ml), 2 volumes of 0.1 M Tris-HCL (pH 7.4), 4 volumes of 0.05 M phosphate buffer (pH 7.4), and 1 volume of 0.1 M $MgCL_2$. Enzyme extract which was heated at 100° C for 2 min. was used for the control. Reactions were stopped by quickly freezing at -90° C and could be stored at this temperature prior to chromatographic separation.

Quantification of the enzyme reaction was performed by thin layer chromatography and counting of separated spots in a liquid scintillation counter. A volume of 10 µl of each reaction mixture which contained added carrier iosine + inosinic acid (IMP) was spotted on polyethyleneimine thin-layer chromatographic plates (Polygram Cel 300 PEI, Brinkman Instruments), which were previously washed with ammonium formate (10^{-3} M). Plates were

resolved for 1 hour with 0.2 M LiCl and dried. The inosine and IMP spots were located by fluorescence, cut out, and eluted with 0.5 M $MgCl_2$. Ten ml. of Triton-toluene scintillation fluid [1 part Triton-X, 2 parts toluene-PPO solution (6 g/l, PPO)] was added and the samples were counted. Quenching was corrected by the channels ratio method. The amount of pmoles of ^{3}H-inosine + ^{3}H-IMP produced during the reaction was used as the value for the total amount of product formed in the reaction catalyzed by HGPRT.

Results

Survival Curves

X-ray survival curves for MGL33C19, MGK8E, and KS3A4 are shown in Fig. 1A. Results of an analysis of variance of the dose-survival values indicated that 8E and 33C19 do not differ significantly in radiation sensitivity. KS3A4, however, is significantly more radiosensitive than either the MGK8E or MGL33C19 lymphoblast lines.

The D_o values estimated from the various survival curves are: MGK8E (105 rads), MGL33C19 (104 rads), and KS3A4 (51 rads). The D_o values for MGL33C19 and MGK8E are within the range of 50-100 rads estimated for skin, spleen, and ovary human fibroblast cells by Puck et al.[15].

The X-ray survival curve for KS3A4 lymphoblasts apparently has no shoulder, indicating that X-ray lethality followed single-hit kinetics. However, the least squares regression lines drawn through the MGK8E and MGL33C19 survival values intercepted the Y-axis above 100% survival, indicating a definite, but small shoulder for MGL33C19 and a questionable shoulder for MGK8E.

A comparison of survival for these same cell lines treated with EMS is shown in Fig. 1B. There are large differences in EMS sensitivities, especially between the MGK8E and the other lymphoblast cell lines. D_o values, estimated from initial slopes, were: MGK8E (284 µg/ml), MGL33C19 (83 µg/ml), and KS3A4 (24 µg/ml). In an additional experiment (not shown), the MGL33B (6,1) lymphoblasts, the parental cells for the MGL33C19 clone, showed a D_o value of 30 µg/ml with EMS. KS3A4 was the most sensitive cell line to either X-rays or EMS, but was similar in EMS sensitivity to MGL33B (6,1).

Growth of Cells Following Mutagen Treatment

The growth of lymphoblast cells following treatment with X-rays and EMS

is shown on Fig. 2A,B. A reduction in growth rate and an extended lag period, prior to resumption of growth, is evident after treatment with increasing doses of each mutagen. After the lag period, the growth rate was identical in all experiments with X-rays, but was slightly decreased in cells treated with high concentrations of EMS.

Mutation data to be presented in this study are based upon expression times given in terms of elapsed generations rather than days after treatment to avoid the obvious problem of the extended effect of high doses of mutagen on the growth of lymphoblasts.

6-TG^R Mutation Frequencies for MGL33C19 Cells

Table 1A. shows the highest mutation frequencies obtained by selecting for 6-TG-resistance at 4-5 generations of growth following treatment of MGL33C19 cells with various doses of EMS. The highest mutation frequencies obtained at expression times of 2 to 6 generations following X-ray treatments are shown in Table 1B. In both tables, the percentage of cell killing obtained in each experiment is given as a means to compare the mutagen efficiencies of X-rays and EMS at equitoxic levels. There is a clear indication that for both X-ray and EMS mutagenesis, mutation frequencies increase as the dose of mutagen is increased. If the average EMS-induced mutation frequencies versus concentration of EMS shown in Table 1B are plotted, an approximately linear dose-response relationship for EMS mutagenesis is observed. Sufficient X-ray data is not yet available to clearly determine whether the X-ray dose-response is linear or logarithmic.

The spontaneous mutation frequency for MGL33C19 cells resistant to 6-TG, estimated from 31 experiments, was 3.74×10^{-5} (S.E. = 0.98×10^{-5}, S.D. = 5.46×10^{-5}). The spontaneous mutation frequency for MGL33C19 is significantly higher than any other of the lymphoblast lines characterized in our laboratory. The significance of this high spontaneous mutability is

not explainable with our current data and possible mechanisms are under investigation.

6-TG^R Mutation Frequencies for HGK8E and KS3A4 Cells

Mutation frequencies for MGK8E and KS3A4 lymphoblasts are shown in Table 2. The spontaneous frequency of 6-TG^R mutants is similar for both cell lines; only a 3-fold higher frequency was found for KS3A4 cells. The cytotoxic effect of X-rays was most apparent with 8E cells and no mutant colonies were observed. Also, there was only a two-fold increase in mutant frequency over spontaneous values obtained for KS3A4 cells treated with X-rays.

Comparison of X-ray and EMS Mutagenesis

In Fig. 3, the highest mutation frequencies attained with various doses of X-rays and EMS are plotted against the % kill values determined on the day of treatment. This plot allows a comparison of the frequency of mutations induced by the two mutagens at equitoxic doses. The data indicate, in contrast to results obtained with MGK8E and KS3A4 cells, that the mutagen efficiencies of EMS and X-rays for MGL33C19 lymphoblasts are very similar, differing by less than two-fold. This result is also in contrast to the results obtained with Chinese hamster V79 cells, in which it has been observed that EMS is 10-12 times more efficient as a mutagen than X-rays[10,21].

Characterization of Mutants

In Tables 3 and 4, a total of 31 6-TG^R clones from X-ray and EMS mutagenesis experiments were assayed for HGPRT activity. All 31 clones were found to have significantly lower HGPRT activities than the MGL33C19 parent and suggest that phenocopies or false mutants were not obtained in our study. Seven of the 31 clones had HGPRT activities that ranged between 1.5% and 6.6% of MGL33C19. When five of these clones with intermediate HGPRT activity were assayed for growth in liquid and soft agarose media, none were capable

of growth in HAT medium and all five grew in 6-TG medium. The growth curves in liquid selective media of the parental and one of the mutant lymphoblasts, 33JX3, are shown in Figs. 4A,B. The growth of the 33JX3 mutant which had 6.6% HGPRT activity of the parent cell line was consistent with the pattern of growth in selective media shown by the four other mutants which were similarly characterized. The 33JX3 mutant grew at identical rates in 6-TG and maintenance media but not in HAT medium. The phenotype of the 33JX3 mutant and the four other mutant clones characterized, was stable after a 3 month period of growth in non-selective medium.

Discussion

The results of our study demonstrated that various human cell lines differ significantly in EMS and X-ray sensitivity and mutability. We observed the following differences in mutagen sensitivity for the three cell lines studied: 1) MGK8E (D_o 284 µg/ml) is much more resistant to EMS than either MGL33C19 (D_o 83 µg/ml) or KS3A4 (D_o 24 µg/ml); 2) MGL33C19 (D_o 104 rads) is not significantly different in X-ray sensitivity from MGK8E (D_o 105 rads); and 3) KS3A4 is significantly different in both EMS and X-ray sensitivity than either MGK8E or MGL33C19.

The reasons for these differences in mutagen sensitivities in such similar human cell lines are not presently known. However, studies by Fox and Fox[9] with Yoshida sarcoma cells, which differ in sensitivity to alkylating agents, indicate that there is a direct relationship between the sensitivity of a particular cell line to a mutagen and the ability of the cell line to repair mutagen-caused damage. Various cell lines treated with nitrogen mustard (HN_2) or methylene dimethanesulfonate (MDMS) show that those with the lowest sensitivities to the mutagen (highest D_o values) had the greatest capability for repair. Repair was measured as unscheduled DNA synthesis by CsCl density gradient centrifugation and autoradiography. Fox and Fox observed that the kinetics of ^{3}H-thymidine incorporation differed for all three mutagens that were used, HN_2, MDMS, and methyl methanesulfonate (MMS). This led them to suggest that different enzymes may be involved in the repair of damage by different mutagens. Clarkson and Evans[5], studying unscheduled DNA synthesis in human lymphocytes, also suggested that this may be the case.

Differential sensitivities of the various lymphoblast lines studied, thus, may be due to inherent differences in repair capabilities of these

cell lines. We offer this possibility as a suggestion at this time since we do not currently have any quantitative data to compare the repair capabilities of these various cell lines. The appearance of biphasic survival curves for the MGL33C19 cell line in a previous study[20], however, does provide some support for the possibility of altered repair capability. Arlett and Potter[1] previously observed biphasic survival curves in Chinese hamster cells exposed to gamma-radiation. They suggested that the biphasic nature of the survival curves may have something to do with repair mechanisms, because they observed less cell killing when irradiation was given in split doses, than in a large single dose. Additional studies with lymphoblasts are necessary to clarify the role of DNA repair in the different mutagen sensitivities we observed in our study.

