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Principal Investigator - Dr. Amy Kronenberg

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Regents of theUniversity of California
Lawrence Berkeley Laboratory
Mailstop 936A
Berkeley, CA 94720

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Summary

The overall goals of this research were to determine the risks of mutation induction and the spectra of mutations induced by energetic protons and iron ions at two loci in human lymphoid cells. During the three year grant period the research has focused in three major areas: 1) the acquisition of sufficient statistics for human TK6 cell mutation experiments using Fe ions (400 MeV/amu), Fe ions (600 MeV/amu) and protons (250 MeV/amu), and 2) collection of thymidine kinase- deficient (tk) mutants or hypoxanthine phosphoribosyltransferase-deficient (hprt) mutants induced by either Fe 400 MeV/amu, Fe 600 MeV/amu, or H 250 MeV/amu for subsequent molecular analysis, and 3) molecular characterization of mutants isolated after exposure to Fe ions (600 MeV/amu). As a result of the shutdown of the BEVALAC heavy ion accelerator in December 1992, efforts were re-arranged somewhat in time to complete our dose-response studies and to complete mutant collections in particular for the Fe ion beams prior to the shutdown. These goals have been achieved. A major effort was placed on collection, re-screening, and archiving of 3 different series of mutants for the various particle beam exposures: tk-ng mutants, tk-sg mutants, and hprt-deficient mutants. Where possible, groups of mutants were isolated for several particle fluences. Comparative analysis of mutation spectra has occured with characterization of the mutation spectrum for hprt-deficient mutants obtained after exposure of TK6 cells to Fe ions (600 MeV/amu) and a series of spontaneous mutants. Molecular analysis of the remaining tk-deficient or hprt-deficient mutants is underway in the laboratory. In addition, we have initiated a series of investigations using the WIL2NS cell line, to examine the effect of differences in DNA damage responsiveness to sparsely ionizing X-rays on cell killing and mutagenesis by densely ionizing radiations.

Cytotoxicity Results for TK6 Lymphoblasts:

Data are presented for two Fe ion beams (400 MeV/amu and 600 MeV/amu initial energy), two proton beams (55 MeV/amu and 250 MeV/amu) , and for X-rays. We would note that no difference was observed between the results obtained for 160 kVp x-rays from our present Pantak x-ray generator as compared with results obtained with a Phillips 100 kVp x-ray generator used for previous studies. The results reported for the 400 MeV/amu (initial energy) and 600 MeV/amu (initial energy) beams come from a minimum of 3 replicate dose-response curves exposed at the BEVALAC heavy ion accelerator at Lawrence Berkeley Laboratory. The data reported for the 250 MeV proton beam (initial energy) is a compilation of three replicate dose-response curves exposed at the Loma Linda University Proton Accelerator. The data reported for the 55 MeV proton beam (initial energy) is preliminary data from two runs at the 88 inch cyclotron at Lawrence Berkeley Laboratory.

LET values for BEVALAC beams were determined based on the residual range of the extracted beam as measured in a water phantom. We can place these data into context by comparison with results obtained for several additional beams obtained at the BEVALAC. In certain instances, the LET values were also measured

directly using a compound LET spectrometer. For all charged particle irradiations, cells were positioned in the plateau region of the Bragg ionization curve. The results are summarized in Table I. Each of the dose-response functions for survival of TK6 cells exposed to charged particle beams is best fitted by a simple exponential curve with the exception of the Fe 400 MeV/amu beam. A small shoulder is apparent on this survival curve (intercept = 1.3). This shoulder is most likely artificial and due to the fact that at the lower doses, not every cell is traversed by an Fe ion. The D_0 (terminal slope) is 57 cGy. We can also calculate the inactivation cross section using the formula 16 x LET (keV/μm) x $1/D_0$. The inactivation cross section for 400 MeV/amu Fe ions is 65.96 μm^2, which is still smaller than the average cross sectional area of the TK6 cell nucleus (85.5 μm^2). Thus, single particle traversals are not uniformly lethal to these cells.

