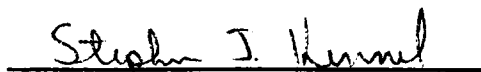
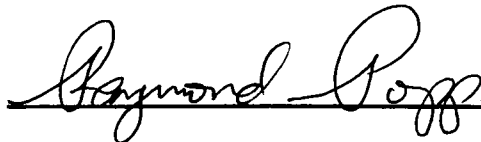
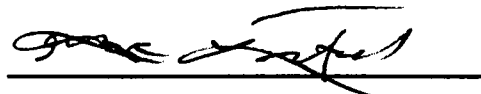


To the Graduate Council:

I am submitting herewith a thesis written by Steven Andrew Plichta entitled "Comparisons of Mutation Frequencies and Cytotoxic Responses of V79 and G12 Cells Exposed to X Rays and Alpha-Particles". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.


Dr. Margaret Terzaghi-Howe, Major Professor

We have read this thesis and
recommend its acceptance:



Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**COMPARISONS OF MUTATION FREQUENCIES AND CYTOTOXIC
RESPONSES OF V79 AND G12 CELLS EXPOSED TO X RAYS AND
ALPHA-PARTICLES**

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

**Steven A. Plichta
August 1995**

DEDICATION

TO MY WIFE

Jill A. Plichta

MY PARENTS

William and Darlene Plichta

AND MY BROTHER

William P. Plichta

ACKNOWLEDGEMENTS

I would like to thank Dr. Margaret Terzaghi-Howe for her support, guidance, advice, and friendship during my research in her laboratory. She has provided me with an invaluable learning experience. I would also like to thank the other members of my committee, Drs. Stephen Kennel, Gayle Littlefield, and Ray Popp for their time involved in reviewing my thesis and their valuable suggestions.

I would also like to express my appreciation to Everline B. Wright and Gen-Win Chang for their technical support and friendship throughout the years.

A very special thanks to my wife, mother, father, and brother who have encouraged, supported, and loved me for being the type of person I am.

Finally, I would like to say thanks to the lord for providing me with a beautiful life and beautiful people to share my life with.

ABSTRACT

Chinese hamster V79 cells and a pSV2*gpt* transformed UV-induced HPRT-deficient V79 cell line (G12) were exposed to alpha-particles or X rays to analyze sensitivity to the induction of mutations at the HPRT and *gpt* loci. Alpha-particles are a type of densely ionizing radiation that mainly interact directly with DNA, while X rays are typical of sparsely ionizing radiation and mainly interact indirectly with DNA through the formation of free radicals. Suspended and attached cells were exposed to alpha-particles in order to study the effects of nuclear shape on the induction of mutations. In addition to analyzing the induction of 6-thioguanine resistant V79 and G12 cells, the cytotoxicity to V79 and G12 cells was also analyzed immediately, 3, and 6 days after exposure to X rays or alpha-particles. There were no significant differences in the mutation frequency in V79 cells induced by exposure to either X rays or alpha-particles at equitoxic doses. In contrast, the mutation frequency of G12 cells exposed to X rays was significantly greater than the mutation frequency of G12 cells exposed to alpha-particles at equitoxic doses. Mutation frequencies of V79 and G12 cells exposed to alpha-particles were similar. The mutation frequency of G12 cells was significantly higher than that of V79 cells when exposed to X rays. These preliminary results suggest possible differences in the sensitivity to the induction of *gpt* mutations in G12 cells when exposed to X rays compared to alpha-particles.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.....	1
Mutation.....	1
Hypoxanthine-Guanine PhosphoribosylTransferase.....	2
Ionizing Radiations.....	3
Survival.....	5
Repair of Double Strand Breaks.....	6
Induction of Mutations.....	7
Purpose.....	9
II. MATERIAL AND METHODS.....	10
Cell Lines.....	10
Alpha-Particle Exposure.....	10
X Ray Exposure.....	11
Assay for Survival.....	12
Assay for Mutations.....	12
Dosimetry.....	13
a. CR-39 detection of alpha-particles.....	13
b. Nuclear Measurements.....	14
III. RESULTS.....	16
Growth Characteristics.....	16
Survival.....	16
Induction of Mutations.....	36
IV. DISCUSSION.....	53
LIST OF REFERENCES.....	62
VITA.....	67

LIST OF FIGURES

FIGURE	PAGE
3.1 A Phase Contrast Picture of Giemsa Stained Colonies of V79 and G12.....	17
3.2 Comparison of Survival of V79 and G12 Hamster Cells After Irradiation with 300 keV X rays.....	19
3.3 Comparison of Survival of Attached V79 and G12 Cells After Irradiation with alpha-particles from americium-241.....	22
3.4 Comparison of Survival of Suspended V79 and G12 Cells After Irradiation with alpha-particles from americium-241.....	24
3.5 Analysis of Survival of V79 and G12 Cells Plated Immediately, 3, and 6 Days After Irradiation with 300 keV X rays.....	30
3.6 Analysis of Survival of Attached V79 and G12 Cells Plated Immediately, 3, and 6 Days After Irradiation to Alpha-Particles from americium-241...	32
3.7 Analysis of Survival of Suspended V79 and G12 Cells Plated Immediately, 3, and 6 Days After Irradiation to Alpha-Particles from americium-241....	34
3.8 Comparison of the induction of Mutants at the HPRT and <i>gpt</i> locus in G12 and V79 Cells by 300 keV X ray.....	37
3.9 Comparisons of the induction of Mutants at the HPRT and <i>gpt</i> locus in attached V79 and G12 Cells Irradiated by Alpha-Particles from americium-241.....	39

3.10	Comparisons of the induction of HPRT and <i>gpt</i> mutants in suspended V79 and G12 cells irradiated to alpha-particles from americium-241.....	41
3.11	Comparisons of the induction of HPRT mutants in V79 cells exposed to X rays (V79#1,V79#2) and attached V79 cells (V79A) exposed to alpha-particles.....	44
3.12	Comparisons of the induction of <i>gpt</i> mutants in G12 cells irradiated with X rays (G12#1,G12#2) and attached G12 cells (G12AA) irradiated with alpha-particles.....	46
3.13	Comparisons of the induction of HPRT mutants in suspended (V79S) and attached (V79) V79 cells irradiated with alpha-particles.....	48
3.14	Comparison of the induction of <i>gpt</i> mutants in suspended (G12S) and attached (G12) G12 cells irradiated with alpha-particles.....	50
4.1	Schematic representation of tracks of alpha-particles from a planar americium-241 source passing through the nucleus of an attached and suspended cell.....	55

CHAPTER I

INTRODUCTION

Mutation

Mutations have been associated with many diseases. Tumor cells frequently exhibit a large number of specific mutations (Loeb, 1991). The spontaneous mutation rate for normal cells estimated at 10^{-6} mutations per gene per division has been considered insufficient as an explanation for the high frequency of mutations observed in tumor cells. It has been hypothesized that the genome of a cancer cell exhibits a mutator phenotype or instability, which results in an increased mutation frequency (Loeb, 1994). Individually mutated genes may give rise to many different types of diseases. A congenital lack of functional hypoxanthine-guanine phosphoribosyl transferase (HPRT) is the fundamental defect in patients with Lesch-Nyhan syndrome (Caskey and Kruh, 1979). Therefore, studies of the induction of mutations could explain the development of tumors.

Mutations are alterations such as single base changes or deletions that occur within the sequence of DNA. Mutations have been studied at specific loci that allow for detection of wild type and mutant forms through metabolic selection. Mutations can occur spontaneously without the influence of external factors as a result of errors in DNA replication and repair. The occurrence of spontaneous mutations has been shown by many investigators to vary depending upon the cell type and locus being observed (Sankaranaryanan, 1991). Molecular analysis of spontaneous HPRT mutations in Chinese hamster V79 cells and Chinese hamster ovary cells has shown a small percentage of deletions and a large proportion (greater than 75%) of no detectable alterations to the gene, which could represent base changes (Schwartz et al., 1994; and

Aghamohammadi et al., 1992). Relative to the endogenous HPRT gene, a higher spontaneous mutation frequency has been observed for the bacterial xanthine-guanine phosphoribosyl transferase (*gpt*) gene when integrated into mammalian cells (Ashman and Davidson, 1984; and Stankowski and Hsie, 1986). Along with a higher spontaneous mutation frequency, a larger proportion of deletions occur in integrated bacterial genes compared to endogenous sequences (Tindall and Stankowski, 1989).

The frequency of mutations can be increased above the spontaneous rate by treatment with various mutagens. Radiation and certain chemical compounds such as alkylating agents are general types of mutagens that damage DNA by different mechanisms. Alkylating agents such as ethyl methanesulphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and β -propiolactone (BPL) produce mainly base pair substitutions, while ionizing radiation produces a variety of mutations with the most common type being deletions (Klein and Rossman, 1990, and Jostes et al., 1994). Some mutagens such as ionizing radiation are also carcinogens.

Hypoxanthine-Guanine Phosphoribosyl Transferase

HPRT is a commonly analyzed locus for mutation studies because one can readily select for either wild type or mutant forms. HPRT is an enzyme involved in the purine salvage pathway that catalyzes the conversion of hypoxanthine and guanine to 5'-inosine monophosphate (IMP) and 5'-guanosine monophosphate (GMP), respectively. Since there are two pathways for purine synthesis (*de novo* and salvage), the HPRT function is not essential to cell survival if mutated. In 1967, HPRT was shown to be defective in patients with Lesch-Nyhan syndrome and inherited as X-linked recessive trait (Caskey and Kruh, 1979). Because of the inactivation of one X chromosome in females and hemizyosity in males, only one functional allele is present and therefore its inactivation is not masked by the presence of a second, non-mutated, allele (Zhu and Hill, 1994). The metabolic selection of HPRT makes it a favorable locus for mutation studies. Cells lacking HPRT activity are unable to survive in medium supplemented with

hypoxanthine, aminopterin, and thymidine (HAT). Aminopterin is a folic acid analog that blocks the formation of tetrahydrofolate from dihydrofolate, thus blocking the *de novo* synthetic pathway. In the presence of aminopterin, the cell becomes dependent upon the synthesis of purines via the salvage pathway, and only cells containing a functional HPRT gene will survive. Cells with a mutated HPRT locus can be selected using 6-thioguanine. This purine analog is a substrate for HPRT and will become incorporated into the DNA of cells carrying a normal gene, and these cells are unable to function and survive with this altered purine base. Cells lacking HPRT activity will not incorporate 6-thioguanine and will continue to divide and function normally.

The HPRT locus has been extensively studied at the molecular level in the genomes of mouse, Chinese hamster, and humans. There are nine exons, the intron/exon junctions are conserved, and the size of the gene ranges from 34kb for mouse to 40kb in humans with Chinese hamster gene of 36kb. There is a 95% identical amino acid sequence among the coding region of the three species (Rossiter et al., 1991; and Konecki et al., 1982). With extensive sequencing information and with the capabilities of Southern blotting and polymerase chain reactions (PCR), the type and size of mutations at the HPRT locus can now be determined (Jostes et al., 1994).

Ionizing Radiations

Ionizing radiations transfer energy through interactions with matter. The way in which energy is transferred depends upon the type of ionizing radiation. The energy transferred is sufficient to eject an orbital electron from an atom or molecule, a process called ionization. X rays are a type of electromagnetic radiation, characterized as sparsely ionizing. Interactions with matter are widely spaced along the tracks of X rays. X rays do not usually interact with DNA directly, but will release their energy upon interacting with atoms or water molecules, which results in the formation of electrons or free radicals. Electrons are then capable of interacting with DNA directly or indirectly by the formation of free radicals. Water molecules may become ionized

from interactions with electrons or X rays, and result in the formation of a reactive hydroxyl radical ($\text{OH}\cdot$). This type of free radical is then capable of damaging DNA by producing strand breaks. Since studies utilizing free radical scavengers have shown a reduction in biological effects such as cell killing and mutation frequency (Grdina et al. 1985), a large proportion of lesions induced by X rays is thought to be caused by the formation of free radicals.