The growth curves following X-ray and EMS treatments, Fig. 2A and B, revealed that there are qualitative differences in the response of cells to these two mutagens. Although in both cases there was a lag period following mutagen treatment before growth resumed, some growth always occurred in the first 2 days after treatment, even after administration of high doses of EMS. Exposure to X-radiation, in contrast, greatly inhibited growth in these first 2 days, especially after treatment with high doses of X-radiation. This result may be due to the differential radiosensitivity during the cell cycle. Cells in the mitotic phase are the most radiosensitive[6]. Consequently, X-radiation preferentially kills cells that are in the process of dividing.

Despite the differences in growth response to EMS and X-rays, preliminary experiments suggest that the expression time for 6-TG^R mutants induced by X-rays was similar to the optimum expression time of 4-5 generations determined previously for cells treated with EMS. This result is in agreement with the data obtained in mouse lymphoma cell systems, in which

the expression times for X-ray and EMS mutagenesis were the same[14]. In mouse lymphoma cells, extremely long expression times of 12 generations were required to produce maximal frequency of 6-TG^R mutants[14]. Additional experiments with X-ray-treated human lymphoblasts are necessary to determine if longer expression times will increase the recovery of mutants.

A potential problem we encountered in this study and inherent in all mutagenesis studies with mammalian cells[19] is the effect of mutation expression time on the frequency of mutants obtained after a certain treatment. We attempted to determine the optimum expression time for lymphoblasts after EMS and X-ray treatments, but apparently a frequency of mutant numbers is attained which does not maintain a stable, plateau value as the cell population continues to grow after treatment[20]. This led us to show in Tables 1A and 1B just the highest mutation frequencies obtained at each dose of mutagen rather than the average mutation frequencies.

A comparison of mutation frequencies induced by X-ray and EMS treatments at equitoxic doses (Fig. 3) indicates that the frequency of 6-TG^R cells induced by each mutagen are not appreciably different for MGL33C19 cells. This result is unexpected, since several investigators found that X-rays and gamma rays are less efficient mutagens than EMS in their cell systems. In Chinese hamster V79 cells, EMS has been found to be as much as 10-12 times more efficient as a mutagen than X-rays[10,21].

In contrast to the results obtained with MGL33C19 cells, X-rays were much poorer than EMS at inducing 6-TG^R mutants in MGK8E and KS3A4 cells. EMS was over 10 times as effective as X-rays at inducing mutants in KS3A4 cells (Table 2). In MGK8E cells, EMS treatment induced a mutation frequency of 2.12×10^{-4}, while X-ray treatment with 200 rads (98% kill) produced no mutants (Table 2).

MGL33C19 appears to be significantly more mutable than either of the two lymphoblast cell lines studied. The spontaneous mutation frequency of

MGL33C19 is $3.74 \pm 0.98 \times 10^{-5}$ (not shown), while that of KS3A4 and MGK8E are 7.76×10^{-6} and 2.10×10^{-6} (Table 2). The spontaneous frequency observed for MGK8E is similar to that obtained for the parental cell line MGK8E by Epstein (2.1 to 2.9 $\times 10^{-6}$)[8]. X-ray and EMS-induced mutation frequencies in MGL33C19 are about 4×10^{-4} or approximately 10 times the spontaneous frequency when the dose of mutagen produces 95% kill. EMS-induced mutation frequencies for 8E and KS3A4, however, are similar to the values obtained for MGL33C19. A mutation frequency of 2.12×10^{-4} was obtained for MGK8E at a % kill level of 97%, while 1.43×10^{-4} was obtained for KS3A4 at a 94% kill level. X-ray induced mutation frequencies for KS3A4 and MGK8E are very low, as stated earlier, and attained values quite similar to spontaneous values obtained with no X-ray treatment.

Differences in sensitivity of our cell lines to X-rays and EMS do not seem to be related to differences in mutability. Although MGK8E and KS3A4 differ greatly in mutagen sensitivities, they exhibit similar induced mutation frequencies. Differences in plating efficiencies, however, do seem to be related to differences in mutability. When data from several separate experiments were pooled to determine the average plating efficiencies of the three cell lines, MGL33C19 had the highest average plating efficiency of $20.6 \pm 12.3 \times 10^{-2}$. The plating efficiencies of MGK8E and KS3A4 were $7.2 \pm 1.6 \times 10^{-2}$ and $8.0 \pm 3.9 \times 10^{-2}$. Although mutation frequencies are corrected for plating efficiencies, it appears that higher mutability in some manner is associated with higher plating efficiency for the human lymphoblasts studied.

6-TG appears to be effective in selecting for mutants deficient in HGPRT in this system. Using 6-TG as a selective agent, we found no evidence of "phenocopies" or false mutant clones containing high levels of HGPRT activity. Of 31 clones isolated from 6-TG-containing plates, the one with the highest amount of residual HGPRT activity contained only 6.6% of the

activity of that exhibited by the parental MGL33C19 cells (Tables 3 and 4). When 5 of the clones were tested for growth in selective media, all five responded as did the 33JX3 clone as shown in Fig. 5B. The 33JX3 clone could grow in 6-TG, but not in HAT medium. All five of the clones tested for growth in selective media had been grown for a period of 3 consecutive months in non-selective media. This suggests that the 6-TG^R trait in these clones is stable.

Table 1A. Frequencies of 6-TG^R colonies of MGL33C19 cells treated with various concentrations of EMS. Maximum mutation frequencies of cells selected in 6-TG at 4-5 generations after treatment are shown. Treatment with EMS was for 4 hours.

Table 1B. Frequencies of 6-TG^R colonies of MGL33C19 cells treated with various levels of X-rays. Maximum mutation frequencies of cells selected in 6-TG at 3-6 generations after treatment are shown.

Table 1A

Dose of EMS	% Kill[1]	Induced[2] Mutation Frequency Per Viable Cell[3] (X 10^{-5})
25 µg/ml	34	3.72
50 µg/ml	55	12.96
100 µg/ml	79	15.12
200 µg/ml	95	26.26
300 µg/ml	99	32.36

Table 1B

Dose of X-rays	% Kill[1]	Induced[2] Mutation Frequency Per Viable Cell[3] (X 10^{-5})
50-Rads	12	3.48
100-Rads	61	12.72
200-Rads	91	39.76

[1]Determined on day of treatment.

[2]Corrected for spontaneous mutation frequency.

[3]Corrected to 100% plating efficiency as discussed in Materials and Methods.

Table 2. MGK8E and KS3A4 mutation frequencies.

Table 2

Cell line	Mutagen[1] Treatment	% Killing[2]	# of Cells Plated ($X 10^5$)	# of TG^R Colonies	TG^R[3] Mutation Frequency Per Viable Cell ($X 10^{-5}$)
MGK8E	0	0	5.4	1	0.2
	EMS (800 µg/ml)	97	55.3	23	21.2
	X-ray (200 Rad)	91	113.0	0	-
KS3A4	0	0	58.2	4	0.8
	EMS (100 µg/ml)	94	5.4	12	14.3
	X-ray (200 Rad)	98	57.4	10	1.6

[1] EMS treatments were for 4 hours.

[2] Determined on day of treatment. See Materials and Methods for additional details.

[3] Values corrected to 100% plating efficiency as discussed in Materials and Methods.

Table 3. HGPRT activities of MGL33C19 lymphoblasts and 6-TG^R clones isolated from X-ray mutagenesis experiments. All clones were derived from cells treated with 100-200 rads of X-radiation except 33JX6 which is a spontaneous mutant. The value given for MGL33C19 is the average of three determinations. Details of enzyme assay are in Materials and Methods.

Table 3

Cell line	Counts/min/ 10^6 cells	HGPRT activity pmoles (I + IMP)/ mg protein/min	HGPRT activity % of control
MGL33C19	47807 ± 4522	1727.6 ± 200.0	100.0
33JX1	0	0.0	0.0
33JX2	0	0.0	0.0
33JX3	3051	113.5	6.6
33JX4	6	0.3	0.0
33JX6	0	0.0	0.0
33JX7	340	22.5	1.3
33JX8	839	40.8	2.4
33JX9	0	0.0	0.0
33JX10	0	0.0	0.0
33JX13	696	25.9	1.5
33JX17	0	0.0	0.0
33JX28	485	12.6	0.7

Table 4. HGPRT activities of 6-TG^{R} clones isolated from EMS mutagenesis experiments. All of the clones were derived from cells that were treated with EMS, except for 33KT19, which is a spontaneous mutant. The value given for MGL33C19 is the average of four determinations. Details of enzyme assay are in Materials and Methods; protein determinations were not done on these cell extracts.