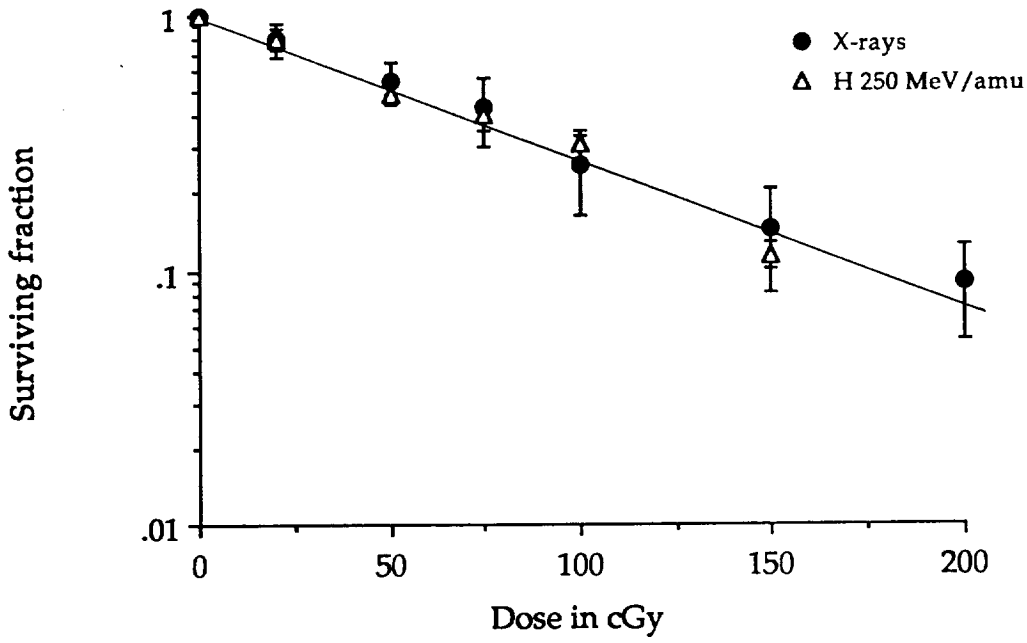
TABLE I

Radiation Type	LET (keV/μm)	D_0 (cGy)	Inactivation Cross Section (μm^2)
X-rays	----	77	-----
H 250 MeV/amu	0.4	75	00.09
Ne 425 MeV/amu	32	48	10.67
Si 670 MeV/amu	50	47	16.91
Si 456 MeV/amu	61	28	34.86
Si 330 MeV/amu	80	38	33.33
Ar 470 MeV/amu	95	34	44.71
Fe 600 MeV/amu	190	51	59.60
Fe 400 MeV/amu	235	57	65.96

The cytotoxicity of 250 MeV protons is compared with the results obtained for 100 kVp X-rays in Figure 1 below. In all cases, cell were irradiated in suspension culture in T-25 flasks or in larger T-75 flasks. Dosimetry was performed with a Victoreen ionization chamber for the appropriate irradiation geometry.