Alpha-particles are a type of particulate radiation composed of two protons and two neutrons. Alpha-particles are about 8000 times more massive than electrons and contain a net positive charge. Because of their mass and charge, alpha-particles transfer their energy in short densely ionizing tracks. Alpha-particles, unlike X rays, mainly produce lesions by interacting directly with DNA. Because of their positive charge, alpha-particles are also capable of releasing electrons from atoms. These electrons are then capable of interacting directly or indirectly with DNA as previously discussed. Since alpha-particles are densely ionizing compared to sparsely ionizing X rays, alpha-particles will transfer more energy per unit track length upon interacting with a cell than X rays.

The relative biological effectiveness (RBE) of ionizing radiation is influenced mainly by the distribution of ionizations or interactions along its track length. Ionizing radiations are qualitatively characterized by their linear energy transfer (LET). LET is the average energy emitted per unit track length. X rays and alpha-particles have significantly different LET values that are associated with marked differences in biological effectiveness. A 5.4 MeV alpha-particle emitted from americium-241 has a track length in water of 43 μm and an average LET of 126 $\text{keV}/\mu\text{m}$. A 300 keV X ray has an average LET of 2-3 $\text{keV}/\mu\text{m}$ in water. Therefore, alpha-particles are characterized as high-LET, while X rays are low-LET. The LET for alpha-particles is not constant but varies along its track. Bragg curves, generated by plotting the energy deposited or absorbed dose as a function of distance travelled through a substance, are used to depict variations in LET for alpha-particles in relation to distance traversed. As an alpha-particle travels through a substance, the number of interactions or ionizations

increases per unit track length as the speed of the particle decreases. A peak in ionizations is reached towards the end of the alpha-particle's track followed by a rapid decline in ionizations. The effectiveness of alpha-particles in killing mammalian cells can be plotted as a function of LET. This plot shows an increase in RBE with increasing LET from 25 to 110 keV/ μ m followed by a decline in RBE with higher LET values (Barendsen, 1964). Therefore, a maximum biological effectiveness is attained around 100-110 keV/ μ m. In general, high-LET ionizing radiations have been shown to be more effective at cell killing and mutation induction than low-LET radiation (Hei et al., 1988).

Although, there are many ways to describe the dose of ionizing radiation, it is commonly expressed in terms of absorbed dose. Absorbed dose is a physical quantity related to the amount of energy absorbed per unit mass of material. One Gray (Gy) is a unit of absorbed dose which equals 1 Joule per kilogram of material. Since the LET varies along the track of an alpha-particle, the absorbed dose from exposure to alpha-particles is dependent upon the thickness and nuclear cross-section of the nucleus. Alpha-particle dose can also be expressed in terms of fluence (number of alpha-particles/ μ m²/minute exposure). The average number of alpha-particles/nucleus can be determined by multiplying the target nuclear cross-section and specific exposure time by the fluence.

Survival

The shape and slope of a particular survival curve provides information on the cytotoxic response of treated cells. Survival curves are generated by plotting the surviving cell fraction as a function of absorbed dose or fluence. Survival curves for cells exposed to X rays exhibit a characteristic shoulder at low doses, suggesting the potential to repair sublethal damage. This shoulder is followed by a log-linear decrease in surviving fraction at higher doses. The width of the shoulder can be quantified by the D_q , the quasi-threshold dose, or extrapolation number(n). The D_q is the dose where the

extrapolated exponential portion of the survival curve crosses at 100% survival, while the extrapolation number is the y-intercept of the exponential portion of the survival curve. The shape of the survival curve for cells exposed to alpha-particles shows a linear decrease in surviving fraction with increasing dose (Thacker et al., 1982; and Barnhart and Cox, 1979). The lack of a shoulder is due to the effectiveness of cell killing by densely ionizing alpha-particles and other forms of high-LET radiation. The D_0 or slope is the dose required to reduce the surviving fraction to 37% on the exponential portion of the survival curve. Therefore, the toxicity of ionizing radiations can be analyzed by the value of D_0 ; the lower the D_0 the more toxic the ionizing radiation.

Repair of Double Strand Breaks

Since double strand breaks have been implicated as being the lesion responsible for cell lethality (Jenner et al., 1993), the ability of cells to repair double strand breaks enhances survival. Repair of double strand breaks can occur through two recombinational pathways (Price, 1993). The ends of a double strand break can be digested by 5'-3' exonuclease to allow for annealing and ligation of exposed complimentary sequences, which allows for a fast repair of the damage but may also cause deletions. The second pathway utilizes homologous pairing between the damage sequence on one chromosome and the identical sequence from the second copy, in order to allow for strand exchange followed by replication of the damage area. This type of double strand break repair process would result in the conservation of the damaged area. Since only one copy of HPRT exists in hemizygous males, this process of repair by homologous pairing is unlikely. The ability to rejoin double-strand breaks has been shown to be less effective for high LET than low LET (Jenner et al., 1993; and Fox and McNally, 1988).

For reasons which are not entirely clear, surviving progeny of cells irradiated with X rays show a persistently reduced colony forming efficiency compared to control levels

for many cell generations postirradiation. This phenomenon has been termed delayed reproductive death (Chang and Little, 1991). Recent studies by Chang and Little (1992) have shown that double strand breaks and the repair of these breaks might be associated with delayed reproductive death. Cells exposed to either X rays or alpha-particles could exhibit delayed reproductive death. Variations in expression of this phenomenon could result from differences in the induction and repair of double strand breaks of cells exposed to either X rays or alpha-particles.

Induction of Mutations

Mutations induced by different types of ionizing radiations can be analyzed by evaluating the induction of mutants as a function of cell survival. For example, the mutation frequency at the HPRT locus of V79 chinese hamster cells exposed to alpha-particles was higher than for V79 cells exposed to X rays at equitoxic doses (Thacker et al., 1982). Although the mutation frequencies were different at equitoxic doses, there was little difference in the mutation spectrum between X rays and alpha-particles at the HPRT locus with over 50% of the mutations being deletions (Aghamohammadi et al., 1992). Schwartz et al. (1994) showed a similar percentage of deletions at the HPRT locus for cells exposed to X rays or alpha-particles, but a larger proportion of intragenic deletions was observed following alpha-particle exposure.

Many genetically altered cell lines have been developed and utilized for mutational analysis. Human-hamster hybrid A_L cells (developed by Puck et al., 1971), which contain a single copy of human chromosome 11 with the S1 human antigenic marker along with a standard set of hamster chromosomes, have been utilized for comparisons of human S1 and hamster HPRT mutants induced by different ionizing radiations (Hei et al., 1994). Another genetically altered cell line used for mutational analysis is AS52, which is a HPRT deficient Chinese hamster ovary cell line transfected with the bacterial xanthine-guanine phosphoribosyl transferase (*gpt*) gene using pSV2*gpt*. Experiments were performed using various mutagens in order to compare mutation induction at the

HPRT locus of wild type Chinese hamster ovary cells with the *gpt* locus of AS52 (Stankowski and Hsie, 1986). In an analogous system, Klein and Rossman (1990) have integrated the bacterial *gpt* gene into UV-induced HPRT-deficient Chinese hamster V79 cells using pSV2*gpt* in order to study the effects of chromosomal location on the induction and repair of mutations in mammalian cells. The HPRT-deficient Chinese hamster V79 cell line used for transfection with pSV2*gpt* was shown to be consistently non-revertible at the HPRT locus with mutagens (Klein and Rossman, 1990). The 0.45kb *gpt* gene, which converts xanthine into XMP (a precursor for GMP) is functionally analogous to the endogenous HPRT gene and the *gpt* gene can be selected for or against in the same manner as the HPRT gene. One particular transformed cell line (G12) was shown to exhibit a relatively low spontaneous mutation frequency (3×10^{-5}). This spontaneous mutation frequency was not associated with direct loss of the plasmid (Klein and Rossman, 1990). G12 cells were shown to contain a single copy of the *gpt* gene, which has been mapped to chromosome 1 (Klein and Snow, 1993).

Once a favorable transgenic cell line was isolated, Klein and Rossman (1990) then analyzed the mutation frequency of V79 and G12 cells exposed to various mutagens. Similar mutation frequencies for the *gpt* and HPRT loci were observed following treatments with alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine and β -propiolactone. In contrast, marked differences in mutation frequencies between cell lines were observed after treatment with UV and X rays. The mutation frequency at the *gpt* locus of G12 was significantly higher (2.5-4.5 times) than the HPRT locus when exposed to X rays. Similar results after X ray exposure were obtained by Stankowski and Hsie (1986) using the AS52 cell line. The mutation frequency at the S1 locus of A_L cells was 16-fold higher than the HPRT locus when exposed to gamma-rays (Hei et al., 1994). Differences in mutation frequency between V79 and G12 cells exposed to X ray were attributed to the possibility that the *gpt* gene was affected by its chromosomal location.

Purpose

Because of the marked difference in mutation frequencies induced in G12 and V79 following exposures to X rays, it was of interest to analyze differences in responses between these two cell lines when exposed to alpha-particles. The purposes of this study were: 1) to determine if the same sensitivity of the induction of mutants occurs for G12 cells exposed to alpha-particles as was shown with X rays; 2) to compare the induced mutation frequencies between V79 and G12 cells exposed to X rays and alpha-particles; and 3) to analyze the effects of nuclear shape on the induction of mutations by alpha-particles.

CHAPTER II

MATERIALS AND METHODS

Cell Lines

Chinese hamster V79 cells and a transgenic cell line (G12), which was derived from V79, were kindly provided by Catherine Klein and Toby Rossman (New York University, NY). Both cell lines were cultured in HAM's F-12 (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) and 50 µg/ml gentamicin (Gibco) at 37°C and 5% CO₂. Stock cultures of V79 and G12 cells were cultured in medium supplemented with HAT (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine, Sigma, St. Louis, MO) until 20 hours before irradiation to reduce the accumulation of spontaneous mutants. Both cell lines were used within a span of five passages. Stocks of G12 and V79 were kept in medium containing 5% DMSO and stored in liquid nitrogen.

Alpha-Particle Exposure

In order to expose suspended V79 and G12 cells to alpha-particles, two semi-confluent cultures of either V79 or G12 were harvested by rinsing with Ca²⁺ and Mg²⁺ free phosphate buffered saline (0.01 M sodium phosphate plus 0.15 M NaCl) and adding 700 µl of 1% trypsin-EDTA (Gibco). Cultured cells were incubated 7 minutes at 37°C in order to release attached cells. Cells were collected using 12 ml of medium and carefully pipetted 30 times at room temperature in order to break apart possible clumps of cells. Cell numbers were determined using a hemocytometer. Nonviable cells

were excluded from counts based upon uptake of erythrosin B (Sigma). Cell suspensions were centrifuged for 6 minutes at 200 xg, and resuspended in 300-500 μ l of medium at a concentration of $8-11 \times 10^5$ cells per 15 μ l. Suspended cells (15 μ l) were placed in the center of a prewetted 6 μ m mylar bottom 60 mm dish. Cells were allowed to settle for 10 minutes in order to form a monolayer. The mylar dish was placed into a 100 mm 3 μ m nucleopore dish (Costar, Cambridge, MA). The nucleopore filter dish containing the mylar bottom 60mm dish was placed on top of an americium-241 source (Isotope Products Laboratories, Burbank, CA). The nucleopore filter eliminated alpha-particles which were not emitted at right angles to the planar source. Exposure times varied from 0-6 minutes depending on the desired dose. The number of dishes used for each exposure varied depending upon the expected cell survival. After exposures, the cells were trypsinized for 2 minutes at room temperature and collected in 2-3 ml of medium. Trypsin was necessary since cells attached during the procedure. The resulting suspension was centrifuged for 6 minutes at 200 xg, resuspended in an appropriate volume of medium, and the number of cells/ml was determined.

In order to expose attached cells to alpha-particles, cells from two semi-confluent 100 mm dishes were harvested and then plated into 60 mm dishes with 6 μ m mylar bottoms at a density of $1.5-2 \times 10^6$. Cells were incubated at 37 °C for 20 hours prior to exposure. The attached cells were then exposed for identical time points as noted for suspended cells. Cells were harvested, centrifuged, resuspended into appropriate volumes, and counted.

