LA-UR-93-1696

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SUBMITTED TO Symposium: An International Seminar on Molecular Mechanisms in Radiation Mutagenesis and Carcinogenesis
Doorwerth, The Netherlands
April 19-22, 1993

Los Alamos National Laboratory
Los Alamos, New Mexico 87545

Involvement of Recombination in X-ray mutagenesis of human cells.

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ABSTRACT: Closely related human lymphoblastoid cell lines derived from WI-L2 differ greatly in their responses to X-irradiation. Compared with TK6 (ATCC CRL 8015), WI-L2-NS (ATCC CRL 8155) has an enhanced X-ray survival. The induction of mutation by X-rays is also markedly different. The hemizygous *hprt* locus is slightly more mutable in WI-L2-NS than in TK6, and the dose response fits best to a linear-quadratic curve rather than the linear fit of TK6. X-ray induced mutation at the autosomal *tk* locus in heterozygotes derived from WI-L2-NS is 20-50 fold higher than in heterozygotes derived from TK6. A larger proportion of WI-L2-NS mutants had lost heterozygosity compared with mutants of TK6. Fluorescence *in situ* hybridization indicated that loss of heterozygosity was due almost uniformly to deletion of an allele in mutants of TK6, and to recombination or gene conversion in mutants of WI-L2-NS. These results indicate that recombinational repair contributes to both cell survival and mutation following exposure to ionizing radiation.

1. INTRODUCTION

The importance of DNA repair for cell survival and mutation has been studied through the use of many different cell lines with different capacities for repair. For example, the *xrs*5 and *xrs*6 mutants of CHO-K1 cells, which are deficient in double strand break (dsb) repair (Kemp *et al.*, 1984), are more sensitive to killing by X-irradiation, show a slight increase in mutation at the *hprt* locus (Darroudi and Natarajan, 1989), and a 3-4 fold increase in mutation at the *tk* locus (Mussa *et al.*, 1990). Several of the *xrs* mutants also have been tested for their ability to carry out homologous recombination of plasmids. *Xrs*1 and *xrs*7 were not found to be deficient in homologous recombination, but showed impairment of integration of plasmid DNA into the genome (Hamilton and Thacker, 1987). *Xrs*5 was also shown to be proficient in recombination of plasmids (Moore *et al.*, 1986). Radiation sensitive mutants of mouse L5178Y cells differ in their mutability. The M10 line is about 4 times more mutable at *hprt* than the parent line (Shiomi *et al.*, 1981), while L5178Y-S (LY-S) cells were about three-fold less mutable by X-rays and ethyl methanesulfonate (EMS) at the *hprt* locus than were the parent L5178Y-R (LY-R) (Evans *et al.*, 1986). Induction of *tk*- mutants, however, was similar or greater in *tk* heterozygotes derived from LY-S than in heterozygotes derived from LY-R (Evans *et al.*, 1986). The LY-S phenotype has been attributed to a defect in topoisomerase II activity (Evans *et al.*, 1989).

Similar studies in human cells have been done in lines derived from patients with cancer prone syndromes. Defects in DNA repair have been associated with many of the complementation groups of these diseases. Cell lines from patients with ataxia telangiectasia (AT) were found to be two to three fold more sensitive to gamma ray induced killing (Taylor, *et al.*, 1975). In comparison to TK6 lymphoblasts, AT lymphoblasts were about two times more sensitive to killing by gamma rays but equally mutable at *hprt* (Tatsumi and Takebe, 1984).