FIGURE 1

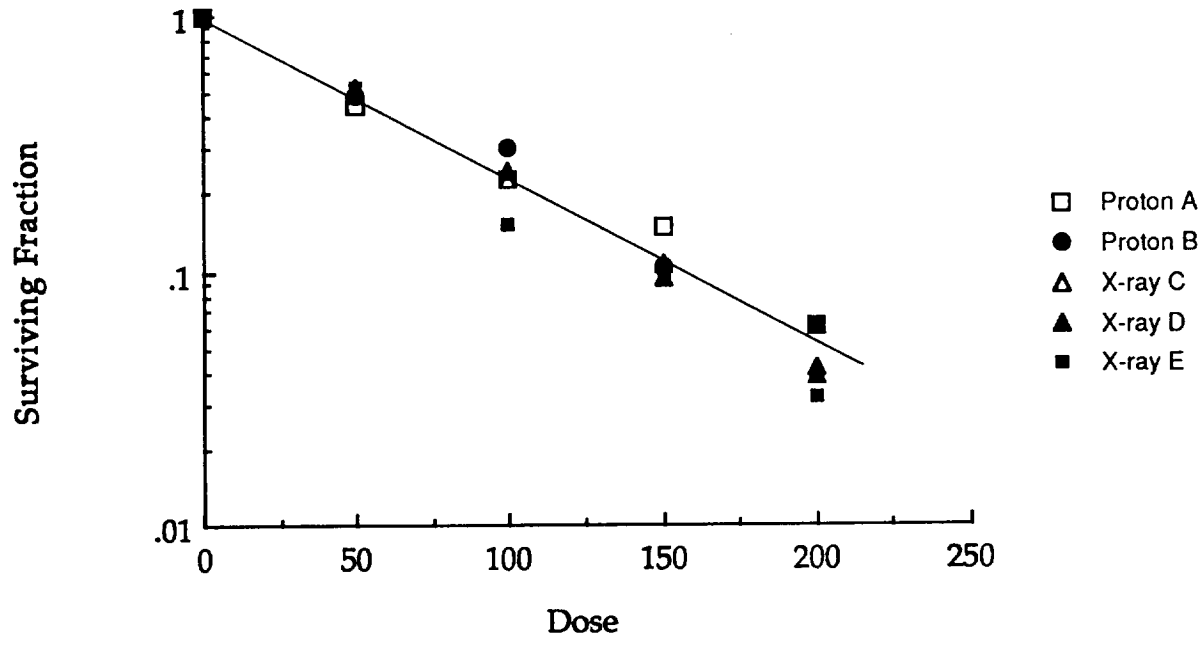
Cytotoxicity of H 250 MeV/amu or 100 kVp X-rays - TK6 cells



Results are shown (Figure 2) for cytotoxicity of 55 MeV protons (initial energy) and 160 kVp x-rays for cells irradiated in Eppendorf tubes. This irradiation geometry is designed to minimize changes in LET through the sample irradiated with the proton beam.

FIGURE 2

Cytotoxicity of 55 MeV protons or 160 kVp x-rays - TK6 cells in Eppendorf tubes



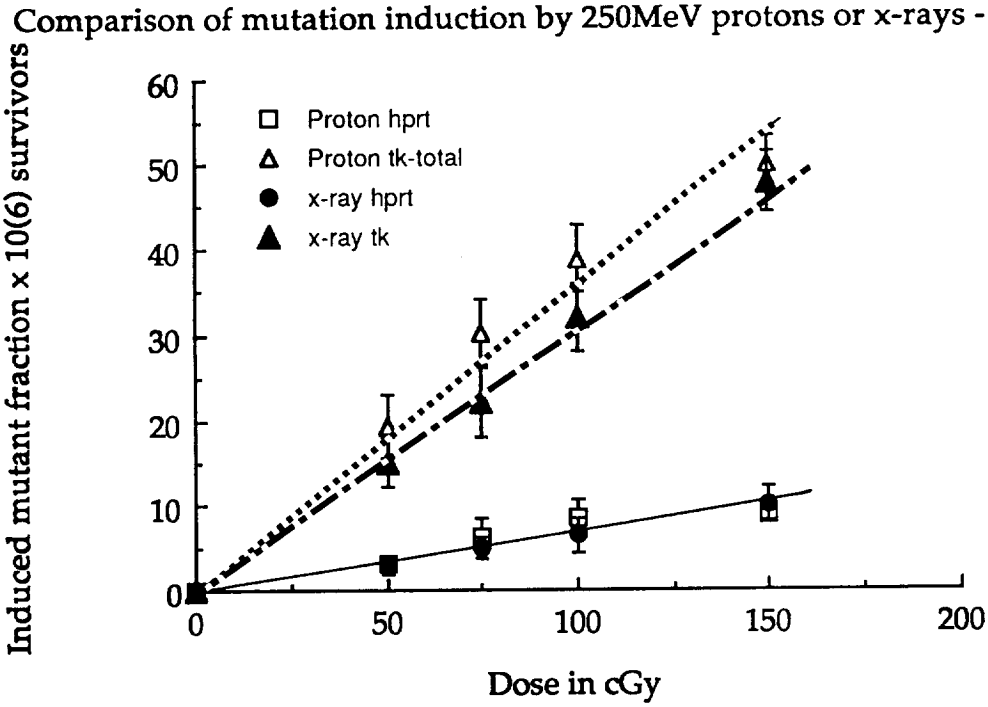
Additional experiments are planned to extend the present results obtained with 55 MeV protons. Initial results suggest that protons in this energy range do not exhibit markedly different cytotoxic effects than the 250 MeV protons or X-rays.