X Ray Exposure

Cells from two semi-confluent dishes were harvested, centrifuged for 6 minutes at 200 xg, resuspended in 10 ml of medium, and counted. The resulting cell suspension ($9-10 \times 10^5$ cells/ml) was placed into separate 17x100 mm plastic tubes (volume of cell suspension depended on expected cell survival after exposure) and irradiated 0-9 minutes with a Gemini Industrial X ray unit operated at 300 keV and 12 mA with 2 mm

of added aluminium filtration resulting in an average radiation of 1.5 Gy/minute. Dose rates were measured using a Victoreen ionization chamber. Total doses for exposures varied from 0-7.5 Gy.

Assay for Survival

Surviving fractions were determined at three time points after X ray and alpha-particle exposure. Cells were plated at clonogenic densities immediately, 3 days, and 6 days after exposure. Cells were seeded in duplicate at 75, 150, and 225 cells/well into 6-well tissue culture plates (Costar). Variations in the number of cells plated were dependent upon expected cell survival. Thus, cells were plated at 150, 300, and 450 cells/well if the expected survival was 50%. The 6-well plates were fixed in methanol and stained with Giemsa (Fisher Scientific, Pittsburgh, PA) on day 7. The number of colonies with 25 or more cells were counted for each well. Average colony forming efficiencies with standard deviations were determined from 6 wells per exposure group by dividing the number of colonies by the number of cells plated. Surviving fractions and corresponding standard deviations were then calculated for each exposure group by dividing the colony forming efficiency of a particular exposure group by the control (unirradiated) colony forming efficiency.

Assay for Mutations

Methods similar to those previously described by Zhu and Hill (1994) were used to evaluate the frequency of X ray and alpha-particle induced 6-thioguanine resistant mutants. Following irradiation, cells (6×10^5 /100 mm dish) were cultured for 6 days to allow for expression of the mutant phenotype, which is referred to as the expression period. The number of cells seeded depended upon the expected surviving fraction. For example, if the expected surviving fraction for an irradiated sample was 0.5 for an experiment with an unirradiated sample plated at 1×10^6 cells/100 mm

dish, then the irradiated sample was plated at 2×10^6 cells/100 mm dish. Cells were harvested after 3 days of expression and replated into a second series of 100 mm dishes at similar cell densities. In this manner cells were maintained in exponential growth throughout the expression period. Following the 6 day expression period, cells were harvested and replated into ten 100 mm dishes at a density of 2×10^5 cells/dish. Cells were plated into medium containing 10 $\mu\text{g/ml}$ of 6-thioguanine (Sigma), which is referred to as the selection medium. Fresh stocks of 6-thioguanine (1 mg/ml) were prepared by dissolving 6 mg of 6-thioguanine in 6 ml of medium followed by addition of 25 μl of 10N NaOH in order to fully dissolve the 6-thioguanine. For sterilization, this solution was passed through a 0.2 micron filter. A final concentration of 10 $\mu\text{g/ml}$ 6-thioguanine was earlier shown by Klein and Rossman (1990) to be appropriate for selection of the mutant phenotype. The selection medium was changed on day 7, and the dishes were stained with Giemsa and scored for large and small mutant colonies on day 10. A 10 day selection period was chosen in order to reduce the problem of reseeding that was observed after 13 days of selection (unpublished observation). Mutation frequencies were calculated by dividing the number of HPRT or *gpt* mutants (6-thioguanine resistant colonies) by the number of colony forming cells plated. Standard deviations were calculated for each mutation frequency. Since variations in spontaneous mutation frequencies were observed between G12 and V79, the spontaneous mutation frequencies for G12 and V79 for each experiment were subtracted from the induced mutation frequencies. The average spontaneous mutation frequency for G12 was 2.26×10^{-5} , while V79 cells exhibited a lower average spontaneous mutation frequency of 8.78×10^{-6} . The spontaneous mutation frequencies were significantly higher for cells not initially plated in HAT-supplemented medium before exposures.

Dosimetry

a. CR-39 Detection of Alpha-particles

The dose from exposures to alpha-particles can be expressed in terms of fluence

(alpha-particle/ $\mu\text{m}^2/\text{min}$). Methods previously described by Ford and Terzaghi-Howe (1993) were used for the detection of alpha-particles with CR-39 (thermoset plastic). A sheet of CR-39 (Homalite, Wilmington, DE) was cut with a scalpel into squares 30 mm x 30 mm and placed into a 6 μm mylar bottom 60 mm dish. The mylar dish with CR-39 was then exposed to alpha particles in the same manner as the cells but for different time periods. The exposed pieces of CR-39 were incubated in 6.5N NaOH for two hours at 70 $^{\circ}\text{C}$ and then transferred into fresh 6.5N NaOH and incubated for another two hours at 70 $^{\circ}\text{C}$. The CR-39 was then rinsed with water and allowed to air dry. Alpha-particles depolymerize areas of the CR-39 upon contact. Depolymerized areas can be dissolved with 6.5N NaOH at 70 $^{\circ}\text{C}$ leaving behind small etched circles on the exposed surface of the CR-39. Samples were viewed using a Zeiss microscope and photographed using a Polaroid Microcam SLR instant camera. Circles representing single alpha-particle events on CR-39 were counted for a defined area in order to calculate the fluence.

b. Nuclear Measurements

Another way of expressing dose is in terms of the number of alpha-particles traversing a cell's nucleus. The number of alpha-particles/nucleus for a specific exposure time was calculated by multiplying the nuclear cross-section of the target cell in μm^2 by the fluence (alpha-particle/nucleus = alpha-particle/ $\mu\text{m}^2/\text{min}$ x time x nuclear cross-section).

In order to measure nuclear size of cells in suspension, V79 or G12 cells were harvested in 5 ml of medium and centrifuged for 6 minutes at 200 xg. Cells were resuspended in 5 ml of phosphate buffered saline with Ca^{2+} and Mg^{2+} , centrifuged, and resuspended in 1ml of phosphate buffered saline containing 14 $\mu\text{g}/\text{ml}$ hydroethidine (Polysciences, Warrington, PA) for 15 minutes at room temperature in the dark. After 15 minutes, cells were resuspended in 5 ml of phosphate buffer saline, centrifuged, and resuspended. A small volume (15 μl) of resuspended cells was added to a 60 mm dish and cells were photographed by a Polaroid Microcam SLR instant camera attached to a

Zeiss fluorescent microscope equipped with a rhodamine filter. Nuclear size measurements were made on the resulting photographs using a hand reticle.

To determine the nuclear dimensions of attached cells, V79 or G12 cells grown in 60 mm dishes were washed three times with phosphate buffered saline followed by the addition of 2 ml of phosphate buffered saline containing 14 $\mu\text{g/ml}$ hydroethidine, and incubated for 15 minutes at room temperature in the dark. The labelled cell monolayers were washed three times with phosphate buffer saline, photographed, and measured as described above.

CHAPTER III

RESULTS

Growth Characteristics

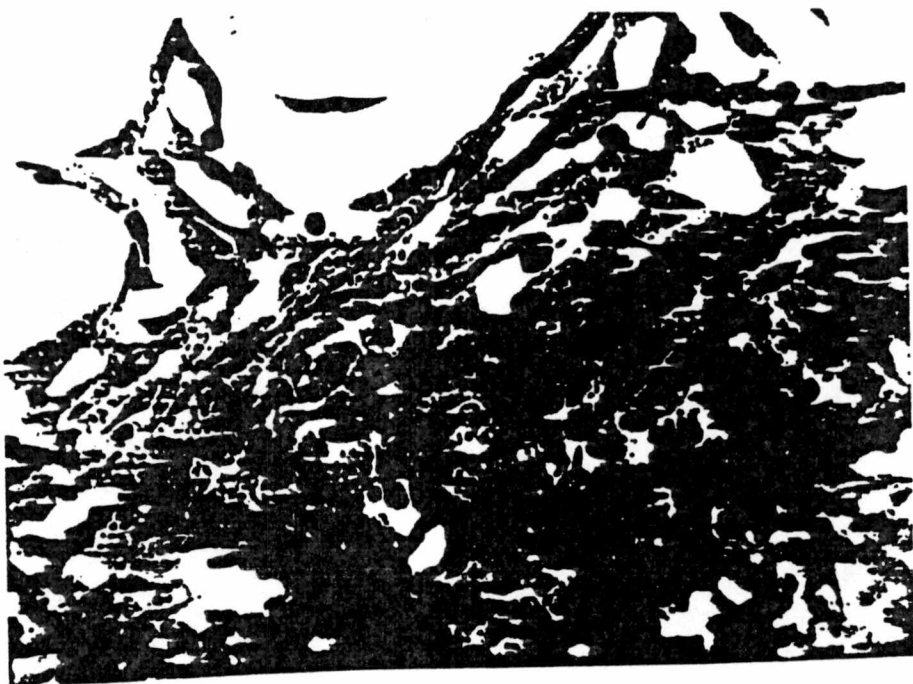
The growth characteristics of V79 and G12 cells were examined. These cell lines exhibited a similar average colony forming efficiency of 69% (51-92%) for G12 cells and an average colony forming efficiency of 62% (44-73%) for V79 cells. There were some morphologic differences between G12 and V79 cells. Colonies of G12 were consistently smaller and higher in cell density than those of V79 cells. Individual V79 cells within colonies were mainly elongated in shape as shown in Figure 3.1 A, while most G12 cells were spherical in shape (Figure 3.1 B).

Survival

The cytotoxic response of V79 and G12 cells exposed to X rays was analyzed by generating dose-response curves. Surviving fractions of V79 and G12 cells plated immediately after X ray exposure were plotted as a function of dose (0-7.5 Gy) as shown in Figure 3.2. Since there were no significant differences in X ray dose response between V79 and G12 cells, a computer best-fitted curve ($R=0.97872$) was drawn for all data points (Figure 3.2). A computer derived best-fitted curve ($R=0.82323$) was then utilized to analyze the exponential portion of the survival curve. The D_0 , which is the dose required to reduce the surviving fraction to 37% along the exponential portion of the survival curve, was found to be 2.9 Gy. The width of the shoulder was measured by the extrapolation number, n , and by the quasi-threshold dose, D_q . The D_q is the dose where the exponential part of the survival curve crosses 100% survival which was at 1.75 Gy. The extrapolation number is the point at which the exponential part of the

Figure 3.1. A phase contrast picture of Giemsa stained colonies of (A) V79 Chinese hamster cells and (B) G12 cells, which is a HPRT deficient V79 cell line transformed with *pSV2gpt*.(204X)

(A)



(B)

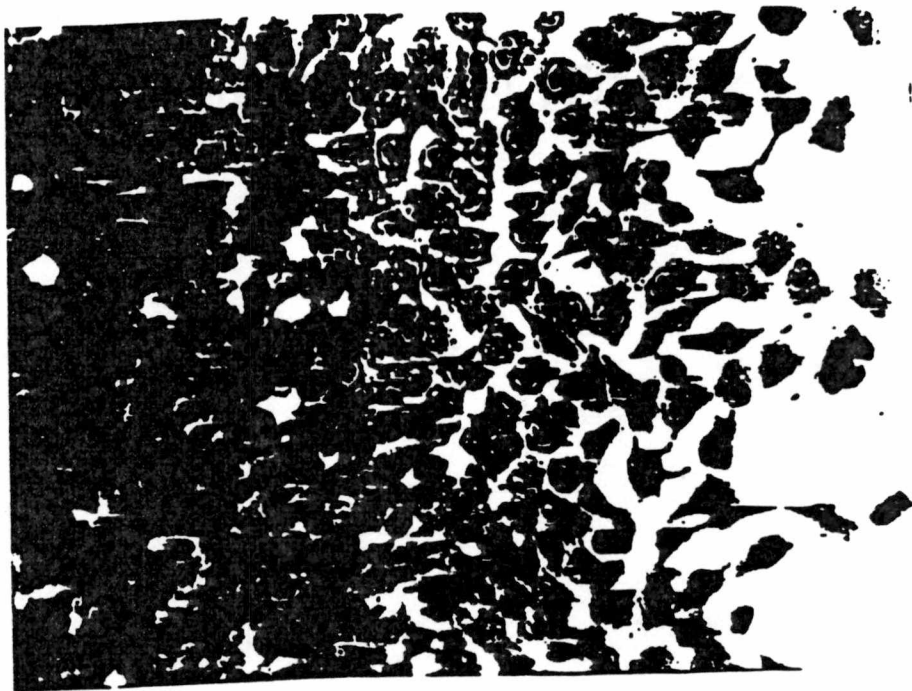
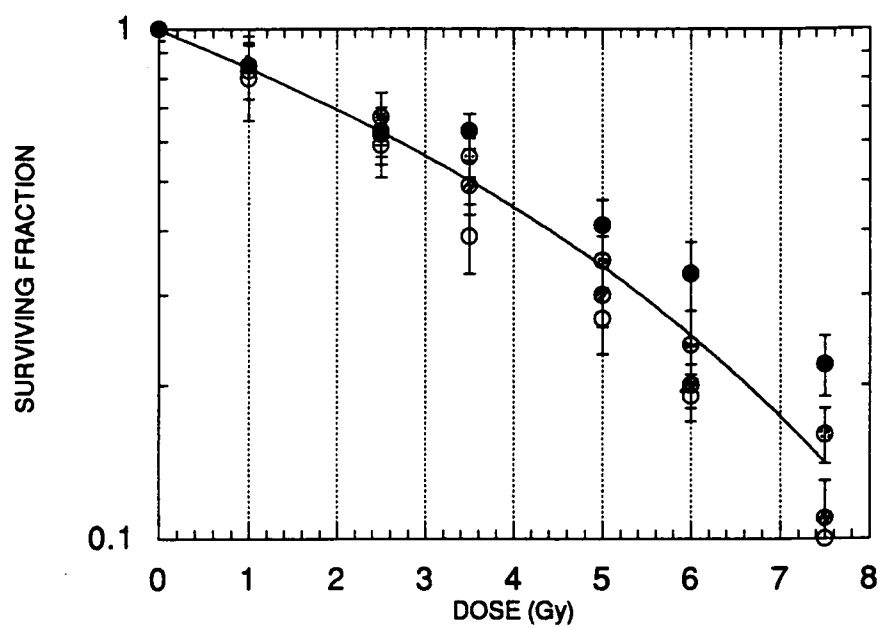