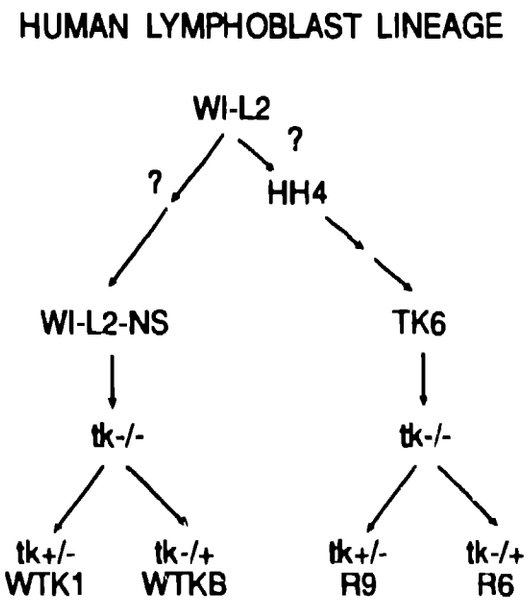
While in some of these systems a decrease in survival following irradiation occurs along with a decrease in recoverable mutants at one or more loci, in others mutation is the same or slightly increased. It seems likely that the various cell lines studied are deficient in different components of a complex DNA repair system or systems.

Mutagenic response also differs between loci and among different cell systems. The difference between mutation at hemizygous and heterozygous loci has been explored in mouse

lymphoma cells, where hemizygous *hprt* was found to be 100 fold less mutable than heterozygous *tk* (Evans *et al.*, 1986). The same loci in human lymphoblastoid cells were found to differ by only about 6 fold (Liber *et al.*, 1989). When the selectable target gene was on a single human chromosome in a human-hamster cell hybrid, very high mutant fractions were observed (Waldren *et al.*, 1979; 1986). Such results led to the assumption that the observed mutant fraction could be influenced by the degree to which the surrounding genetic material was nonessential. It followed that heterozygous essential genes may also affect the number of mutants recovered from linked heterozygous targets, and we have demonstrated a 10 fold difference between X-ray mutability of the two alleles of the *tk* locus in a human cell line (Amundson and Liber, 1991). This difference was due to a lack of large scale loss of heterozygosity events recovered from one of the alleles. We have since extended this work to syngeneic *tk* heterozygotes with decreased radiation cytotoxicity and increased mutability (Amundson *et al.*, 1993) and now offer a possible mechanism to explain the effects observed.

2. MATERIALS AND METHODS

Cell lines WI-L2 was isolated from a human spleen by Levy *et al* (1968). This nonclonal isolate was widely distributed and has since passed through a number of laboratories. WI-L2-NS (ATCC CRL 8155) is a subclone of WI-L2 deposited at the ATCC by T.A. Coons. A different unselected clone, HH4, was used to derive the TK6 cell line (Skopek *et al.*, 1978) (See figure 1). A *Sac*I polymorphism distinguishes the two alleles of the *tk* gene in WI-L2-NS and TK6 cells (Yandell *et al.*, 1986). TK6 was designated as *tk*+ -, referring to the 14.8/8.4 alleles, and heterozygotes with the other allele active were referred to as *tk*- +. Two *tk* heterozygotes (WTK-1, a *tk*+ - line and WTK-B, a *tk*- + line) derived from WI-L2-NS cells were obtained from M.B. Benjamin (Benjamin *et al.*, 1991).



Cells were maintained as exponentially growing cultures in RPMI 1640 medium supplemented with 10% horse serum (heat treated for 2 hours at 56°C). The cultures were incubated at 37°C in 5% CO_2 and 100% humidity and maintained at densities of 1-12 x 10^5 cells/ml.

FIGURE 1 Relationships between the cell lines used in these studies. WI-L2-NS and HH4 are removed from the original WI-L2 isolate by an unspecified number of cloning steps which did not involve selection or mutagenic treatment.

Mutagen treatment CHAT (deoxycytidine, hypoxanthine, aminopterin, and thymidine) treated cultures were treated with mutagens as previously reported (Amundson and Liber, 1991). Irradiations were performed with a Philips MG-102 X-ray generator operating at 9.6 mA with 1 mm Al added filtration. The dose-rate to the cells was determined to be approximately 76 cGy/min using a Victoreen ionization chamber and thermoluminescent dosimetry. Doses to the cultures ranged from 0-600 cGy. Cultures were plated for survival and mutation fraction using standard protocols, and mutant fractions were calculated using the method of Furth *et al.* (1981).