Mutation Induction at the hprt and tk loci in TK6 cells

Mutation experiments were carried out with 250 MeV protons at the Loma Linda University Medical Center, 55 MeV protons at the 88 inch cyclotron at Lawrence Berkeley Laboratory, and with 600 MeV iron ions and 400 MeV/amu iron ions at the Bevalac accelerator at Lawrence Berkeley Laboratory.

The results of a comparison between mutation induction by 250 MeV protons and mutation induction following exposure to 160 kVp X-rays is shown in Figure 3.

FIGURE 3



The shape and magnitude of the dose response curves is quite similar for the two different types of radiations. The data were analyzed by zero-intercept linear regression and represent the results of a minimum of 3 independent experiments for each type of radiation. The results are displayed in Table II below. We also initiated a series of mutation experiments using 55 MeV protons (initial energy) extracted from the 88 inch cyclotron at Lawrence Berkeley Laboratory. Preliminary results are presented for the first two experiments using this new beam line. Cells are irradiated in suspension Costar T-25 flasks positioned so that the 1.7 cm thickness of the flasks is maximum thickness of water-equivalent material through which the beam passes. LET will vary through the flask thickness and the range was verified on film exposures through defined thickneses of polyethylene sheets. Energy measurements are in process. Absorbed dose is estimated at the center of the

flask position. The results of the first experiments are displayed below in Figure 4 and results of the second experiments are shown in Figure 5.