Figure 3.2. Comparison of survival of V79 and G12 hamster cells after irradiation with 300 keV X rays. The results from 4 independent experiments are reported (V79#1 open circles, V79#2 closed circles, G12#1 dashed circles, and G12#2 dotted circles). Each point represents the average surviving fraction from six colony forming efficiencies relative to the control colony forming efficiency for graded doses of X rays. Bars represent the standard deviation.



survival curve intersects with the y-axis (surviving fraction) which was found to be 1.8.

Survival curves were generated for alpha-particle irradiation of V79 and G12 cells. Surviving fractions were calculated and plotted as a function of exposure time (0-6 minutes). There was a log-linear decrease in surviving fraction with increasing exposure time for attached V79 and G12 cells exposed to alpha-particles as shown in Figure 3.3. No significant differences in survival between G12 and V79 cells were observed. The survival curves of suspended V79 and G12 cells exposed to alpha-particles exhibit an exponential decrease in surviving fraction with increasing exposure time as shown in Figure 3.4. Similarly, there were no marked differences in surviving fraction between suspended V79 and G12 cells.

Although the survival curves of suspended and attached cells both exhibit an exponential decrease in surviving fraction with no shoulder, a difference was noted in the slope of the survival curves. The slope of the survival curve for suspended cells exposed to alpha-particles (Figure 3.4) was 2-fold greater than the slope of the survival curve for attached cells (Figure 3.3). The toxicity of alpha-particles for suspended and attached cells was further analyzed by calculating the number of alpha-particles/nucleus required to reduce survival to 37%. The average (and one standard deviation) fluence of the ^{241}Am source was 0.0133 (0.0016) alpha-particles/ μm^2 /minute exposure (Table 3.1). The fluence was determined by counting the holes of 11 random 0.1mm^2 sections from a photograph (kindly provided by Gin Wen Chang University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences) of CR-39 exposed for 15 seconds to alpha-particles. The average (and one standard deviation) nuclear cross-section (μm^2) of suspended V79 and G12 cells was found to be 66.5 (21.2 μm^2) and 64.4 (21.4 μm^2) respectively, while the nuclear cross-section of attached V79 cells was 106.7 (19.5 μm^2) (Table 3.2). Nuclear cross-sections for attached G12 cells were not obtained because of difficulties with staining and photographing. It required 3.1 and 6 minutes of exposure to the ^{241}Am source to reduce survival to 37% for attached and suspended cells, respectively (Figure 3.3 and 3.4). The average fluence of the ^{241}Am source was multiplied by the average nuclear cross-section and exposure time in order to estimate the average number of alpha-

Figure 3.3. Comparison of survival of attached V79 and G12 cells after irradiation with alpha-particles from americium-241. The results from two independent experiments are reported. Each point represents the average surviving fraction with standard deviation bars. Surviving fractions were plotted as a function of exposure time (0-6 minutes).

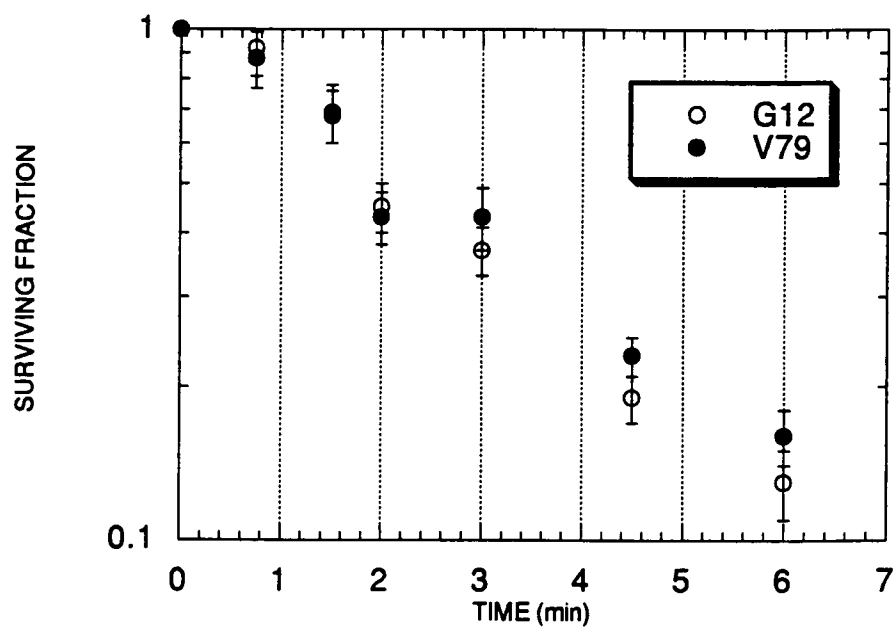


Figure 3.4. Comparison of survival of suspended V79 and G12 cells after irradiation with alpha-particles from americium-241. The results from two independent experiments are reported. Each point represents the average surviving fraction plotted as a function of exposure time (0-6 minutes). Bars represent standard deviations.

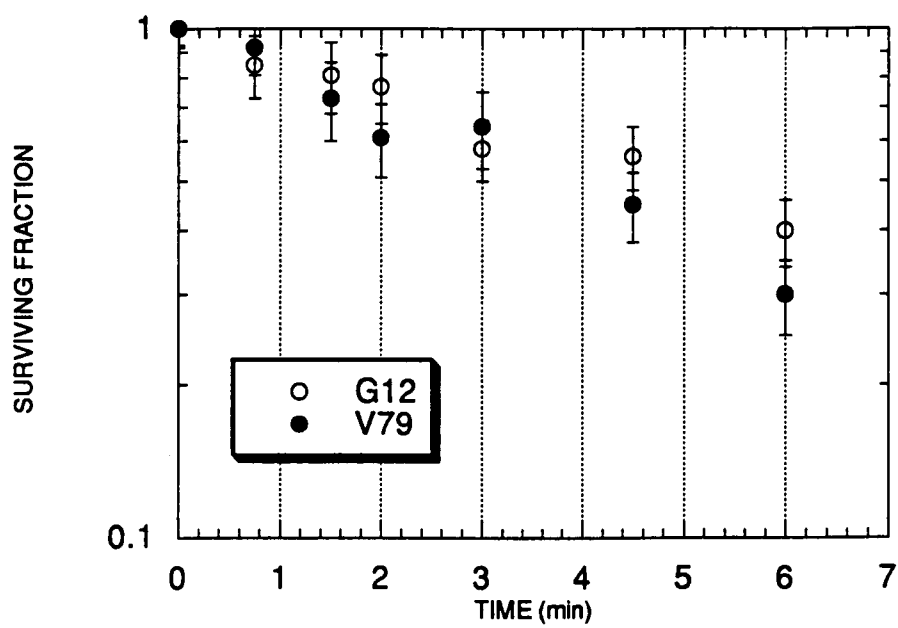


TABLE 3.1. Fluence Measurements from Alpha-Particle Exposed CR-39

SAMPLE NUMBER	FLUENCE ^a MEASUREMENTS (ALPHA-PARTICLE/ μm^2 /MIN EXPOSURE)
1	0.0136
2	0.0136
3	0.0148
4	0.0108
5	0.0144
6	0.0144
7	0.0136
8	0.0120
9	0.0104
10	0.0140
11	0.0148
AVERAGE (STANDARD DEVIATION)	0.0133 (0.0016)

a The average (standard deviation) fluence of the americium-241 source was determined by dividing the number of holes per 0.1 mm^2 sections of exposed CR-39 by the specific exposure time (15 seconds). Fluence = number of holes/ $1 \times 10^4 \mu\text{m}^2$ /15 seconds.

TABLE 3.2. Nuclear Cross-Section of Hydroethidine Labelled Cells

SAMPLE NUMBER	NUCLEAR CROSS- SECTION ^a (um ²) OF SUSPENDED V79 CELLS	NUCLEAR CROSS- SECTION (um ²) OF SUSPENDED G12 CELLS	NUCLEAR CROSS- SECTION (um ²) OF ATTACHED V79 CELLS
1	94.6	65.7	100.8
2	65.7	42.0	130.5
3	42.0	84.4	122.7
4	74.8	65.7	84.2
5	84.4	141.0	97.3
6	49.4	65.7	97.3
7	49.4	42.0	87.4
8	117.0	57.2	77.9
9	57.2	49.4	115.2
10	74.8	42.0	87.4
11	57.2	74.8	111.5
12	65.7	57.2	111.5
13	42.0	57.2	84.2
14	57.2	57.2	126.6
15		74.8	122.7
16		74.8	90.6
17		65.7	138.5
18		57.2	134.5
19		57.2	
20		57.2	
AVERAGE (STANDARD DEVIATION)	66.5 (21.2)	64.4 (21.4)	106.7 (19.5)

^a Measurements using a hand reticle of hydroethidine labelled cells from photograph. (164X)

particles/nucleus. Thus, an average of 4.4 (1) alpha-particles/nucleus were required to reduce survival to 37% for attached V79 cells, while an average of 5.1 (1.8) and 5.3 (1.8) alpha-particles/nucleus were required for suspended G12 and V79 cells, respectively (Table 3.3).

The phenomena of delayed reproductive death was analyzed by evaluating changes in surviving fraction 0-6 days after exposure to X rays. Since there were no significant differences between the surviving fractions of V79 and G12 cells, the data were pooled together for each plating period. There were no significant differences in surviving fraction for cells plated immediately or 3 days after X ray exposure, while a significant increase in surviving fraction was observed for cells plated after 6 days (Figure 3.5). The surviving fraction for cells plated immediately, 3 and 6 days after exposure to 7.5 Gy of X ray was 0.15, 0.14, and 0.49, respectively. There was a recovery in colony forming efficiency to control levels after 6 days from exposure for cells that received an initial dose of 2.5 Gy. Immediately following exposure to 2.5 Gy a surviving fraction of 0.65 was observed.

Delayed reproductive death was observed for attached and suspended V79 and G12 cells following alpha-particle exposure. There was an increase in surviving fraction from initial survival data for attached and suspended cells plated 3 and 6 days after exposure (Figures 3.6 and 3.7). The surviving fraction for attached cells plated immediately, 3, and 6 days after 6 minutes of alpha-exposure was 0.15, 0.19, and 0.6, respectively (Figure 3.6). There was a recovery in colony forming efficiency to control levels after 6 days from exposure for attached cells that initially received 2 minutes of exposure. Immediately following two minutes of alpha-exposure a surviving fraction of 0.5 was observed. The surviving fraction for suspended cells plated immediately, 3, and 6 days after 6 minutes of alpha-exposure was 0.35, 0.72, and 0.91, respectively (Figure 3.7). Nearly a full recovery in colony forming efficiency to control levels was attained at all exposure times for suspended cells plated 6 days after exposure. Overall, the slope of the survival curve was shown to decrease with increasing time after exposure to alpha-particles for attached and suspended cells indicating cell recovery.