Table 4

Cell line	Counts/min/ 10^6 cells	HGPRT activity % of control
MGL33C19	45515 ± 13861	100.0
33KT1	220	0.5
33KT2	180	0.4
33KT3	285	0.6
33KT4	715	1.6
33KT5	180	0.4
33KT6	30	0.1
33KT7	890	2.0
33KT8	25	0.1
33KT9	635	1.4
33KT10	505	1.1
33KT11	625	1.4
33KT12	185	0.4
33KT13	575	1.3
33KT14	715	1.6
33KT15	480	1.1
33KT16	0	0.0
33KT18	1105	2.4
33KT19	430	0.9
33KT21	510	1.1

Fig. 1A. Survival curves of three human lymphoblast cell lines treated with various levels of X-radiation. Data represent the percent survival observed in 1 to 5 experiments of 4-5 plates each. The curves are plotted as the linear regressions of the mean values. The r^2 values for MGL33C19 = 0.991; MGK8E = 0.977; KS3A4 = 0.999.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

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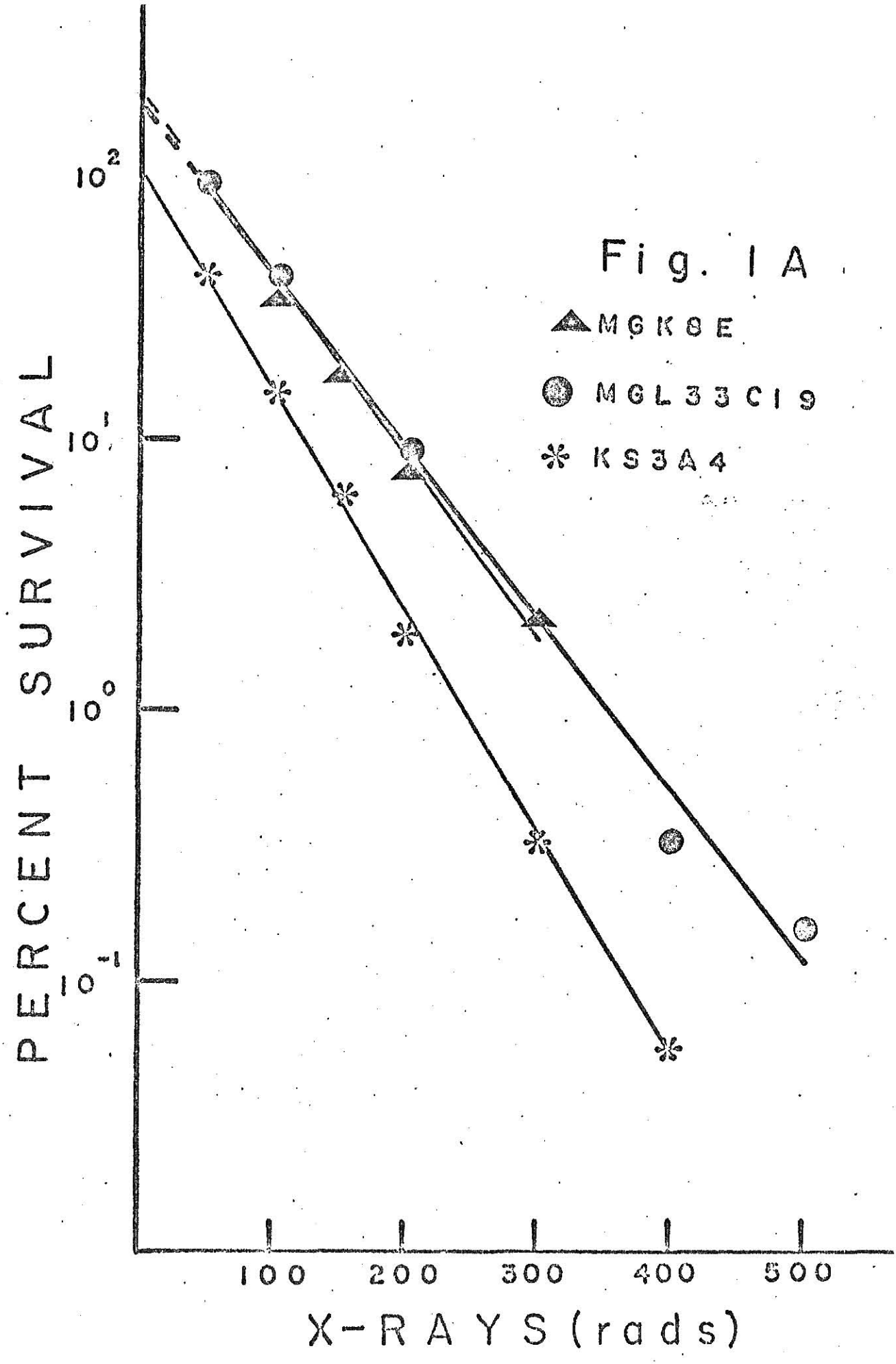
Fig. 1A
MGK8E
MGL33C19
KS3A4
PERCENT SURVIVAL
10^2
10^1
10^0
10^{-1}
100
200
300
400
500
X-RAYS (rads)

Fig. 1B. Survival curves of three human lymphoblast cell lines treated with various concentrations of EMS. Treatment times were 4 hours and data represent the mean of 1 to 5 experiments of 4 to 5 plates each. The surves are plotted as the linear regressions of the mean survival values. The r^2 values for each cell line was greater than 0.95.

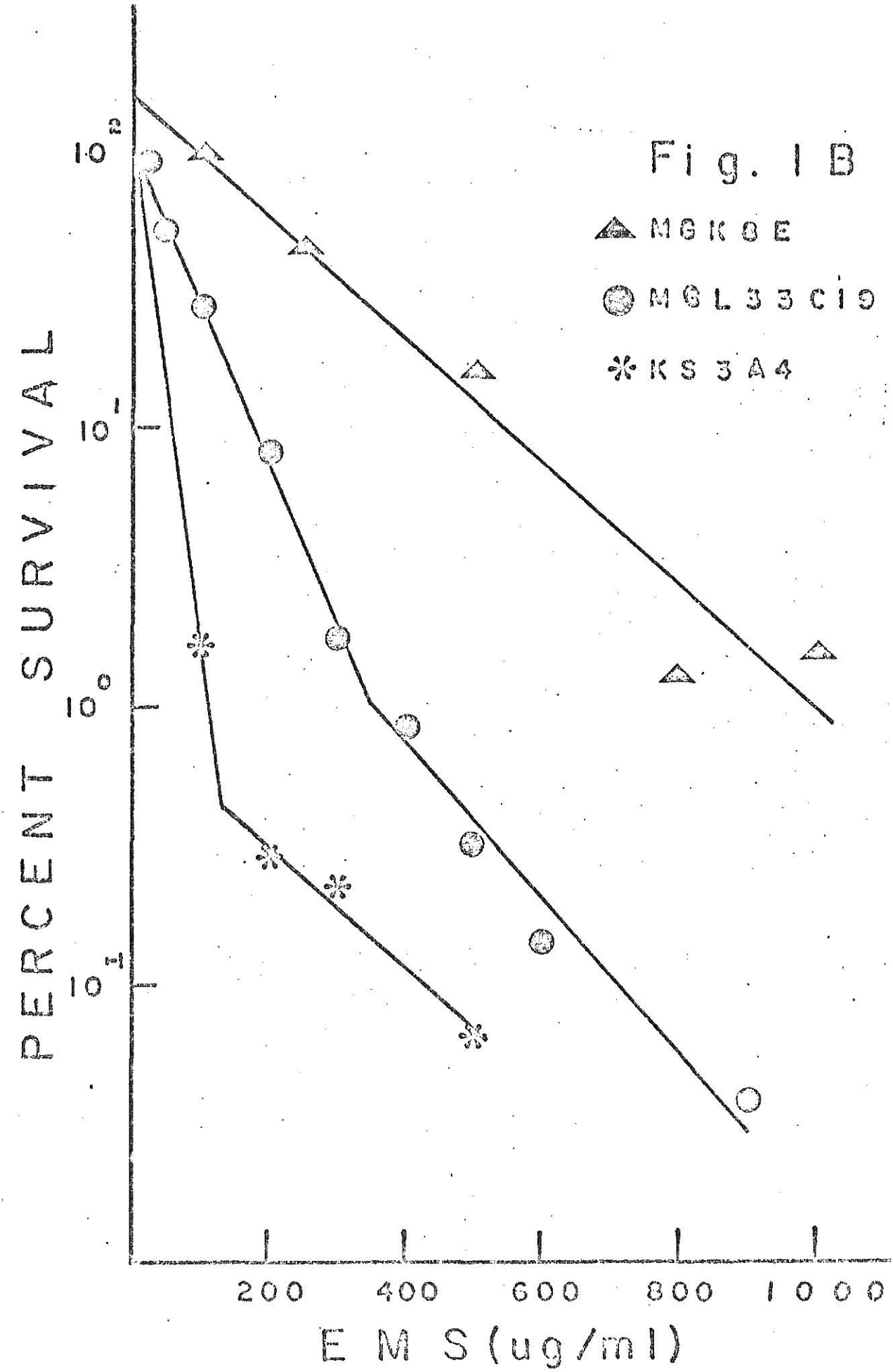
Fig. 1B
MGKOE
MGL33C10
KS3A4
PERCENT SURVIVAL
10²
10¹
10⁰
10⁻¹
200
400
600
800
1000
EMS (ug/ml)

Fig. 2A. Growth curves of MGL33C19 lymphoblasts after treatment with various levels of X-radiation. Cells were treated as described in Materials and Methods. Data are the mean of treatments done in duplicate.