FIGURE 4

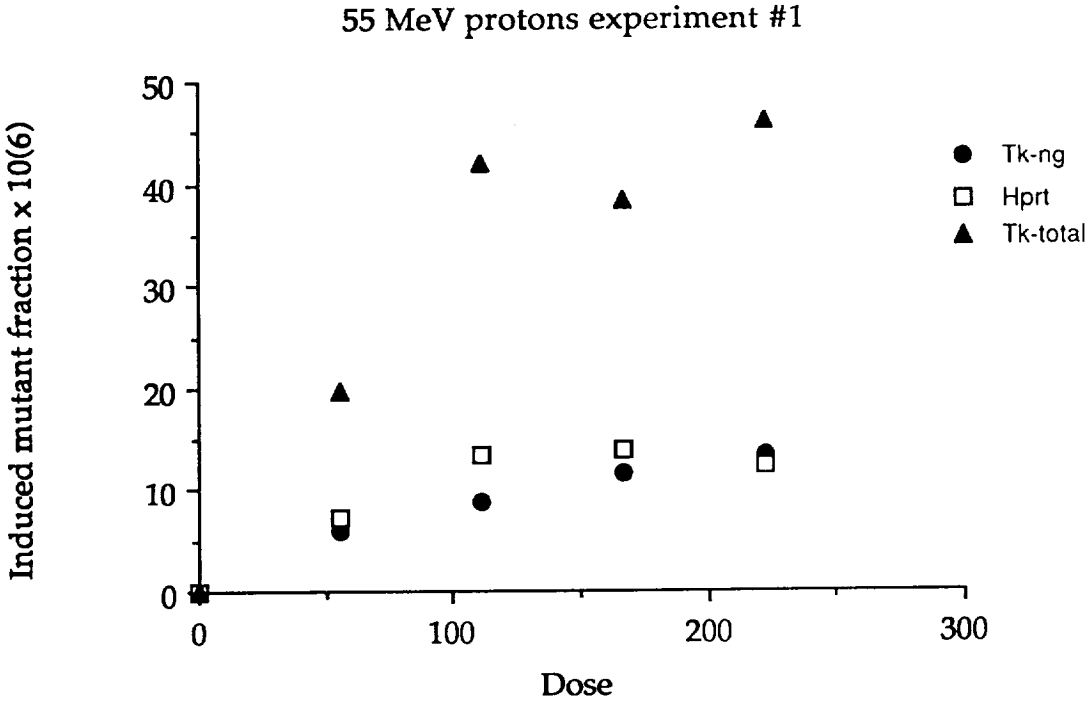
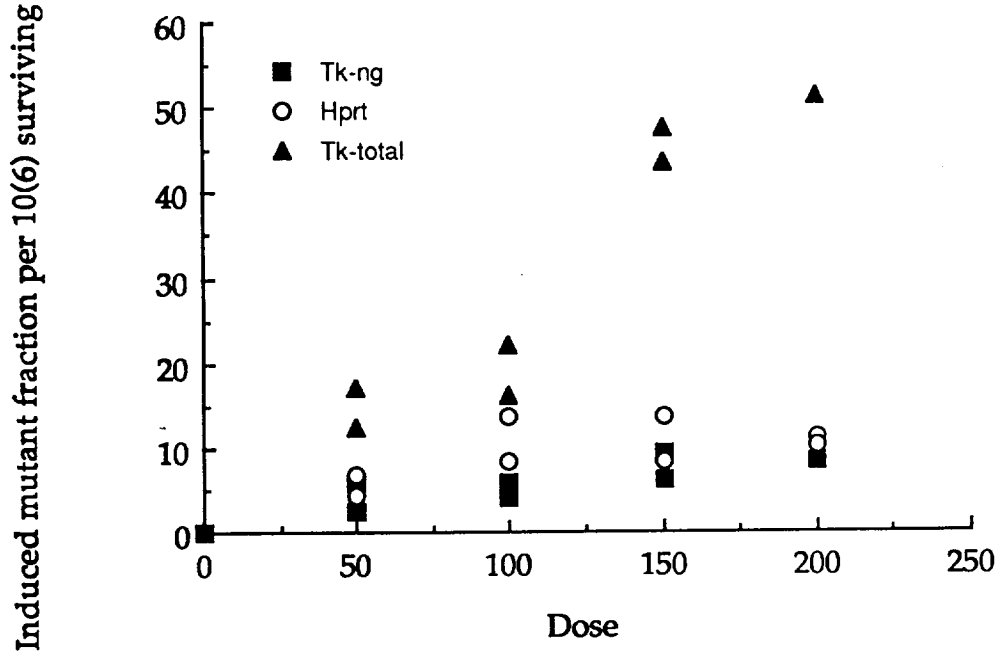