TABLE 3.3. Determining the Average Number of Alpha-Particles/Nucleus Required to Reduce Survival to 37%.

CELL SHAPE	TIME (MINUTES) AT D ₀	FLUENCE ^a (ALPHA- PARTICLES/μm ²) AT D ₀	AVERAGE ALPHA- PARTICLE TRAVERSAL/ NUCLEUS ^b AT D ₀
ATTACHED V79 CELLS	3.1	0.041 (0.005)	4.4 (1)
SUSPENDED G12 CELLS	6	0.08 (0.009)	5.1 (1.8)
SUSPENDED V79 CELLS	6	0.08 (0.009)	5.3 (1.8)

a Alpha-particle/μm² were calculated by multiplying the various measured fluences of the americium source (Table 3.1) by the exposure time. An average (standard deviation) is reported.

b The average alpha-particle/nucleus was calculated by multiplying the average fluence of the americium source (Table 3.1) by the exposure time and by the average nuclear cross-section. An average (standard deviation) is reported.

Figure 3.5. Analysis of survival of V79 and G12 cells plated immediately, 3 and 6 days after irradiation (G12&V79, G12&V79-3, and G12&V79-6) with 300 keV X rays. Results from one experiment are reported. Each point represents the average surviving fraction of V79 and G12 cells at specific doses (0-7.5 Gy). Bars represent one standard deviation.

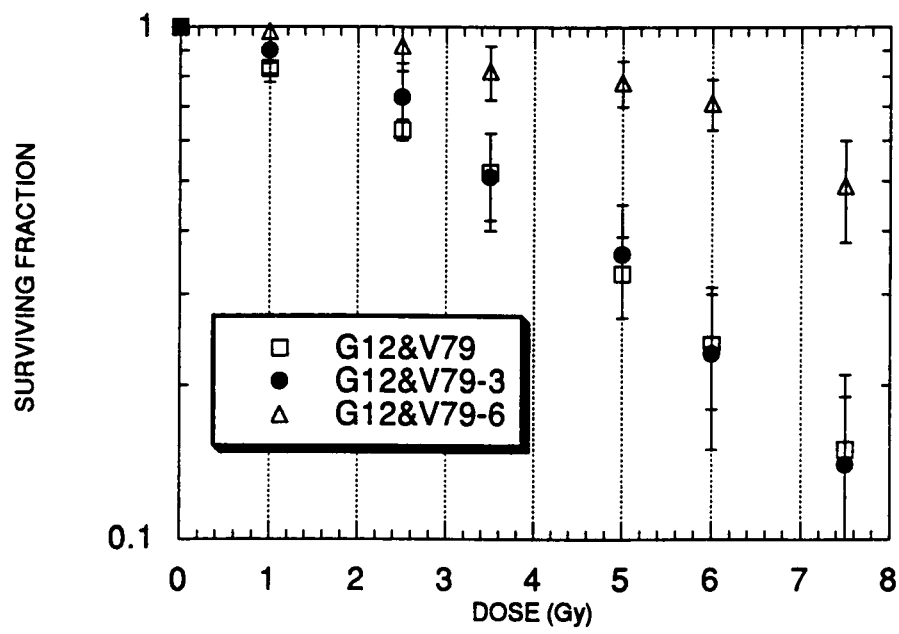


Figure 3.6. Analysis of survival of attached V79 and G12 cells plated immediately, 3 and 6 days after irradiation to alpha-particles from americium-241. Results from one experiment are reported. Each point represents the average surviving fraction of G12 and V79 cells at specific exposure times (0-6 minutes). Bars represent one standard deviation.

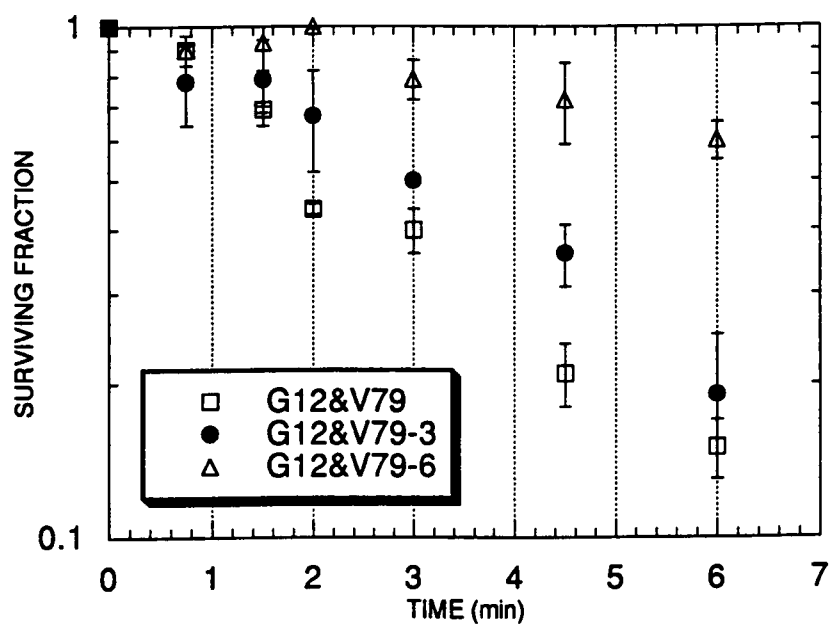
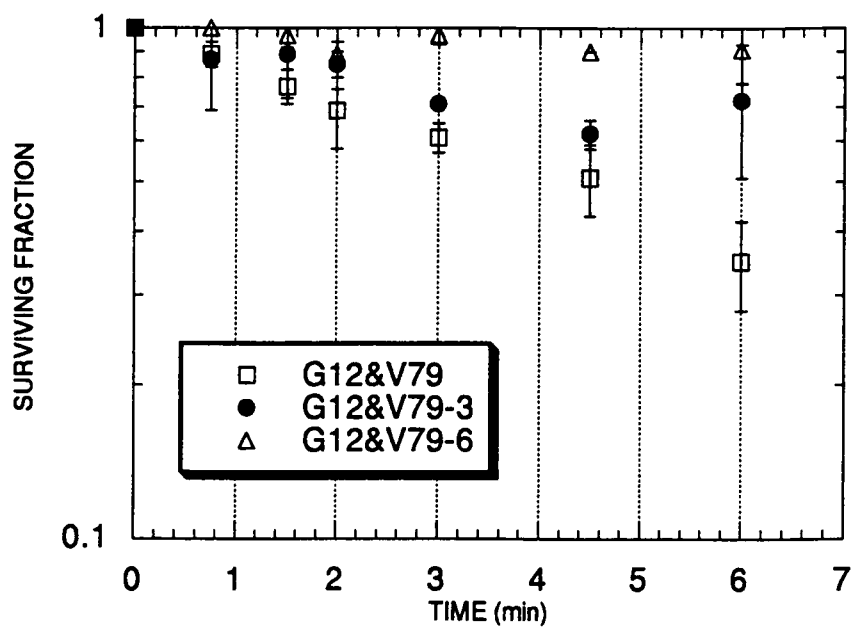


Figure 3.7. Analysis of survival of suspended V79 and G12 cells plated immediately, 3 and 6 days after irradiation to alpha-particles from americium-241. Results from one experiment were reported. Each point represents the average surviving fraction of V79 and G12 cells as a function of exposure time. Bars represent standard deviations.



Induction of Mutations

The induction of *gpt* and HPRT mutants from X ray-exposed G12 and V79 cells was analyzed. The frequencies of 6-thioguanine resistant V79 and G12 mutant cells were plotted as a function of dose as shown in Figure 3.8. There was an increase in the mutation frequency with increasing dose of X ray for both cell lines, although the increase in mutation frequency was more pronounced for G12 cells (Figure 3.8). The most pronounced difference in mutation frequency was observed at 7.5 Gy with an approximate 5.5-fold higher frequency in *gpt* induced mutants compared to HPRT mutants. Thus, there was a significant difference in the induced mutation frequency between V79 and G12 cells exposed to X rays.

The mutation frequencies of attached V79 and G12 cells exposed to alpha-particles were determined. The induction of mutations for attached V79 and G12 cells exposed to alpha-particles was plotted as a function of exposure time as shown in Figure 3.9. There was an increase in mutation frequency with increasing exposure time for both attached V79 and G12 cells. There were no significant differences between the frequencies of induced *gpt* and HPRT mutants (Figure 3.9). The induced *gpt* mutation frequency observed following 6 minutes of exposure was 1.61×10^{-4} mutants/viable cell, while the frequency of HPRT mutants was 1.25×10^{-4} . At 4.5 minutes of exposure, there was a low mutation frequency for V79 cells that did not coincide with the trend of the other mutation frequencies. In contrast to the difference in induction of *gpt* and HPRT mutants between G12 and V79 cells when exposed to X rays, there were no significant differences in mutation frequencies between attached V79 and G12 cells exposed to alpha-particles.

Comparisons were made between the induction of *gpt* and HPRT mutants from alpha-exposed suspended G12 and V79 cells (Figure 3.10). There was a gradual increase in the induction of *gpt* mutants with increasing exposure time (Figure 3.10). The induced mutation frequencies of suspended V79 cells fluctuated with increasing exposure time and could not be curve-fitted for analysis (Figure 3.10). The induction of *gpt* and HPRT mutants of suspended G12 and V79 cells was not significantly different. The induced mutation frequency for suspended G12 cells at 4.5 and 6 minutes

Figure 3.8. Comparisons of the induction of mutants at the HPRT and *gpt* locus in V79 and G12 cells by 300 keV X ray. Cells were cultured for 6 days in nonselective medium, and replated into 10 $\mu\text{g/ml}$ of 6-thioguanine in order to select for mutants. The results from four independent experiments are reported. Each point represents the mutation frequency, the number of 6-thioguanine-resistant mutants per viable cell plated, at a specific dose (0-7.5 Gy). Bars represent standard deviations.

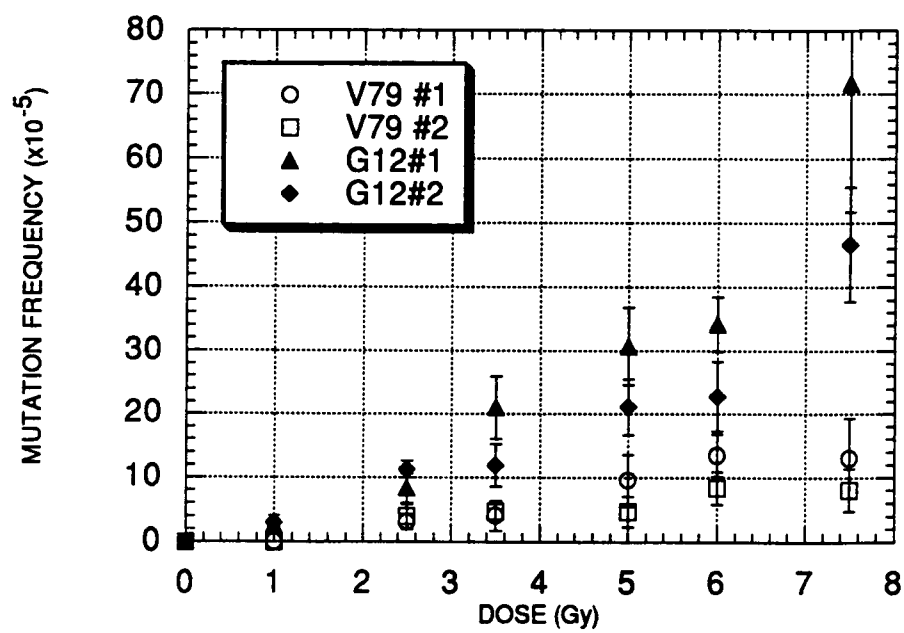


Figure 3.9. Comparisons of the induction of mutants at the HPRT and *gpt* locus in attached V79 and G12 cells irradiated by alpha-particles from americium-241. Results from two independent experiments are reported. Each point represents the mutation frequency at a specific exposure time for either V79 or G12 cells. Standard deviations are represented by bars.