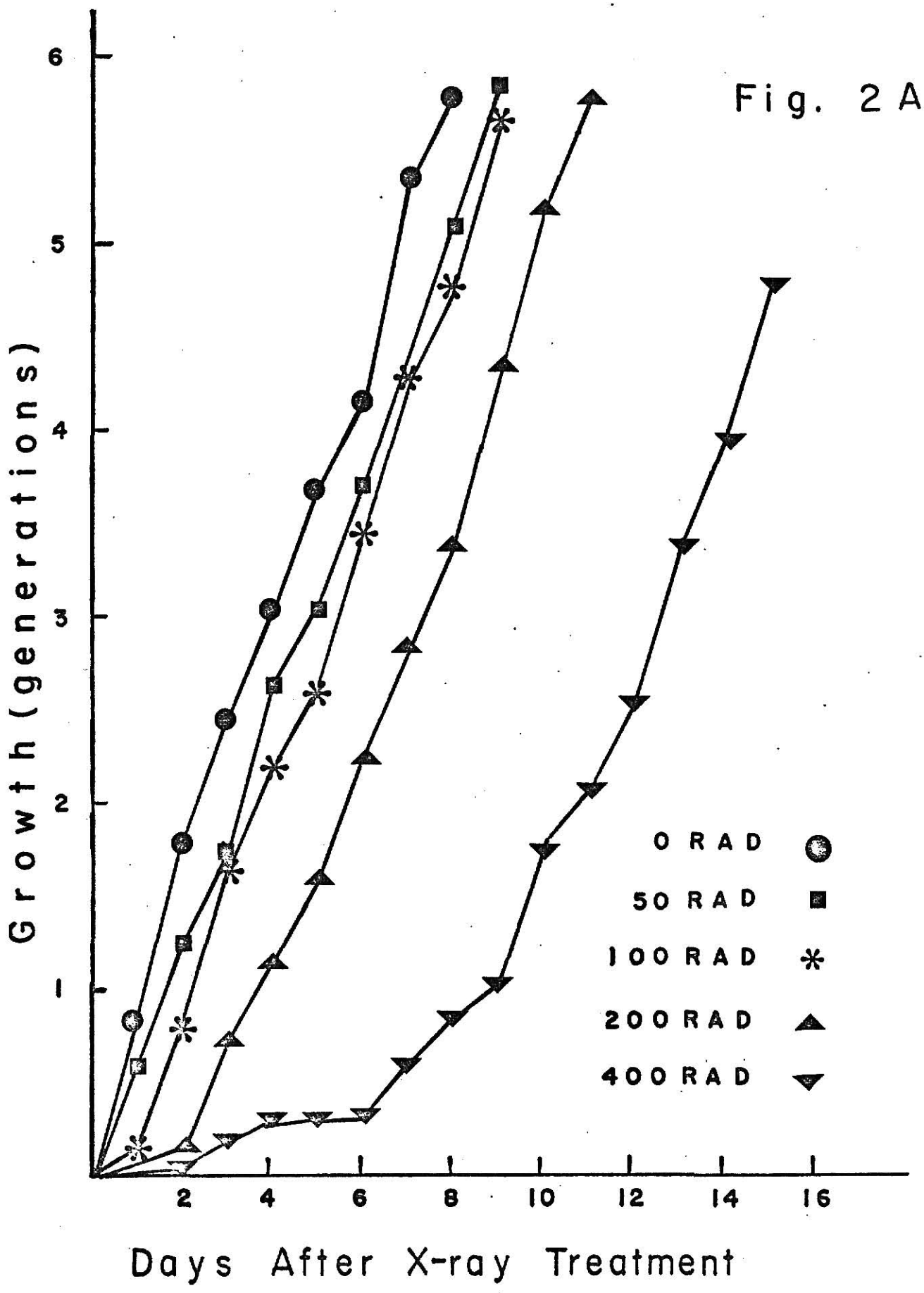
Growth (generations)
6
5
4
3
2
1
2
4
6
8
10
12
14
16
Days After X-ray Treatment
0 RAD
50 RAD
100 RAD
200 RAD
400 RAD

Fig. 2A

Fig. 2B. Growth curves of MGL33C19 lymphoblasts after treatment with various concentrations of EMS. Treatment times were 4 hours and data are the means of experiments done in duplicate. Details of EMS treatments are in Materials and Methods section.

Fig. 2B

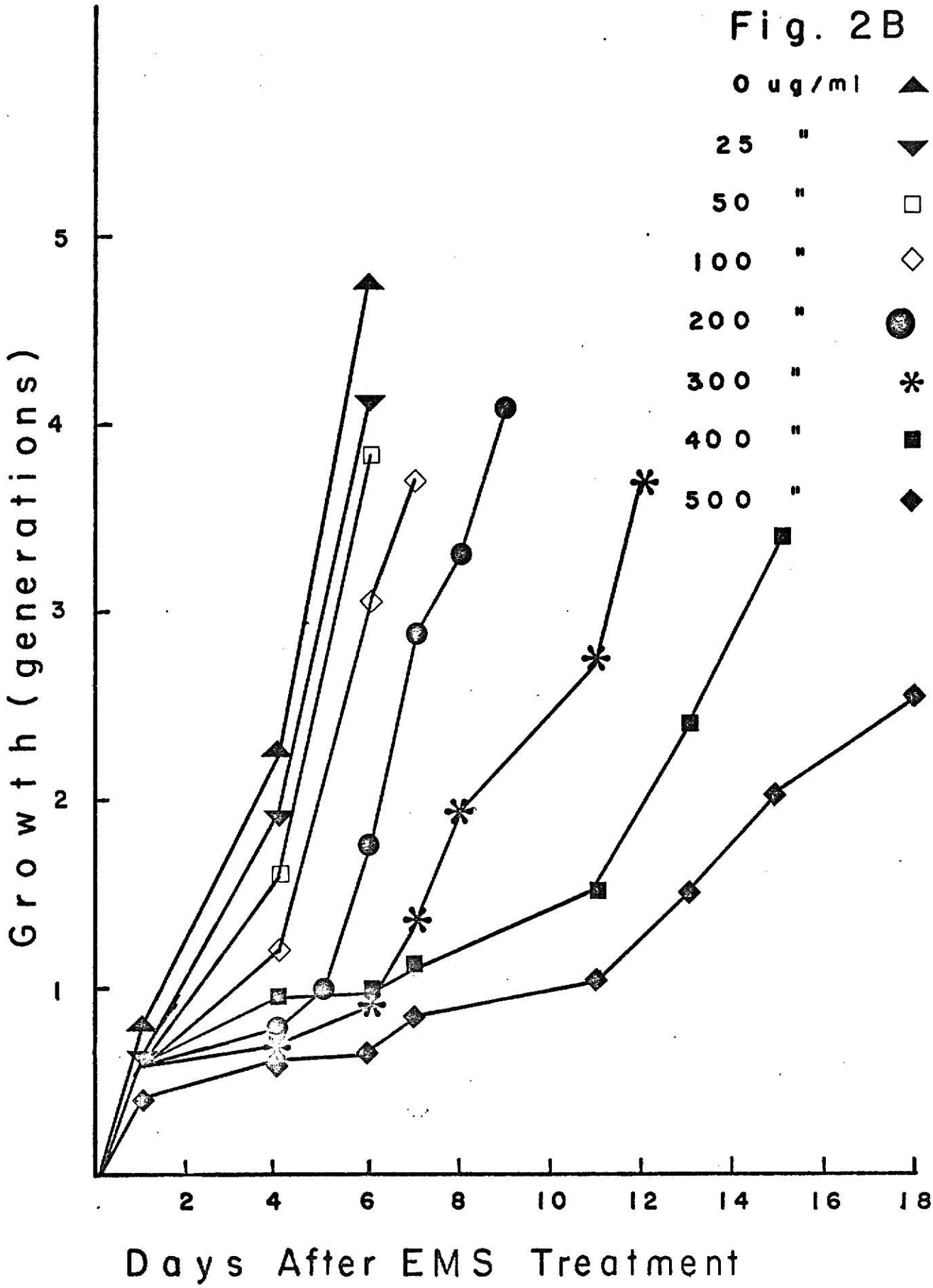
0 ug/ml
25 "
50 "
100 "
200 "
300 "
400 "
500 "
Growth (generations)
1
2
3
4
5
2
4
6
8
10
12
14
16
18
Days After EMS Treatment

Fig. 3. Maximum frequency of 6-TG^R colonies of MGL33C19 cells plotted against percentage of cell killing following X-ray and EMS treatment. Selection of mutants was performed at 3 to 6 generations after treatment. Cell killing was determined by rinsing treated cells in fresh media and plating in soft-agarose on the day of treatment. The highest mutation frequency observed in 2-5 experiments is shown. Additional details are in Materials and Methods.