FIGURE 5

Mutation induction by 55 MeV protons in TK6 cells



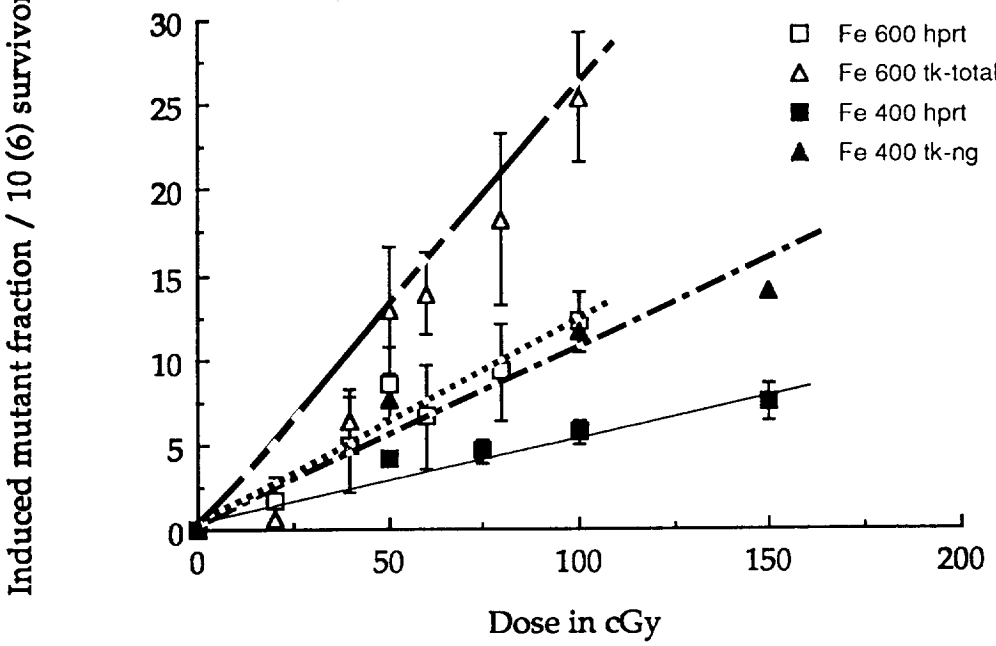
We have quantitated mutations induced at the hprt and tk loci for Fe ions with an initial energy of 600 MeV/amu (190 keV/μm), and Fe ions with an initial energy of 400 MeV/amu (235 keV/μm) in TK6 cells. The results are compared with results obtained for x-rays and other several other charged particle beams (Table II). The results are presented in as a function of dose or as action cross-sections A graphical representation of the dose-response for Fe ions is shown in Figure 6.

TABLE II

Radiation Type	LET (keV/μm)	Locus	Induced Mutant Fraction x (10-7/cGy)	Action Cross Section $x10^{-4}$
100 kVp X-ray		hprt	0.6± 0.1	N/A
		tk-total	3.0± 0.3	N/A
H 250 MeV/amu	0.4	hprt	0.6 ± 0.1	0.004
		tk-total	3.5 ± 0.3	0.022
Ne 425 MeV/amu	32	hprt	2.5± 0.1	1.3
		tk-total	4.5 ± 0.2	2.3
Si 670 MeV/amu	50	hprt	2.1± 0.1	1.7
		tk-total	4.7± 0.3	3.8
Si 456 MeV/amu	61	hprt	4.2 ±0.4	4.1
		tk-total	16.1 0.9	15.8
Si 330 MeV/amu	80	hprt	4.3± 0.3	3.4
		tk-total	8.2± 0.5	10.5
Ar 470 MeV/amu	95	hprt	3.0± 0.3	4.60
		tk-total	6.5± 0.5	9.8
Fe 600 MeV/amu	190	hprt	1.2± 0.1	3.7
		tk-total	2.3± 0.3	7.0
Fe 400 MeV/amu	235	hprt	0.6 ± 0.1	1.5
		tk-total	1.1 ± 0.1	2.7

FIGURE 6

Comparison of Fe-ion-induced mutation in TK6 cells:
600 MeV/amu initial energy or 400 MeV/amu initial energy