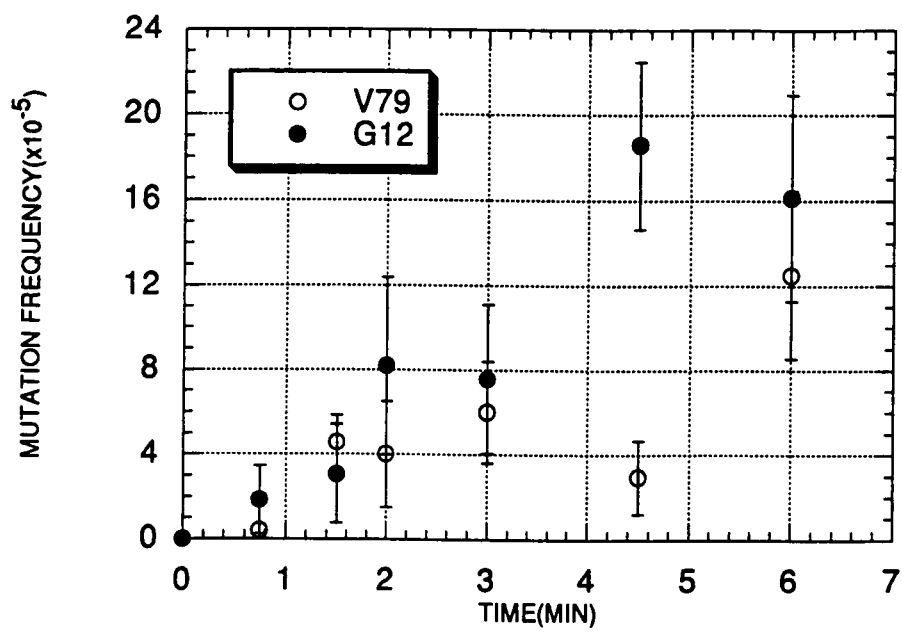
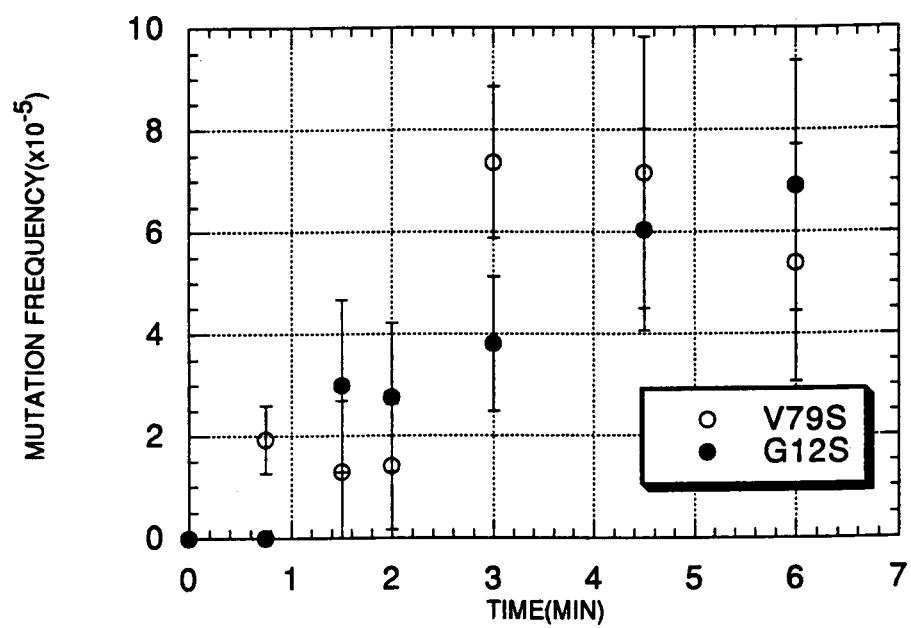


Figure 3.10. Comparisons of the induction of HPRT and *gpt* mutants in suspended V79 and G12 cells irradiated to alpha-particles from americium-241. Results of two independent experiments. Each point represents the mutation frequency of either V79 or G12 cells at specific exposure times. Bars represent standard deviations.



was 6.04×10^{-5} and 6.9×10^{-5} , while the induced mutation frequency for suspended V79 cells at 4.5 and 6 minutes was 7.16×10^{-5} and 5.39×10^{-5} . As shown with attached cells, there were no significant differences in mutation frequencies between suspended V79 and G12 cells exposed to alpha-particles.

The mutation frequencies of attached cells exposed to alpha-particles (high-LET) were then compared to the mutation frequencies of V79 and G12 cells exposed to X rays (low-LET). Since the survival was similar for V79 and G12 cells exposed to X rays and alpha-particles, comparisons of X ray and alpha-particle induced mutations at the HPRT and *gpt* locus were plotted as a function of surviving fraction as shown in Figures 3.11 and 3.12, respectively. There were no significant differences in mutation frequencies between attached V79 exposed to alpha-particles and V79 cells exposed to X rays (Figure 3.11). For example, the induced mutation frequency of V79 cells exposed to X rays at 0.19 surviving fraction was 1.35×10^{-4} , while the induced mutation frequency of attached V79 cells exposed to alpha-particles at 0.16 surviving fraction was 1.25×10^{-4} . There was an outlying mutation frequency at 0.23 surviving fraction for V79 cells exposed to alpha-particles. In contrast to HPRT mutants, there was a 2- to 3-fold increase in *gpt* mutants when G12 cells were exposed to X rays compared to alpha-particles at equitoxic doses (Figure 3.12). Thus, there were no significant differences between the induced mutation frequencies of V79 cells exposed to X rays or alpha-particles at equitoxic doses, while there was a significant increase in the induced mutation frequencies of G12 cells exposed to X rays compared to alpha-particles.

The induction of mutations was compared between suspended and attached V79 and G12 cells exposed to alpha-particles. Since there were variations in the survival data between attached and suspended V79 and G12 cells, comparisons of the induction of HPRT mutants between attached and suspended V79 cells and the induction of *gpt* mutants between suspended and attached G12 cells were plotted as a function of surviving fraction as shown in Figures 3.13 and 3.14. Although some mutation frequencies of suspended and attached V79 cells were variable at equitoxic doses, the induction of HPRT mutants for suspended and attached V79 cells did not appear to be significantly different as shown in Figure 3.13. The mutation frequency of suspended

Figure 3.11. Comparisons of the induction of HPRT mutants in V79 cells exposed to X rays (V79#1,V79#2) and attached V79 cells (V79A) exposed to alpha-particles. Results from three independent experiments are presented. Each point represents the mutation frequency at a specific surviving fraction. Bars represent standard deviations. Surviving fraction was plotted logarithmically and mutation frequency linearly.

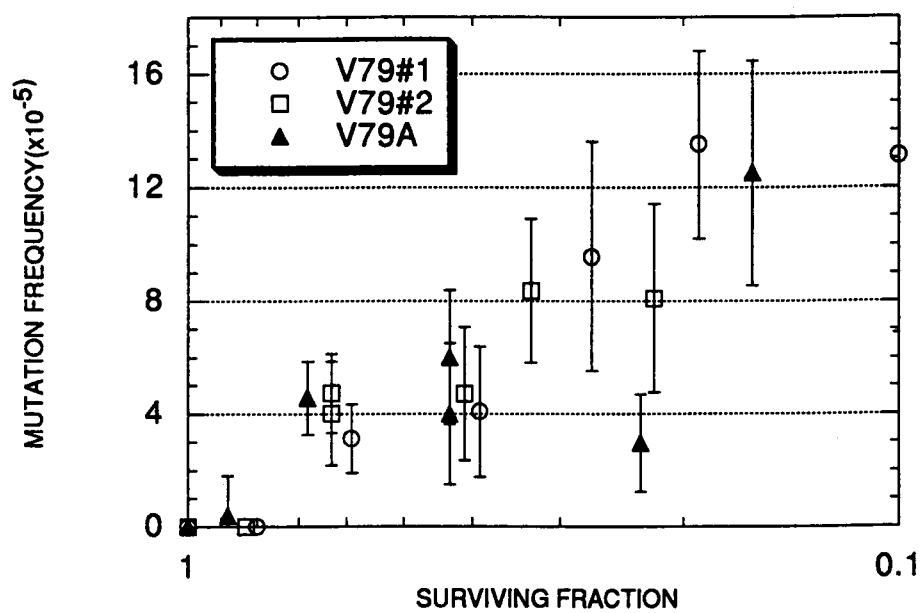


Figure 3.12. Comparisons of the induction of *gpt* mutants in G12 cells irradiated with X rays (G12#1,G12#2) and attached G12 cells (G12AA) irradiated with alpha-particles. Results from three independent experiments are reported. Each point represents the mutation frequency at a specific surviving fraction. Bars represent standard deviations.

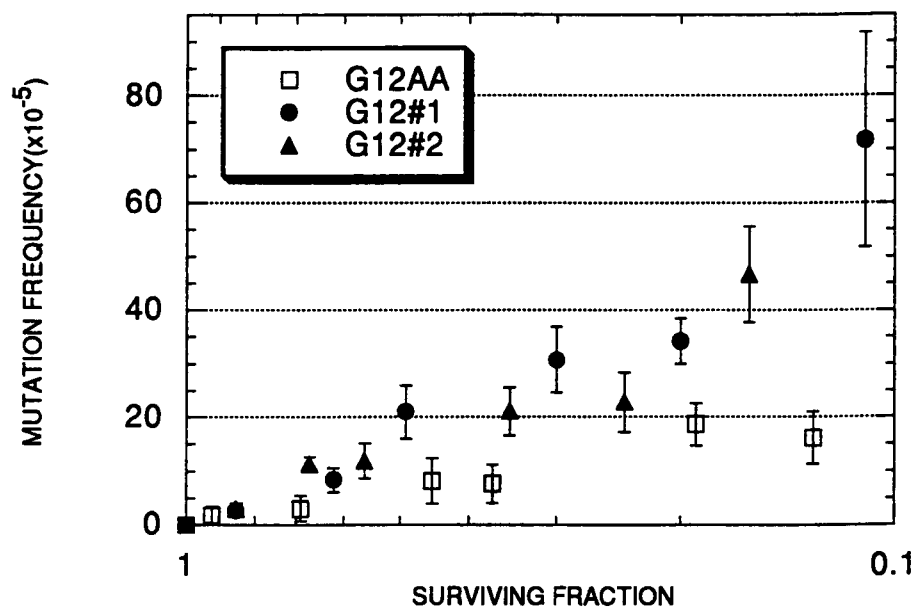


Figure 3.13. Comparisons of the induction of HPRT mutants in suspended (V79S) and attached (V79) V79 cells irradiated with alpha-particles. Results from two independent experiments are reported. Each point represents the mutation frequency at a specific surviving fraction. Bars represent standard deviations.