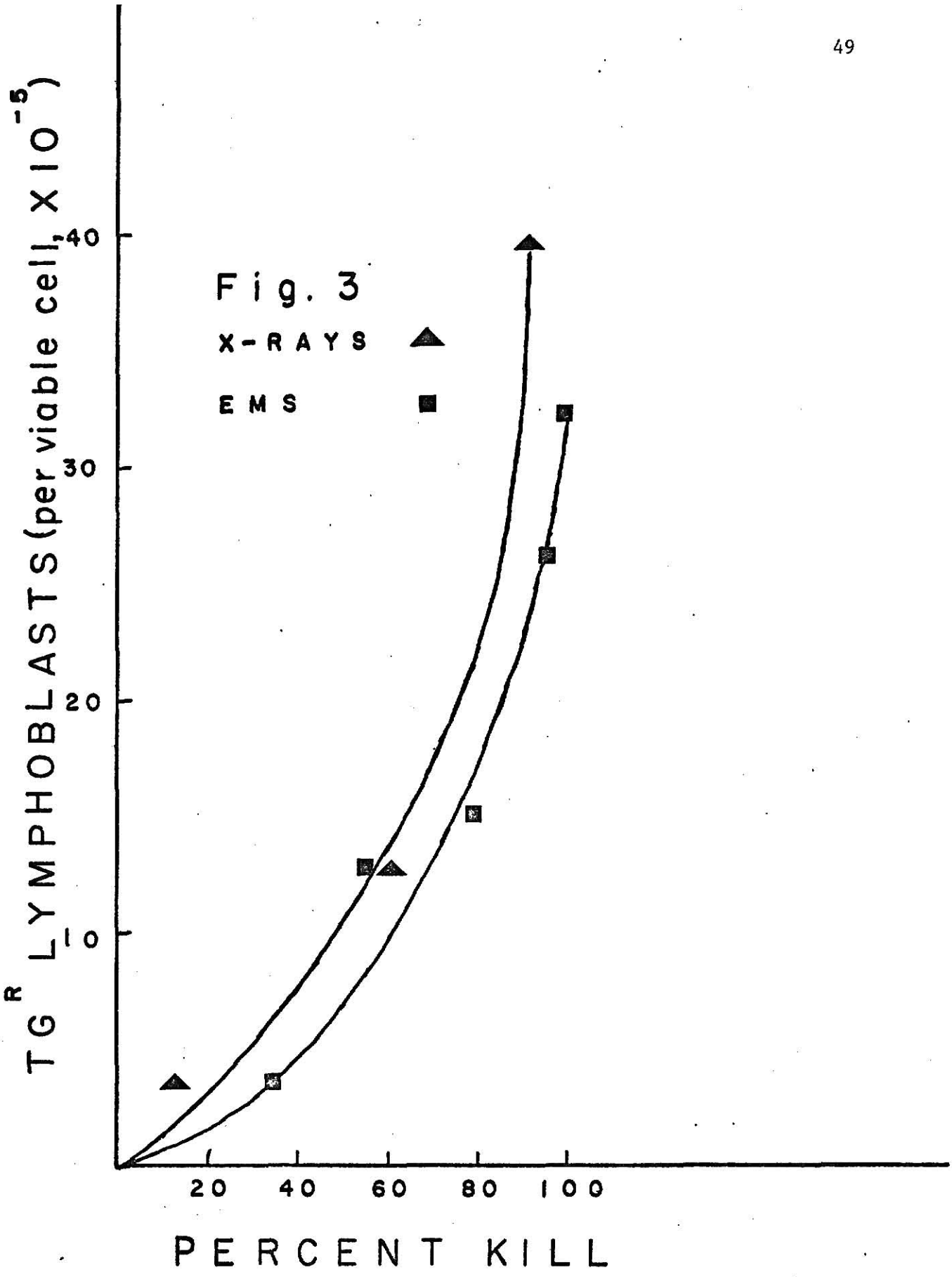
Fig. 3
X-RAYS
EMS
TG^R LYMPHOBLASTS (per viable cell, X 10^-5)
0
10
20
30
40
20
40
60
80
100
PERCENT KILL

Fig. 4A. Comparisons of growth of MGL33C19 ($HGPRT^+$) lymphoblasts in suspension cultures in R.P.M.I. 1640 media, 6-TG and HAT selective media. Data are the averages of duplicate flasks. Experimental details in Materials and Methods.

Fig. 4B. Comparisons of growth of 33JX3 ($HGPRT^-$) lymphoblasts in three selective media as described in Fig. 4A.

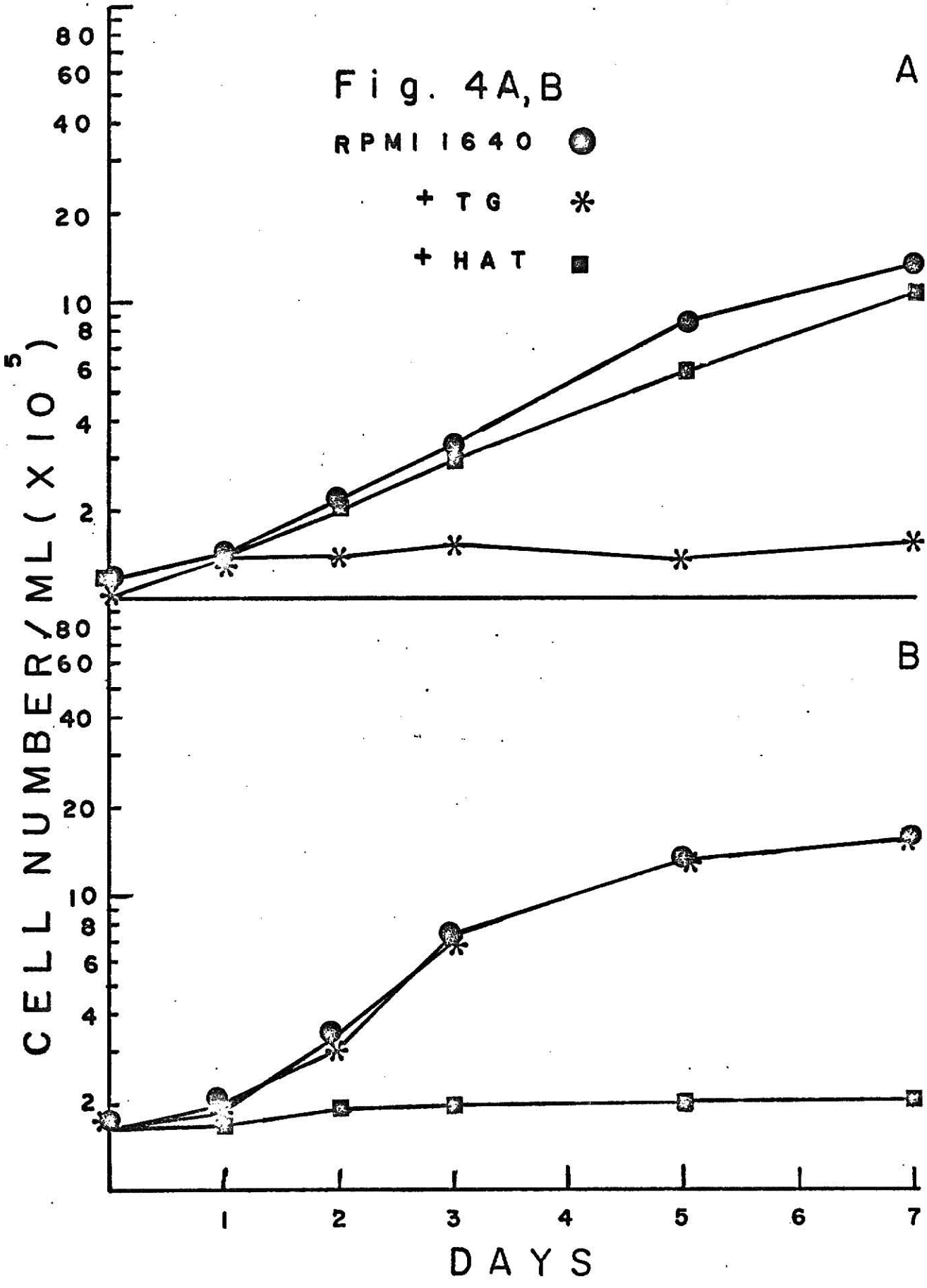
Fig. 4A,B
RPMI 1640
+ TG
+ HAT
A
B
CELL NUMBER/ML (X10^5)
80
60
40
20
10
8
6
4
2
1
2
3
4
5
6
7
DAYS

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Paper: SOME COMPARISONS BETWEEN THE USE OF 6-THIOGUANINE AND 8-AZAGUANINE FOR THE SELECTION OF HUMAN LYMPHOBLASTS DEFICIENT IN HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

Summary

6-Thioguanine (6-TG) and 8-azaguanine (8-AZG) were compared as selective drugs for selection of human lymphoblast cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT). 8-AZG appeared less suitable than 6-TG for this purpose, because some clones having 100% of the HGPRT activity of the parental lymphoblast cells were selected with 8-AZG (30 µg/ml). In contrast, no clones having greater than 7% HGPRT activity were selected with 6-TG (5 µg/ml) in previous studies. An attempt to distinguish between $HGPRT^+$ and $HGPRT^-$ colonies growing in 8-AZG medium on the basis of size alone was unsuccessful. Although $HGPRT^-$ colonies tended to be larger than $HGPRT^+$ colonies, colonies containing cells of both phenotypes were found that were within a common size range (0.3 to 0.6 mm in diameter).