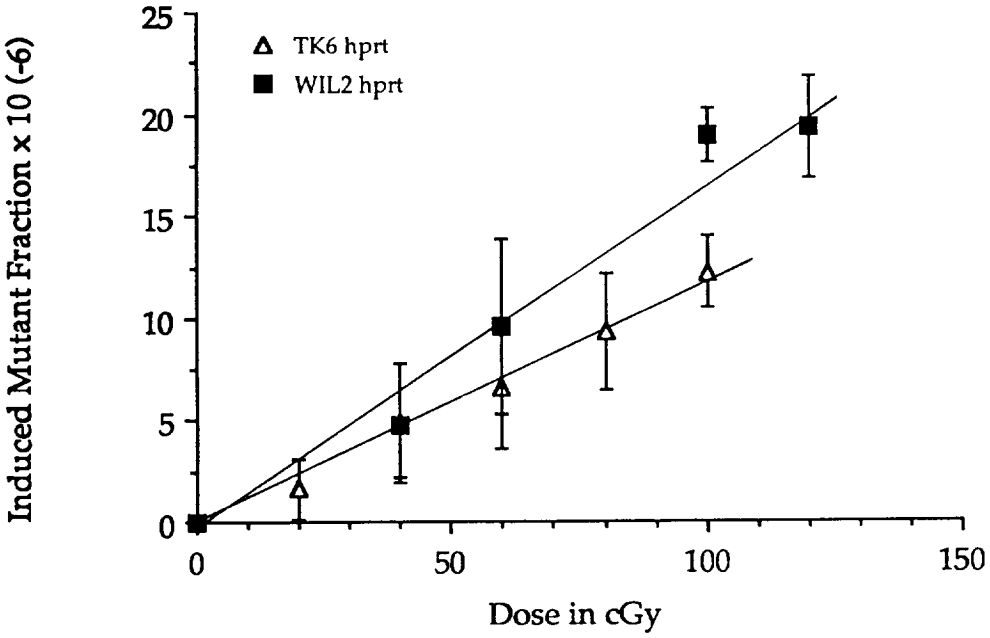
The results obtained for TK6 cells exposed to 400 MeV/amu Fe ions were somewhat surprising as there was no demonstrable dose response for the induction of slowly growing tk-deficient mutants. Although some tk-sg mutants were observed in the irradiated cultures, the frequencies were so low as to be indistinguishable from the background. If we place these results into context, it is known that the slowly growing tk-deficient mutants appeared to be preferrentially induced by particles with LET's up to about 61 keV/μm, and that for more densely ionizing particles the efficiency of induction of the slow growth variants declines rapidly. The data obtained for this very densely ionizing beam (235 keV/μm) demonstrates that we cannot reliably detect tk slow-growth variants above the spontaneous incidence. Further studies on the maximum deletion size associated with chromosome 17 mutations inclusive of the tk locus may tell us whether the failure to induce slow growth mutants is due to the placement of essential genes along chromosome 17 or whether the processing of the initial damage arising after the most densely ionizing radiation exposures differs from that arising after sparsely ionizing radiation. We would note that a similar difficulty in recovering slow-growth tk-deficient mutants has been reported by Metting, et al., (Radiation Research 1992) in the case of TK6 cells exposed to ^{212}Bi alpha particles (approximately 60-240 keV/μm).

In addition, we have carried out preliminary experiments to compare mutation induction at the hprt locus in TK6 cells with that obtained for WIL2NS cells (Figure 7). WIL2NS and TK6 cells are derived from the same initial donor (Levy, et al., 1968). WIL2NS cells are the direct parent of WTK1 cells and differ from TK6 cells in their ability to carry out recombination. WIL2NS cells are not

heterozygous at the tk locus, and further studies will utilize WTK1 cells for comparison against TK6 cells.

Figure 7

Hprt mutation yields WIL2 NS and TK6 cells- Fe 600 MeV/amu (190 keV/μm)



Molecular characterization of hprt-deficient mutants isolated after Fe ion exposure

We have completed the analysis of intragenic alterations in a series of hprt-deficient mutants isolated from cultures exposed to 100 cGy of Fe ions (600 MeV/amu, LET=190 keV/μm). This exposure level was chosen as it represents a 5-7X increase over the spontaneous incidence, and we were particularly interested to compare the effects of particle exposures of the same fluence but different ionization densities in order to determine whether the nature of the mutational spectrum was similar or different as a function of LET. We had earlier examined the mutation spectrum for a series of hprt-deficient mutants isolated after exposures to 50 cGy of Ar ions (470 Mev/amu, LET=95 keV/μm), and the comparative results are given below. These two particle exposures were of equal fluence (slightly less than 3 particles/cell nucleus on average). The absorbed dose for the Fe ion exposure is twice that for the Ar ion exposure as each Fe ion traversal is twice as densely ionizing as each Ar ion exposure. The results are also compared with results obtained for our own series of spontaneous hprt-deficient mutants and a larger series of spontaneous hprt-deficient mutants assembled from the TK6 cell literature. The level of induction in the case of the Fe ion exposed cells was 1.2×10^{-5} while for the Ar ion exposed cells it was 1.5×10^{-5}. Background mutant frequencies ranged from $2\text{-}3 \times 10^{-6}$.