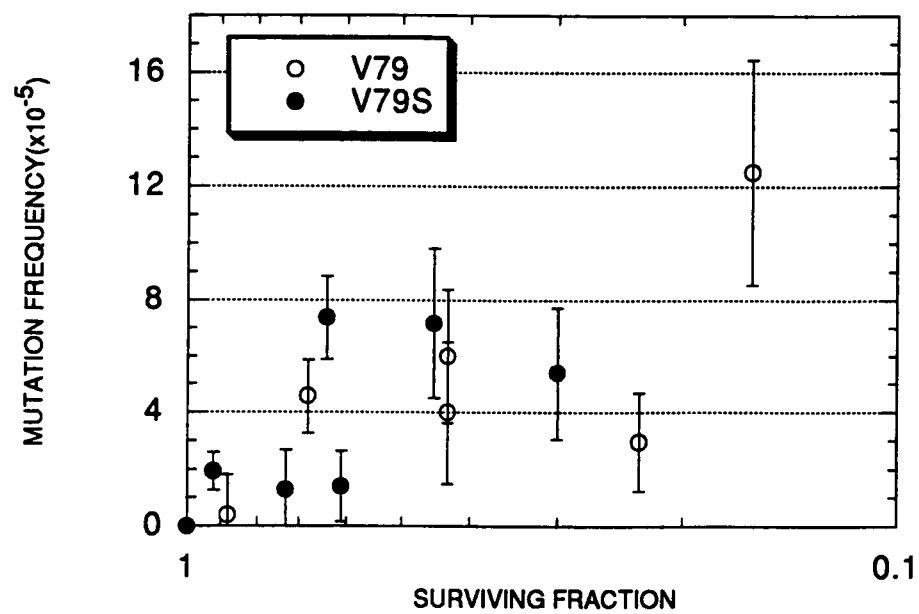
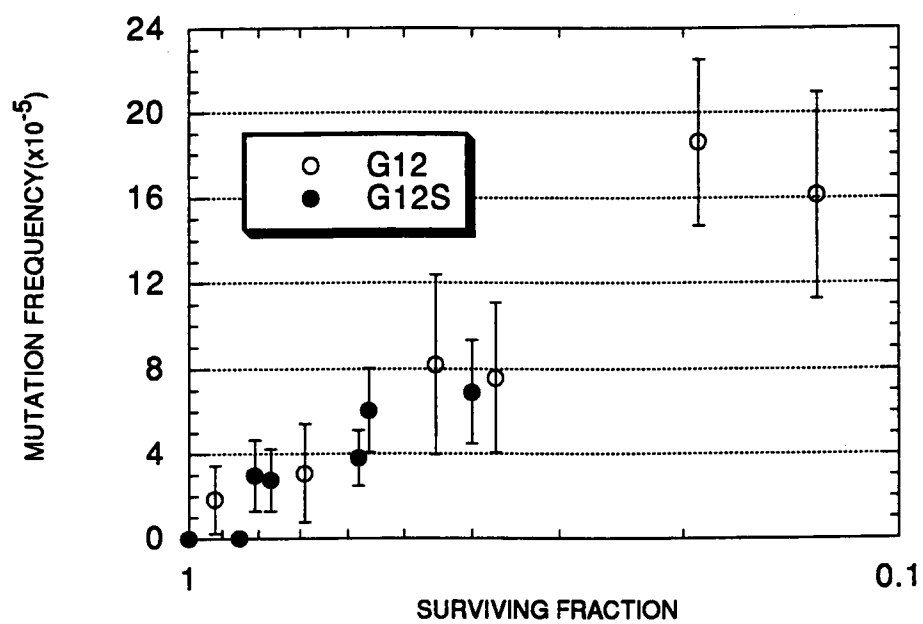


Figure 3.14. Comparison of the induction of *gpt* mutants in suspended (G12S) and attached (G12) G12 cells irradiated with alpha-particles. Results from two independent experiments are reported. Each point represents the mutation frequency at a specific surviving fraction. Bars represent standard deviation.



V79 cells at 0.45 surviving fraction was 7.16×10^{-5} , while the mutation frequency of attached V79 cells at 0.43 surviving fraction was 6×10^{-5} . There was a linear increase in mutation frequency for suspended and attached G12 cells with decreasing surviving fraction as shown in Figure 3.14. The mutation frequency of suspended G12 cells at 0.4 surviving fraction was 6.9×10^{-5} , while the mutation frequency of attached G12 cells at 0.37 surviving fraction was 7.58×10^{-5} . Thus, the induction of *gpt* mutants for suspended and attached G12 cells exposed to alpha-particles was not significantly different at equitoxic doses.

CHAPTER IV

DISCUSSION

Many investigators have utilized genetically altered cell lines to study mutagenesis. Thacker et al., (1983) and Stankowski and Hsie (1986) have utilized pSV2*gpt* transformed HPRT deficient cell lines in order to study the sensitivity of the induction of mutations by various mutagens between wild-type and transformed cells. Mutations in the *gpt* and HPRT genes allow for metabolic selection of mutant and wild-type forms through medium supplemented with either 6-thioguanine or HAT, respectively. Recently, Klein and Rossman (1990) have transformed a UV-induced HPRT-deficient Chinese hamster cell line (V79) with pSV2*gpt*. One selected transformant (G12) was shown to carry a single stably integrated pSV2*gpt* and to exhibit a low spontaneous mutation frequency of 3×10^{-5} mutants/viable cell. This spontaneous mutation frequency is slightly higher than the endogenous HPRT gene, $\sim 10^{-6}$, but significantly lower than many *gpt* transformed cell lines (Thacker et al., 1983; and Klein and Rossman 1990). Klein and Rossman (1990) have shown an increase sensitivity for the induction of *gpt* mutants compared to HPRT mutants when G12 and V79 cells were exposed to X rays. Therefore, the purpose of this study was to analyze the sensitivity of the induction of mutants in G12 and V79 cells exposed to X rays (low-LET) and alpha-particles (high-LET).

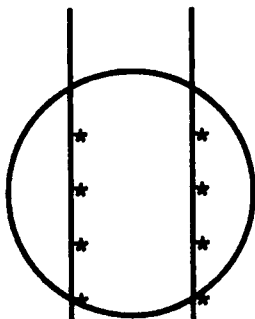
The data confirms that the cytotoxic response of V79 and G12 cells exposed to X rays was not significantly different (Klein and Rossman 1990). The average surviving fraction of V79 and G12 cells at 7.5 Gy X ray was previously found to be 0.1 (Klein and Rossman, 1990), while the average surviving fraction from Figure 3.2 was found to be 0.15. The survival curve of V79 cells exposed to 250 keV X rays (Thacker et al., 1983) exhibits a large D_q value of approximately 6 Gy compared to the D_q value of

1.75 Gy presented here, and a small D_0 value of approximately 1.75 Gy compared to the D_0 of 2.9 Gy determined from the exponential portion of the survival curve. An average surviving fraction of 0.15 was shown for V79 cells exposed to 7.5 Gy of X rays (Figure 3.2) which is greater than the 0.07 surviving fraction obtained by Thacker et al. (1983). The difference between the data presented here and Thacker et al. (1983) could result from the use of different clones of V79 cells. More experiments with doses greater than 7.5 Gy are necessary in order to confirm the shoulder and exponential portions of the survival curve.

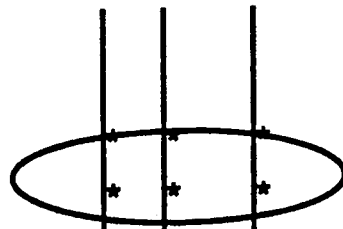
The surviving fractions were similar between G12 and V79 cells when exposed to alpha-particles in a specific morphology (suspended or attached). However, there was a significant increase in the surviving fraction of suspended cells compared to that of attached cells when exposed at similar times to alpha-particles. The difference in surviving fraction between suspended and attached cells exposed to alpha-particles might be explained by differences in nuclear shape. Since alpha-particles are densely ionizing, suspended cells, which exhibit a smaller nuclear cross-section but a greater nuclear thickness, would receive fewer average alpha-particle traversals/nucleus than attached cells as shown schematically in Figure 4.1. Therefore, the number of ionizations would be greater for a suspended cell compared to an attached cell for one alpha-particle traversal. The nuclear cross-section of suspended and attached V79 cells was 66.5 ($21.2 \mu\text{m}^2$) and 106.7 ($19.5 \mu\text{m}^2$), respectively. Although the standard deviations are large, the average nuclear cross-section is substantially different between suspended and attached V79 cells. Suspended G12 and V79 cells required an average of 5.1 (1.8) alpha-particles/nucleus and 5.3 (1.8) alpha-particles/nucleus, respectively, to reduce survival to 37%, while an average of 4.4 (1) alpha-particle/nucleus was required for attached V79 cells. Since the standard deviations for the average number of nuclear measurements were large and overlapped between suspended and attached V79 cells, the difference in sensitivity to alpha-particles between suspended and attached V79 cells could not be compared effectively. Crawford-Brown and Hofmann (1991) showed, through a mathematical formulation, that a higher

Figure 4.1. Schematic representation of tracks of alpha-particles from a planar americium-241 source passing through the nucleus of an attached and suspended cell. Asterisks represent regions of dense ionizations.

NUCLEAR SIZE FOR
SUSPENDED CELL



NUCLEAR SIZE FOR
ATTACHED CELL



Americium-241

average number of alpha-particles/nucleus was required to reduce survival to 37% for attached cells compared to suspended cells. The mathematical formulation does not take into account possible interactions between lesions. The probability of hitting a target associated with cell killing for attached and suspended cells exposed to alpha-particles would be similar at LET values less than 100 keV/ μ m, but smaller for attached cells compared to suspended cells at higher LET (Crawford-Brown and Hofmann, 1991). Thus, the average alpha-particles/nucleus could vary between suspended and attached cells depending upon the LET of the alpha-particles. Monolayers of suspended V79 and G12 cells when exposed to alpha-particles are difficult to generate because cells tend to clump thus increasing the chance of unsettled cells, which could result in higher surviving fractions than expected. One way to reduce cell clumping is by exposing cells at a lower cell density. In addition, more precise nuclear measurements are required in order to reduce the large standard deviations.

Many cell lines show a persistently reduced colony forming efficiency even after 30 population doublings following exposure to X rays, a phenomenon that has been referred to as delayed reproductive death (Chang and Little, 1991). This data demonstrates an increase in surviving fraction after 3 days from exposure for V79 and G12 cells exposed to alpha-particles. In contrast, the cytotoxic dose-response curves were shown to be similar for V79 and G12 cells plated immediately and 3 days after exposure to X rays. Six days from exposure, a significant (2- to 3-fold) increase in surviving fraction was shown for both cell lines exposed to either X rays or alpha-particles. In addition, there was a recovery in colony forming efficiency to control levels for attached V79 and G12 cells plated 6 days after a 2 minute exposure to alpha-particles, which represents an initial surviving fraction of 0.5. The recovery in colony forming efficiency for cells plated 6 days after exposure to X rays was shown at 2.5 Gy which corresponds to an initial surviving fraction of 0.65. The difference in recovery between cells exposed to X rays compared to alpha-particles could be explained by the difference in the induction and repair of double strand breaks. Chang and Little (1992) have shown that Chinese hamster ovary cells defective in the repair of double strand breaks

did not exhibit delayed reproductive death when exposed to X rays, and that CHO cells exposed to UV, which does not produce a significant number of double strand breaks, did not show delayed reproductive death. Therefore, Chang and Little (1992) have shown that the induction and repair of double strand breaks are associated with the onset of delayed reproductive death in Chinese hamster ovary cells. The rejoining of double strand breaks has been shown to be less efficient for cells exposed to alpha-particles compared to X rays (Jenner et al., 1993). Jenner et al. suggested that the clustering of damage from ionizations or energy deposition increases with increasing LET of radiation. Thus, the clustered damage to DNA induced by alpha-particles compared to X rays could result in the inefficient repair of double strand breaks, which could affect the recovery in colony forming efficiency to control levels for cells exposed to alpha-particles compared to X rays. These possible differences in recovery between V79 and G12 cells when exposed to X rays and alpha-particles could be further analyzed by producing more survival curves for various plating periods after exposure as well as by analyzing the induction and rejoining of double strand breaks between cells exposed to alpha-particles and X rays.

There was a significant increase in the induction of *gpt* mutants compared to HPRT mutants when G12 and V79 cells were exposed to X rays. These results are similar to the 2- to 4.5-fold increase in the induction of *gpt* mutants compared to HPRT mutants obtained by Klein and Rossman (1990). Stankowski and Hsie (1986) also showed a significant increase in the induction of *gpt* mutants in AS52 cells compared to HPRT mutants from wild type CHO cells exposed to X rays. There is a considerable size difference between HPRT (46kb) and *gpt* (0.45kb). Thacker et al. (1983) transformed a HPRT deficient V79 cell line, α M1, with either pSV2*gpt* or genomic hamster DNA in order to address the size difference between the two genes. Their results showed a 3-fold increase in the induced mutation frequency for cells containing an integrated HPRT gene compared to cells containing an integrated bacterial *gpt* gene. Integration of exogenous DNA has been shown to be favorable for sites containing repetitive sequences (Kato et al., 1986). Since repair of damage is affected by

chromosomal location (Hanawalt, 1987), the increase induction of *gpt* mutants compared to HPRT mutants could reflect an inefficient repair process at the chromosomal location of the *gpt* gene. If repair is inefficient at the *gpt* locus, there might be an increase in the induction of point mutations compared to deletions, which has been shown to be the major type of mutation within cells exposed to X rays (Bao et al., 1995). Experiments utilizing G12 cells exposed to X rays in the presence of free radical scavengers could shed light on the possible role of free radicals in the induction of mutations.