Mutant analysis Genomic DNAs were digested with *Sst*I following the recommendations of the supplier (Bethesda Research Laboratories), electrophoresed through 0.8% agarose and transferred to nylon using standard blotting techniques (Maniatis *et al.*, 1982). Hybridization was carried out following the protocol of Glaser *et al.* (1986). Dr. P. Deminger (University Medical Center, New Orleans, LA, USA) provided pTK11, the probe for the TK1 locus (Bradshaw and Deminger, 1984). FISH was carried out on interphase cells using modifications of the protocol of Dr. D. Ward (Lichter *et al.*, 1988) and the probe pHTKB, provided by Dr. Lau (VA Medical Center, San Francisco, CA, USA).

3. RESULTS

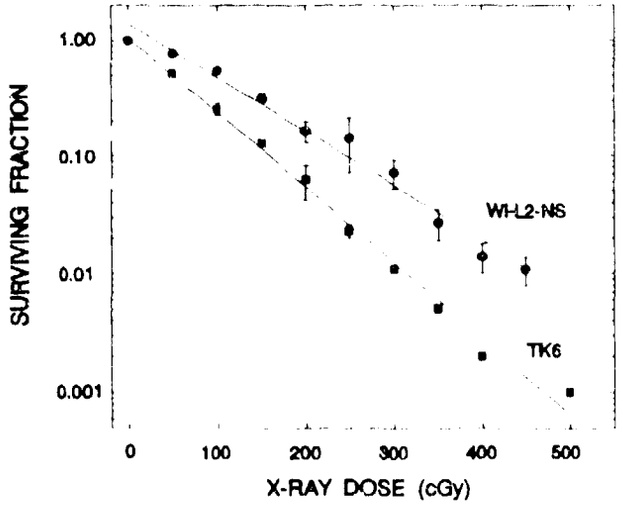
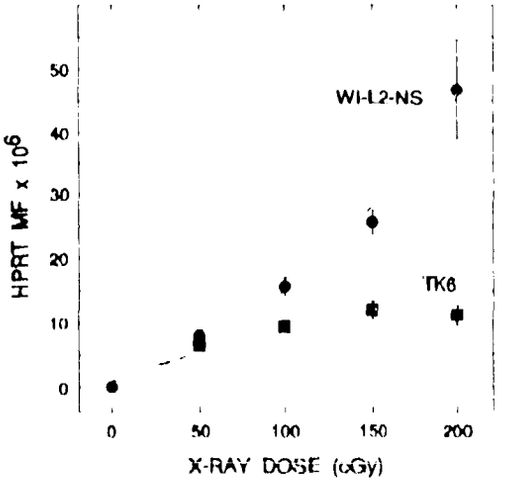


FIGURE 2 X-ray survival of cell lines derived from TK6 and of lines derived from WI-L2-NS. Each point is the average of 7-16 experiments with TK6 lines and 3-13 experiments with WI-L2-NS lines. Error bars are standard error of the mean.

WI-L2-NS and the cell lines derived from it, WTK-1 and WTK-B, exhibited the same dose-response curve for X-ray survival, but this response differed significantly from that of TK6 (figure 2). This difference was due in part to a more pronounced shoulder on the WI-L2-NS survival curve than was observed for TK6. The slopes of the dose response lines also differed for the two cell lineages. The D_0s were calculated to be 67 cGy for TK6 and 93 cGy for the WI-L2-NS lines.

WI-L2-NS, WTK-1, and WTK-B exhibited the same dose-response for X-ray-induced *hprt* mutants (pooled t-test, $p>0.9$). However, this response differed significantly and reproducible from that of TK6 derived cell lines ($p<0.001$). Moreover, the X-ray induction of hprt mutants in the WI-L2 derived lines followed a linear quadratic curve, as opposed to TK6 and its derivative cell lines, which fit very well to a straight line (Figure 3).