Introduction

The use of either 8-azaguanine (8-AZG) or 6-thioguanine (6-TG) as a selective drug for mammalian cells deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) has led to some controversy over the suitability of each respective purine analogue[1,3,5,6]. At least one study concluded that 8-AZG is a more suitable selective agent in Chinese hamster V79 cells, because it appeared to be less affected by cell density than is 6-TG[3]. Results of other studies, however, with V79 and other cells suggest that 8-AZG is unsuitable as a selective agent because it is less toxic at low doses than 6-TG and appears to be susceptible to degradation or detoxification by serum components in culture media[6]. Frequent changes of the selective medium were recommended as one means to prevent the decrease in the 8-AZG concentration due to degradation by serum[6]. However, changes of the medium are not possible, or at least extremely difficult, in cell systems such as mouse lymphoma and human lymphoblast cells that require the use of a matrix of soft agarose for cloning[1].

The present study compares the use of 8-AZG and 6-TG for selection of human lymphoblast cells deficient in HGPRT. Studies on the relative mutation frequencies obtained with 8-AZG or 6-TG and the enzyme levels of the isolated "mutants" are presented. We also attempted to correlate colony size with the presence or absence of HGPRT activity to determine if the putative $HGPRT^-$ colonies fell into certain size classes.

Materials and Methods

Cell Lines

The MGL33C19 lymphoblast cell line (modal chromosome number = 46/47), is an aneuploid clone of the PGL33H culture initiated by Dr. P. R. Glade from the peripheral blood of a female patient with infectious mononucleosis. LN326 human fibroblasts deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) were obtained from a patient with the Lesch-Nyhan syndrome and provided for our use by Dr. J. W. Littlefield. LN326 cells were resistant to both 6-TG and 8-AZG and were used as "feeder" cells in all soft-agarose plating experiments.

Cell Culture Maintenance and Cell Plating Procedures

Lymphoblast cells were maintained in liquid media and plated in soft agarose[2].

X-ray Mutagenesis

Cells were centrifuged and resuspended in fresh growth medium at a concentration of 2.5×10^6 cells/ml. Two mls. of cells, suspended in standard R.P.M.I. 1640 medium or phosphate buffered saline (PBS), were added to 60 mm petri dishes at a concentration of 2×10^6 cells per ml, exposed to X-radiation and plated in soft-agarose medium to determine the percentage of surviving cells[2].

Mutant Selection with 8-AZG or 6-TG

Previous work with EMS indicated that optimum expression of 6-TG resistance occurs at 4-5 generations of growth following treatment with the mutagen[4]. Selection of mutants induced by X-rays was similarly begun at 4-5 generations in either 6-TG (5 µg/ml) or 8-AZG (30 µg/ml). Preliminary experiments indicate that expression time does not appear to differ significantly in EMS vs. X-ray treated cells. Mutant frequencies were corrected to 100% plating efficiency

by dividing the number of colonies which grew in selective medium by the plating efficiency of the same cells in regular (non-selective) medium.

Putative mutant colonies, which grew in media with 8-AZG often varied from small colonies, less than 0.1 mm, to large colonies, often as large as 1.0 mm in diameter. The number of colonies in two broad size ranges were recorded in each experiment: a) colonies greater than 0.1 mm in diameter, and b) those greater than 0.3 mm in diameter.

Growth in Selective Media

The drug-resistance and cross-resistance of selected mutant clones to two guanine analogues was studied. Cells were seeded into routine growth medium and medium supplemented with 8-AZG (Sigma) (30 µg/ml), 6-TG (Sigma) (5 µg/ml), or HAT (2 X 10^{-4} M hypoxanthine, 1 X 10^{-4} M amethopterin, and 4 X 10^{-4} M thymidine). The cell concentration was determined in each flask at the time of seeding and after a seven day period of growth. The increase in cell number in each test medium was recorded as the % of growth in standard medium (control) according to the following formula:

$$\text{Percentage of growth} = (N_t - N_o)/(N_s - N_o) \times 100\%$$

where N_o = number of cells seeded initially,

N_s = number of cells after growth in standard medium,

N_t = number of cells after growth in test medium.

Hypoxanthine-guanine Phosphoribosyltransferase Assay

Procedures for preparing cell extracts and assaying for HGPRT activities are described elsewhere[2].

In Vivo Incorporation of Radioactive Hypoxanthine

Incorporation of (^{3}H) hypoxanthine into DNA was measured by quantifying the appearance of TCA (5%) insoluble material on GF/C-glass fiber filters as a function of time. A 5 ml. aliquot of each actively growing culture was placed into test and 50 µl of (^{3}H) hypoxanthine (1 X 10^{-5} M; 2 Ci/mMole) was

added. The tubes were incubated at 37° C for 60 to 90 min. and incorporation was stopped by placing tubes in ice. A total of 1 X 10^6 cells was pipetted in duplicate on each of two Whatman GF/C filters. The cells were rinsed twice with 5 ml. of PBS containing 10^{-4} M hypoxanthine, then 6 ml. of ice cold 5% TCA was added and allowed to drip slowly. Incorporation of (^{3}H) hypoxanthine into TCA insoluble material was determined by placing the filters into Triton-toluene scintillation fluid [1 part Triton-X, 2 parts toluene-PPO solution (6 g/l, PPO)] and counting in a Beckman Model LS230 scintillation spectrometer. Filters were kept at least 12 hrs. at room temperature before counting since this procedure enhanced counting efficiencies. Quenching was corrected by the channels ratio method.

Results

Relative Sensitivity of MGL33C19 to 8-AZG and 6-TG

Sensitivity of MGL33C19 lymphoblasts to the purine analogues was determined by seeding MGL33C19 cells in media containing various concentrations of 8-AZG or 6-TG and recording the number of population doublings in 8 days. The results shown in Fig. 1 indicate that 6-TG is more toxic than 8-AZG. There was no measurable growth at concentrations greater than 2.5 µg/ml of 6-TG or in concentrations of 8-AZG greater than 5 µg/ml.

The response of five 6-TG^R clones and MGL33C19 to 8-AZG (30 µg/ml) in both solid and liquid media was compared with growth in the presence of 6-TG (5 µg/ml) in Table 1. In four of the five 6-TG^R clones, the plating efficiency in 8-AZG medium was about 30% of that obtained when cells were growing in 6-TG medium. In all of the 6-TG^R clones, however, the plating efficiencies in 6-TG medium were very close to the values obtained in standard medium. In liquid medium, all of the 6-TG^R clones again grew more poorly in 8-AZG than in 6-TG, indicating that the response of the cells to the selective agents was similar to that observed in soft-agarose medium. As expected, MGL33C19 cells, which are HGPRT$^+$, did not grow in 6-TG or 8-AZG media, but were capable of growth in HAT medium.

Selection of HGPRT$^-$ Cells Using 6-TG

A concentration of 5 µg/ml of 6-TG appears to be satisfactory for selection of 6-TG^R mutants, since only lymphoblasts with complete resistance to 6-TG apparently survive and form colonies at this concentration[29-31]. Five clones originally selected by their ability to grow in liquid media containing 5 µg/ml of 6-TG were grown for a period of three consecutive months in non-selective medium. The results shown in Table 1. indicate that all five of these clones were stable. We previously examined a total of 33 6-TG^R clones resulting from spontaneous mutations and those induced by EMS and X-rays for

HGPRT activities in another study[29]. Only 7 of 33 clones had HGPRT activity greater than 1.5% of the parental MGL33C19 cells. The activity of these clones ranged from 1.5% to 6.6%.

Comparison of 8-AZG and 6-TG Selection

The relative numbers of 8-AZG^R and 6-TG^R cells induced by X-rays were compared in the experiments shown in Table 2. Experiments A, B and C in Table 2, respectively, were performed by irradiation of 2 X 10^6 cells/ml in volumes of either 2, 4 or 6 ml. of R. P. M. I. 1640 medium. In experiment D, cells were irradiated in 2 ml. of PBS instead of growth medium. Experiments A to D studied the effect of varying the conditions upon cell killing and mutant induction. Variations in volume and type of medium appeared to influence the effective dose of radiation, as evidenced by the differences in the amount of cell killing. The percent of cells surviving treatment with 200 rads increased with the volume of medium in experiments A, B and C. Experiment D suggested that irradiation in 2 ml of PBS caused less killing than irradiation in 2 ml of standard medium. The relationship between the variation in the X-irradiation treatments and the resultant mutation frequencies was unclear. From the results of previous studies[2], we expected that the treatments which produced the greatest initial cell killing would produce the greatest 6-TG^R mutation frequencies. However, experiment A, with the greatest cell killing had the lowest mutation frequency.