There were no significant differences between the mutation frequencies of attached G12 and V79 cells exposed to alpha-particles. Three factors that could influence the induction of mutations at the *gpt* and HPRT loci are target size, location of essential genes, and location of nonessential genes to the target locus. First, the HPRT gene (36kb) is 80 times larger than the bacterial *gpt* gene (0.45kb). Thus, it would seem more likely for an alpha-particle, which mainly interacts directly with DNA, to have a greater probability of interacting with the HPRT gene compared to the *gpt* gene. Secondly, the linkage of essential genes to the target locus could affect the induction of mutations. If essential genes are in proximity to the HPRT locus, then large deletions at the HPRT locus would not be expressed because the cell would not be able to survive (Kronenberg and Little, 1989). Since recombinational repair of double strand breaks appears to be unlikely for the hemizygous HPRT gene, large deletions at the HPRT locus would not be efficiently repaired (Zhu and Hill, 1994). Finally, the location of the target locus being in a nonessential region for cell survival could influence the induction of mutations. A 20-fold increase in the induction of S1 mutants compared to HPRT mutants was shown in human hamster hybrid (A_L) cells irradiated with helium⁴ ions (Hei et al., 1994). These cells (A_L) have a copy of human chromosome 11, containing the S1 surface antigen, and a complete copy of the hamster genome. One postulated reason for the difference between the induction of S1 and HPRT mutants is due to the nonessential role of the genes on chromosome 11 to the cell's survival (Hei et al., 1994). Therefore, mutational damage to the S1 locus on chromosome 11 is not

likely to be lost due to cell killing. The *gpt* locus might be located within a nonessential region, which would result in the expression of large deletions occurring at the *gpt* locus. Only one experiment was performed on attached V79 and G12 cells exposed to alpha-particles. Therefore, further experiments are necessary in order to support the current results.

There were no significant differences in the mutation frequency between suspended and attached V79 and G12 cells exposed to alpha-particles when plotted as a function of surviving fraction. As previously stated, difficulties in obtaining a monolayer of suspended cells due to cell clumping could result in cells being unevenly exposed to alpha-particles, which could cause a decrease in the mutation frequency for suspended cells. Crawford-Brown and Hofmann (1991) have shown by mathematical formulation that the probability per unit track length of a mutation target being hit is identical for suspended and attached cells exposed to alpha-particles with LET of less than 100 keV/ μ m, but the probability will be smaller for attached cells with LET above 100 keV/ μ m. Therefore, differences in the LET of alpha-particles could explain differences in the induction of mutations between suspended and attached cells. Because of variations in the LET of alpha-particles at various distances from the source, further experiments of suspended and attached cells exposed to alpha-particles at various distances from the source could elucidate the effect of LET on the induction of mutations between attached and suspended cells. More experiments with longer exposure times to alpha-particles are necessary for suspended cells in order to analyze the induced mutation frequency at lower surviving fractions.

The data presented here show no significant differences in the induction of HPRT mutants when V79 cells are exposed to alpha-particles or X rays at equitoxic doses, while there was a significant increase in *gpt* mutants for cells exposed to X rays compared to alpha-particles at equitoxic doses. Thacker et al. (1982) have shown an increase in HPRT mutants for V79 cells exposed to alpha-particles compared to X rays at equitoxic doses. However, Bao et al. (1995) have shown a similar induced HPRT mutation frequency in TK-6 cells exposed to either alpha-particles or X rays at

equitoxic doses. The maximum effectiveness for cell killing has been shown for alpha-particles with an LET at 100 keV/ μ m with a rapid reduction in effectiveness at higher LET. Therefore, the effectiveness for the induction of mutants could be influenced by the LET of alpha-particles. Since X rays interact indirectly with DNA through formation of free radicals compared to directly acting alpha-particles, the location of the *gpt* gene might be more susceptible to free radicals which would increase the mutation frequency of G12 cells exposed to X rays compared to alpha-particles.

In summary, the preliminary results discussed here show no significant differences in the mutation frequency of V79 cells exposed to either X rays or alpha-particles at equitoxic doses. In contrast, there was a significant increase in mutation frequency of G12 cells exposed to X rays compared to alpha-particles at equitoxic doses. Although there was a difference in the mutation frequency of V79 and G12 cells exposed to X rays, the current results suggest no significant differences between the induction of *gpt* and HPRT mutants when cells are exposed to alpha-particles. Future experiments are required at higher doses in order to analyze further the mutation frequencies and cytotoxic responses of V79 and G12 cells irradiated with alpha-particles or X rays.

LIST OF REFERENCES

REFERENCES

- Aghamohammadi, S.Z., T. Morris, D.L. Stevens and J. Thacker. 1992. Rapid screening for deletion mutations in the *hprt* gene using the polymerase chain reaction: X-ray and α -particle mutant spectra. *Mutation Res.* 269:1-7.
- Ashman, C.R., and R.L. Davidson. 1987. High spontaneous mutation frequency in shuttle vector sequences recovered from mammalian cellular DNA. *Mol. Cell Biol.* 4:2266-2272.
- Bao, C.-Y., A.-H. Ma, H.H. Evans, M.F. Horng, J. Mencl, T.E. Hui and W.D. Sedwick. 1995. Molecular analysis of hypoxanthine phosphoribosyltransferase gene deletions induced by α - and X-radiation in human lymphoblastoid cells. *Mutat. Res.* 326:1-15.
- Barendsen, G.W. 1964. Impairment of the proliferative capacity of human cells in culture by α -particles with differing linear-energy transfer. *Int. J. Rad. Biol.* 8:453-466.
- Barnhart, B.J., and S.H. Cox. 1979. Mutagenicity and cytotoxicity of 4.4-MeV α particles emitted by plutonium-238. *Radiat. Res.* 80:542-548.
- Caskey, C.T., and G.D. Kruh. 1979. The HPRT locus. *Cell.* 16:1-9.
- Chang, W.P., and J.B. Little. 1991. Delayed reproductive death in X-irradiated Chinese hamster ovary cells. *Int. J. Radiat. Biol.* 60:483-496.
- Chang, W.P., and J.B. Little. 1992. Evidence that DNA double-strand breaks initiate the phenotype of delayed reproductive death in Chinese hamster ovary cells. *Radiat. Res.* 131:53-59.
- Crawford-Brown, D.J., and W. Hofmann. 1991. An effect-specific track-length model for radiations of intermediate to high LET. *Radiat. Res.* 126:162-170.

- Ford, J.R., and M. Terzaghi-Howe. 1993. Effects of ^{210}Po alpha particles on survival and preneoplastic transformation of primary rat tracheal epithelial cells irradiated while in suspension or in the intact tissue. *Radiat. Res.* 136:89-96.
- Fox, J.C., and N.J. McNally. 1988. Cell survival and DNA double-strand break repair following X-ray or neutron irradiation of V79 cells. *Int. J. Radiat. Biol.* 54:1021-1030.
- Grdina, D.J., B. Nagy, C.K. Hill, R.L. Wells and C. Peraino. 1985. The radioprotector WR1065 reduces radiation induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in V79 cells. *Carcinogenesis*. 6:929-931.
- Hanawalt, P.C. 1987. Preferential DNA repair in expressed genes. *Environ. Health Perspect.* 76:9-14.
- Hei, T.K., L.X. Zhu, D. Vannais and C.A. Waldren. Molecular analysis of mutagenesis by high LET radiation. *Adv. Space Res.* 14:355-361.
- Jenner, T.J., C.M. deLara, P. O'Neill and D.L. Stevens. 1993. Induction and rejoining of DNA double-strand breaks in V79-4 mammalian cells following γ - and α -irradiation. *Int. J. Radiat. Biol.* 64:265-273.
- Jostes, R.F., E.W. Fleck, T.L. Morgan, G.L. Stiegler and F.T. Cross. 1994. Southern blot and polymerase chain reaction exon analyses of HPRT⁻ mutations induced by radon and radon progeny. *Radiat. Res.* 137:371-379.
- Kato S., R.A. Anderson and R.D. Camerini-Otero. 1986. Foreign DNA introduced by calcium phosphate is integrated into repetitive DNA elements of the mouse L cell genome. *Mol. Cell. Biol.* 6:1787-1795.
- Klein C.B., and T.G. Rossman. 1990. Transgenic Chinese hamster V79 cell lines which exhibit variable levels of *gpt* mutagenesis. *Environ. Mol. Mutagen.* 16:1-12.
- Klein C.B., and E.T. Snow. 1993. Localization of the *gpt* sequence in transgenic G12 cells via fluorescent in-situ hybridization. *Environ. Mol. Mutagen.* 21:35.

- Konecki, D.S., J. Brennand, J.C. Fuscoe, C.T. Caskey and A.C. Chinault. 1982. Hypoxanthine-guanine phosphoribosyltransferase genes of mouse and Chinese hamster: construction and sequence analysis of cDNA recombinants. *Nucleic Acid Res.* 10:6763-6775.
- Kronenberg, A., and J.B. Little. 1989. Locus specificity for mutation induction in human cells exposed to accelerated heavy ions. *Int. J. Radiat. Biol.* 55:913-924.
- Loeb, L.A. 1991. Mutator phenotype may be required for multistage carcinogenesis *Cancer Res.* 51:3075-3079.
- Loeb, L.A. 1994. Microsatellite instability: Marker of a mutator phenotype in cancer. *Cancer Res.* 54:5059-5063.
- Price, A. 1993. The repair of ionising radiation-induced damage to DNA. *Cancer Biology.* 4:61-71.
- Puck, T.T., P. Wuchier, C. Jones and F.T. Kao. 1971. Genetics of somatic mammalian cells: Lethal antigens and genetic markers for study of human linkage groups. *Proc. Natl. Acad. Sci.* 68:3102-3106.
- Rossitier, B.J.F., J.C. Fuscoe, D.M. Muzny, M. Fox and C.T. Caskey. 1991. The Chinese hamster HPRT gene: Restriction map, sequence analysis, and multiplex PCR deletion screen. *Genomics.* 9:247-256.
- Sankaranarayanan, K. 1991. Ionizing radiation and genetic risks III. Nature of spontaneous and radiation-induced mutations in mammalian in vitro systems and mechanisms of induction of mutations by radiation. *Mutat. Res.* 258:75-97.
- Schwartz, J.L., J. Rotmensch, J. Sun, J. An, Z. Xu, Y. Yu and A.W. Hsie. 1994. Multiplex polymerase chain reaction-based deletion analysis of spontaneous, gamma ray- and alpha-induced *hprt* mutants of CHO-K1 cells. *Mutagenesis.* 9:537-540.
- Stankowski, L.F., and A.W. Hsie. 1986. Quantitative and molecular analyses of radiation-induced mutation in AS52 cells. *Radiat. Res.* 105:37-48.
- Thacker, J., A. Stretch and D.T. Goodhead. 1982. The mutagenicity of α particles from plutonium-238. *Radiat. Res.* 92:343-352.

Thacker, J., P.G. Debenham, A. Stretch and M.B.T. Webb. 1983. The use of a cloned bacterial gene to study mutation in mammalian cells. *Mutat. Res.* 111:9-23.

Tindall, K.R., and L.F. Stankowski Jr. 1989. Molecular analysis of spontaneous mutations at the *gpt* locus in Chinese hamster ovary (AS52) cells. *Mutat. Res.* 220:241-253.

Zhu, L.X., and C.K. Hill. 1994. Neutron-energy-dependent mutagenesis in V79 Chinese hamster cells. *Radiat. Res.* 139:300-306.

VITA

Steven A. Plichta was born in Chicago, Illinois on November 29, 1969. He attended Loyola Academy High School in Wilmette, Illinois and graduated in May of 1987. He then attended Florida Institute of Technology in Melbourne, Florida where he received a Bachelor of Science degree in Molecular Biology in May 1992. In August of 1992, he entered the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences. He was married to Jill A. Leifheit on September 3, 1994. He received a Master of Science degree with a concentration in radiation mutagenesis in May 1995, while performing research under the direction of Dr. Margaret Terzaghi-Howe.