TABLE III

SUMMARY OF HPRT MUTANT SPECTRA

Mutant Type	Ar ions 50 cGy	Fe ions 100 cGy	Spontaneous Mutants	
Total deletion	9	32	2	13
Partial deletion rearrangement	11	30	6	48
No detectable alteration	20	8	22	99
TOTAL	40	60	30[1]	160[2]

1 Spontaneous hprt mutants from parallel control cultures.
2. Spontaneous hprt mutants from this series and other published TK6 data (Gennett and Thilly, 1990; Whaley and Little, 1990).

The pairwise comparisons are summarized in Table IV:

TABLE IV

Statistical Analysis of HPRT mutant spectra

Mutants Compared	$\chi 2$ (2 d.f.)	p-value
Ar ions vs. 160 spontaneous	6.84	$p < 0.05$
Fe ions vs. 160 spontaneous	64.17	$p<0.001$
Ar ions vs. Fe ions	20.17	$p<0.001$
30 spontaneous vs. 160 spontaneous	1.47	$p>0.25$

The spectrum of mutants isolated after exposure to the more densely ionizing Fe ions was strongly biased towards large scale alterations (total gene loss and partial deletion) and was distinct from the spectra obtained for either Ar ion-induced mutants or spontaneous mutants. The maximum deletion size tolerated in the vicinity of the hprt locus may be quite large (up to a few Mb) and it is likely that the maximum observed deletion size will not be governed by the ionization density of the incident particle but by the position of essential genes along the X chromosome. Very large deletion mutations (in excess of 50 Mb) have been isolated along a largely non-essential human chromosome following exposure of A_L cells to a 2X higher fluence of the same particles in our collaborative studies with Dr. Charles Waldren funded under the NSCORT in Radiation Health. In that study, the frequency of hprt mutations was 50-fold lower than mutations for the S1 locus on the non-essential chromosome. In addition to DNA polymerase α on the short arm of the X chromosome, there are likely additional essential genes on the long arm of the X that are required for viability of both human and hamster cells.

Table V summarizes the collection of TK6 derived mutants archived and in the process of analysis:

TABLE V

Radiation Type	Locus	Doses	Number of Mutants Archived
H 250 MeV	hprt	150 cGy	90
	tk-ng	150 cGy	90
	tk-sg	150 cGy	90
Fe 600 MeV/amu	hprt	100 cGy	80
	tk-ng	50, 100 cGy	61 at 50 cGy, 66 at 100 cGy
	tk-sg	100 cGy	39
Fe 400 MeV/amu	hprt	150 cGy	78
	tk-ng	100, 150 cGy	73 at 100 cGy, 54 at 150 cGy

We have also isolated 68 hprt-deficient mutants of WIL2NS cells for comparative analysis.

Early disintegration of DNA in TK6 and WIL2NS cells following exposure to Fe ions

Cell counts in the first several days following exposure of TK6 cells to most types of ionizing radiations show a dose-dependent lag in growth. This may be a function of cell cycle delay or cell loss or a combination thereof. In collaboration with Dr. Bjorn Rydberg and Dr. Priscilla Cooper (under the auspices of the NSCORT in Radiation Health), we performed one preliminary experiment with irradiated TK6 and WIL2NS cells with 50 Gy of 600 MeV/amu Fe ions to examine the rejoining of double strand breaks using quantitative pulsed-field electrophoresis techniques over the period from 0-8 hours post irradiation. We observed two major bands in each of the lanes below the position of the wells. These represent limits of resolution for the pulse-sequence used in the electrophoresis with the lower band corresponding to molecular weight of about 150kb. In contrast to what Dr. Rydberg has observed for fibroblasts, there was evidence of degradation of DNA within 1-2 hours to sizes below 150kb (the resolution in this area of the gel is such that these pieces may be quite small). These changes are consistent with observations of apoptosis. Apoptosis is a common feature of irradiated lymphoid cells, and has been associated with the presence of wild-type p53 (Livingstone, et al, 1992).

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