The same relative differences in the size and number of colonies of 8-AZG vs. 6-TG containing plates was observed, no matter which X-ray treatment was used. The size of colonies in media with 8-AZG was significantly different than colonies which grew in 6-TG. Colonies in 8-AZG media were often between 0.1 and 0.3 mm in diameter. The minimum size of colonies on 6-TG containing plates was 0.3 mm and often as large as 1.0 mm. Numerous colonies in 8-AZG media were less than 0.1 mm in diameter, which made enumera-

tion of colony numbers very difficult.

Because the minimum size of 6-TG^R colonies was 0.3 mm, only colonies greater than that size on 8-AZG-containing plates were counted in calculating the 8-AZG^R mutant frequencies, which were in all cases greater than the 6-TG^R mutant frequencies.

Characteristics of the 8-AZG-selected Colonies

The HGPRT activities of 14 colonies selected in 8-AZG are shown in Table 4. In general, two phenotypes were evident:

1) Clones that contained little or no HGPRT activity, and

2) Clones that contained full HGPRT activity relative to control values.

Cells which had normal HGPRT levels grew in HAT, did not grow in 6-TG or 8-AZG (Table 3) and could take up and incorporate (^{3}H) hypoxanthine into TCA-precipitable material. The clones which contained little or no HGPRT activity could not grow in HAT, but could grow in 6-TG and 8-AZG. These clones, like the 6-TG^R clones discussed earlier, grew poorly in liquid media with 8-AZG as compared to liquid media with 8-AZG as compared to liquid media with 6-TG. In addition, these mutants could neither take up nor incorporate (^{3}H) hypoxanthine.

XAZ-9, unlike the other drug-resistant clones, grew in all three selective media. In addition, XAZ-9 had a greater amount of HGPRT activity than the other mutant clones. However, recloning of XAZ-9 cells, previously grown in HAT medium and then transferred to standard medium for several generations, revealed that these secondary clones did not grow in 6-TG or 8-AZG media. Further characterization of XAZ-9 is necessary, but it appears at this time that XAZ-9 represented a mixture of the two classes of phenotypes described earlier, and is not an intermediate phenotype. Alternatively, the XAZ-9 may be unstable and we did not test a sufficient number of secondary clones to eliminate this possibility.

All clones isolated from 8-AZG-containing plates of non-mutagenized cells

were found to have full HGPRT activity and none were resistant to 8-AZG or 6-TG when later subjected to these two selective media. In contrast, all clones isolated from 8-AZG-containing plates of mutagenized cells with the exception of XAZ-17, were resistant to the analogues and had little or no HGPRT activity. This result suggests that the 8-AZG^{R} HGPRT^{-} clones were mutants, since the frequency of their appearance increased following mutagen treatment.

A relationship between colony size in 8-AZG medium and the presence or absence of HGPRT activity appears to exist. None of the clones with HGPRT activity grew larger than 0.6 mm. Most of the clones lacking HGPRT activity were derived from colonies that were larger than 0.6 mm. However, it was observed that a small colony (XAZ-7 = 0.30 mm) could also lack HGPRT activity.

Discussion

8-AZG does not appear to be as suitable as 6-TG for selection of MGL33C19 human lymphoblast cells that are $HGPRT^-$. Not only are higher concentrations of 8-AZG than 6-TG required to produce the same amount of cell killing, but colonies containing 100% HGPRT activity of control values appeared in 8-AZG-containing plates that were not evident in previous experiments using 6-TG as the selective agent. The appearance of such cells makes estimation of $8\text{-}AZG^R$ mutation frequencies very difficult.

Using a criterion of only counting colonies over a certain diameter in size in estimating $8\text{-}AZG^R$ mutation frequencies is not acceptable, because this can lead to either an over or under-estimation of mutation frequencies. Because of the difficulty of isolating and growing very small colonies, no colonies less than 0.3 mm in size were characterized. Therefore, it is not known if any of these colonies lacked HGPRT activity. Excluding them may have resulted in an underestimation of $8\text{-}AZG^R$ mutation frequencies. The observation that $HGPRT^+$ colonies can be as large as 0.60 mm and $HGPRT^-$ colonies can be as small as 0.30 mm makes determination of the phenotype of colonies within that size range difficult. Counting all colonies greater than 0.30 mm in estimating $8\text{-}AZG^R$ mutation frequencies would include some $HGPRT^+$ colonies and result, possibly, in an overestimation of $8\text{-}AZG^R$ mutation frequencies.

The conclusion that 8-AZG is not suitable as an agent for selection of $HGPRT^-$ MGL33C19 cells is in agreement with the results obtained by Clive[1], who studied mouse lymphoma cells that also required the use of a soft agarose matrix system for cloning. He observed that the frequency of colonies on plates containing 8-AZG increased as the number of cells plated was increased. As a possible explanation, he suggested that this observation may be due to

the ability of cells to detoxify the 8-AZG. In the mouse lymphoma cell system, as well as in the human lymphoblast cell system, it is not possible to completely remove media from soft-agarose plates and replace it with fresh media. Therefore, a detoxification effect cannot easily be corrected for by adding fresh 8-AZG medium.

The appearance of $HGPRT^+$ colonies could also be explained by degradation of 8-AZG due to serum components[6], since the serum used in this was not dialyzed. Perhaps 8-AZG would be a satisfactory selective agent in this system if the serum were dialyzed. However, this procedure would be an inconvenience that does not appear to be necessary if 6-TG is used as the selective agent.

Table 1. HGPRT activities and growth in selective media of MGL33C19 and five 6-TG^R clones.

Table 1

Cell Line	HGPRT Activity[a] (% of control)	Growth in Selective Media (% of growth in) standard liquid media			Plating Efficiencies in Soft Agarose Selective Media (% of P.E. in standard media)	
		TG	AZG	HAT	TG	AZG
33C19	100.0	3.7	3.8	75.6	0.0	0.0
33JX3	6.6	84.4	31.6	3.0	100.0	39.1
33JX6	0.0	99.9	31.3	ND	120.0	32.0
33KT1	0.5	93.2	16.7	3.9	84.9	32.1
33KT12	0.4	105.0	20.3	ND	127.3	27.3
33KT19	0.9	94.4	24.8	ND	73.3	6.7

[a]These values are from Tables 3 and 4 of another study.

Table 2. Quantification of numbers of AZG^R and TG^R lymphoblast colonies after treatment with X-rays under various irradiation conditions. In experiments A, B and C, 2, 4 and 6 mls. of cells, respectively, were suspended and irradiated in standard growth media at a concentration of 2×10^6 cells per ml. in 60 mm petri dishes. The thickness of the suspensions in experiments A, B, and C were approximately 0.9, 1.8, and 2.7 mm respectively. In experiment D, 2 mls. of cells suspended in phosphate buffered saline (PBS) were irradiated under the same conditions as A, B and C. Cells were plated in soft-agarose containing standard medium immediately after irradiations to determine survival, and then plated in standard and selective media after an expression time of 4-5 generations of growth. The number of colonies greater than 0.1 and 0.3 mm in diameter are shown.

Table 2

Treatment	% Kill[a]	Average # of AZG^R colonies/plate >0.1 mm	>0.3 mm	AZG^R Mutation frequency/surviving cell X 10^{-4}	Average # of TG^R colonies/plate >0.1 mm	>0.3 mm	TG^R Mutation frequency/surviving cell X 10^{-5}
0-Rad	0.0	162.7	22.7	1.74	4	4	3.01
200-Rad[A]	97.5	255.2	51.2	2.09	2	2	0.74
200-Rad[B]	96.6	130.2	34.2	4.16	13	13	15.80
200-Rad[C]	94.6	176.6	32.8	3.16	7	7	6.75
200-Rad[D]	97.1	226.4	48.6	2.93	37	37	22.10

Table 3. Size of colonies and growth characteristics of clones isolated from plates containing 8-AZG.

Table 3

Cell Line	Size (diameter) of colony	Growth in Selective Media (% of control) TG	AZG	HAT
[a]MGL33C19	--	2.2	0.0	68.3
[b]XAZ-1	1.50 mm	130.3	30.5	6.4
XAZ-3	0.75 mm	118.0	15.3	4.5
XAZ-4	0.75 mm	100.0	18.1	4.5
XAZ-9	1.10 mm	79.5	19.7	36.5
XAZ-10	1.00 mm	95.2	59.3	3.4
XAZ-11	0.75 mm	68.9	31.1	3.5
XAZ-17	0.30 mm	4.6	2.3	65.6
XAZ-7	0.30 mm	ND	ND	ND
[c]XAZ-21	0.50 mm	4.4	4.4	76.1
XAZ-22	0.30 mm	9.3	6.9	65.6
XAZ-23	0.60 mm	7.0	5.7	86.1
XAZ-24	0.50 mm	6.3	5.0	67.2
XAZ-28	0.45 mm	3.3	2.4	75.6
XAZ-29	0.30 mm	7.5	4.8	77.7

[a]Parental cells.

[b]Cells treated with 200-Rad[B] (see Table 2).

[c]Cells not treated with X-radiation.

Table 4. HGPRT activities and in vivo incorporation of ^{3}H-hypoxanthine of MGL33C19 and clones isolated from plates containing 8-AZG.

Table 4

Cell Line	HGPRT Activity pmoles (I + IMP)/ mg protein/min	% of control	In Vivo Incorporation of ^{3}H-hypoxanthine pmoles of Hx/ 10^{6} cells/hr	% of control
[a]MGL33C19	839.7	100.0	44.75	100.0
[b]XAZ-1	3.4	0.4	0.66	1.5
XAZ-3	3.5	0.4	0.00	0.0
XAZ-4	1.9	0.2	0.13	0.3
XAZ-7	ND	ND	0.14	0.3
XAZ-9	5.1	0.6	0.17	0.4
XAZ-10	0.0	0.0	0.00	0.0
XAZ-11	0.7	0.1	0.00	0.0
XAZ-17	662.6	78.9	44.98	100.5
[c]XAZ-21	453.4	54.0	49.71	111.1
XAZ-22	981.8	116.9	39.40	88.0
XAZ-23	1186.1	141.3	42.66	95.3
XAZ-24	902.0	107.4	45.37	101.4
XAZ-28	722.9	86.1	45.85	102.5
XAZ-29	ND	ND	47.34	105.8

[a]Parental cells.

[b]Cells treated with 200-Rad^{B} (see Table 2).

[c]Cells not treated with X-radiation.

Fig. 1. Growth of MGL33C19 cells after 8 days in liquid media containing various concentrations of 6-TG or 8-AZG.

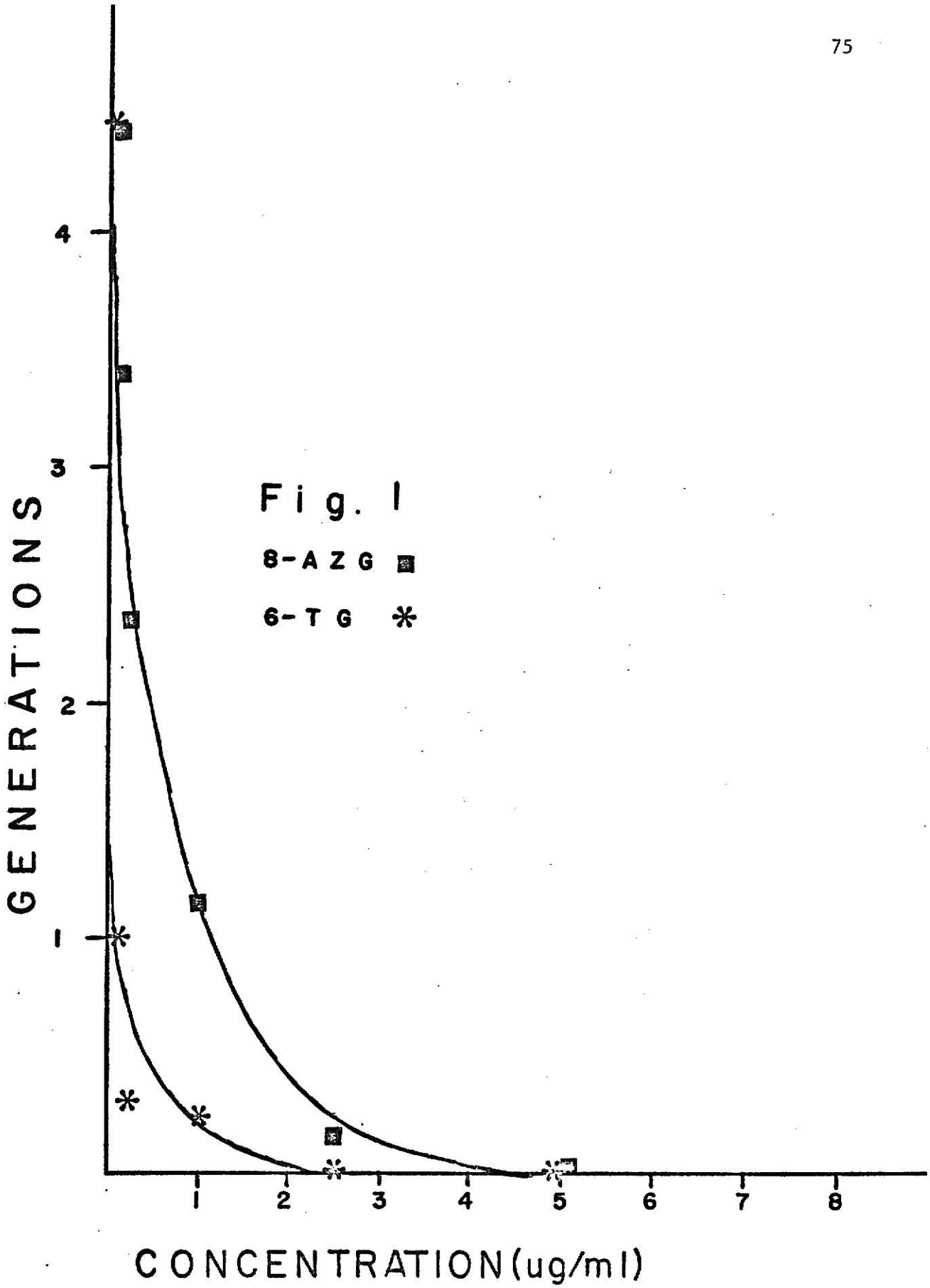

Fig. 1

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Acknowledgements

The author wishes to express sincere appreciation to Dr. Ronald Slesinski, major professor, for his aid, advice and support during the course of this study and in the preparation of this thesis. Appreciation is expressed to Dr. Thomas Manney and Dr. Richard Consigli, committee members, for their assistance. The author also wishes to express gratitude to Mike Riley for assistance in calibrating the X-ray machine, to Dr. Kenneth Kemp, K. S. U. Dept. of Statistics, for statistical advice, and to Ms. Kathy Smith and Lynne Whitlock for their technical help. Appreciation is also extended to good friends, Joseph Arruda, David Lemes, John Tabor, and Martin Stapanian, for their moral support.

MUTAGENESIS OF HUMAN LYMPHOBLAST CELLS

by

JOSEPH JOHN KOTEK

B. S., University of Illinois, 1973

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1976

The goals of this research were to study the frequency and characteristics of human lymphoblasts lacking hypoxanthine-guanine phosphoribosyltransferase (HGPRT) using X-rays as a mutagen and 6-thioguanine (6-TG) as a selective agent, and to compare the results with those already obtained using ethyl methanesulfonate (EMS). I also compared the purine analogs 8-azaguanine (8-AZG) and 6-TG as selective agents for HGPRT deficient lymphoblasts.

The cytotoxicities and mutagenic efficiencies of X-rays and EMS were compared in three lymphoblastoid lines (MGL33C19, MGK8E, and KS3A4), which appeared to differ in their spontaneous mutation frequencies of 6-TG resistant cells. The average spontaneous mutation frequency of MGL33C19 was found to be $3.74 \pm 0.98 \times 10^{-5}$, while values found for KS3A4 and MGK8E were 7.76×10^{-6} and 2.10×10^{-6}, respectively. KS3A4 was significantly more sensitive to both mutagens than the other two lymphoblast lines; MGK8E was similar to MGL33C19 in radiosensitivity, but was more resistant than MGL33C19 to EMS. The optimum time necessary for expression of the 6-TG^R phenotype for MGL33C19 cells treated with either X-rays or EMS appeared to be 4-5 generations. The expression time was similar for each mutagen despite obvious differences in the growth response following X-ray and EMS treatments. Some growth occurred in the first 2 days after EMS treatment, even after high doses (500 µg/ml), while high doses of X-rays (200-400 rads) were much more inhibitory. The frequency of 6-TG^R mutants increased as the dose of mutagen was increased and, at least for EMS, this dose-response relationship appeared to be linear. When compared at equitoxic doses, the effectiveness of X-rays and EMS at inducing 6-TG^R mutations of MGL33C19 cells appeared very similar and values differed by less than two-fold. In contrast, EMS appeared to be greater than ten times as effective as X-rays at inducing 6-TG^R mutants in KS3A4 and MGK8E. Differences in mutability among these cell lines do not appear to be related to mutagen sensitivities,

since both KS3A4 and MGK8E, which had similar mutation frequencies, each differed greatly in X-ray and EMS sensitivity. A correlation between mutability and plating efficiency was observed. MGL33C19, which had an average plating efficiency greater than twice that of the other two lines, was the most mutable cell line, despite correction of mutation frequencies for differences in plating ability.

A comparison of 6-TG and 8-AZG as selective agents for HGPRT deficient mutants indicated that 8-AZG is less suitable than 6-TG. Several colonies selected in 8-AZG (30 μg/ml) medium had nearly 100% of the HGPRT activity of the parental MGL33C19 cells. In contrast, no clones having greater than 7% HGPRT activity were selected with 6-TG (5 μg/ml). An attempt to distinguish between $HGPRT^+$ and $HGPRT^-$ colonies growing in 8-AZG medium on the basis of size alone was unsuccessful.