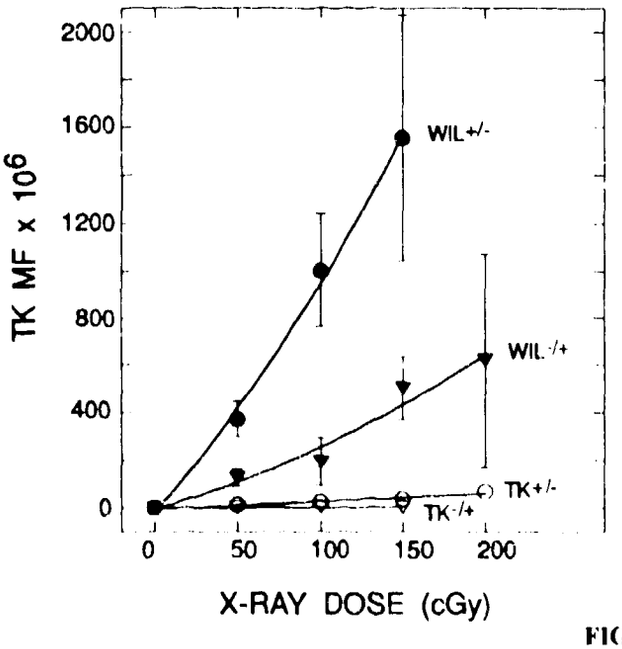
WTK-1 and WTK-B did not appear to be unusually sensitive to mutation at *hprt* induced by ICR-191 ($p>0.9$) or EMS ($p>0.7$), when compared to TK6 (data not shown). The lack of differential response of WI-L2-NS lines to point mutagens suggested that the observed increase in X-ray-induced mutants was likely due to an increased induction of large scale events above the frequency recovered from TK6 cells surviving the same treatment.

FIGURE 3 X-ray dose response for *hprt* mutation in cell lines derived from WI-L2-NS (WTK-1 and WTK-B) and from TK6 (HR3, R6, R7, R9, HR18). Each point is the average of 11-37 (TK6 derived lines) or 9-29 (WI-L2-NS derived lines) experiments. Background mutant fractions were 2-5 x 10^{-6} in all cell lines. Error bars are standard error of the mean.

The background mutant fractions at the *tk* locus were about 130 x 10^{-6} for WTK-1 and 30 x 10^{-6} for WTK-B (figure 4). Following exposure to X-rays, WTK-1 (*tk*+/-) yielded approximately three fold more *tk*- mutants than did WTK-B (*tk*-/-) (see figure 4). This difference was highly significant ($p<0.005$). The *tk*+/- heterozygotes derived from TK6 previously were shown to be 6-10 fold more mutable than the TK6-derived *tk*-/- heterozygotes (Amundson and Liber, 1991). The mutability of the two tk alleles in the WI-L2 derived heterozygotes was therefore qualitatively similar to that seen in TK6 heterozygotes, but of a smaller magnitude. The overall response of WI-L2 tk+/- was 20 fold higher than that of the same allele in TK6, and for the tk-/- heterozygotes, 50 fold higher. The chemical point mutagens EMS and ICR-191 did not produce a significant elevation over the background of

spontaneous *tk* mutants (data not shown). When the background mutant fractions were subtracted, the induced mutant fractions were not significantly different from those in TK6 (EMS, $p>0.2$; ICR-191, $p>0.9$).

WI-L2-NS, WTK-1, WTK-B, and TK6 had no differences in the pattern of heterozygous alleles of five chromosome 17 long arm markers (D17S21, D17S4, D17S77, D17S24, and TK1). Since the 14.8/8.4 TK1 polymorphism was a fortuitous restriction fragment length variation not known to exist elsewhere in the human population (Yandell *et al.*, 1986), its presence in WI-L2-NS reconfirmed the direct relationship of this cell line to TK6.



Southern blot analysis of Tk mutants from TK6 and WI-L2 derived lines was used to determine the extent of loss of heterozygosity. In TK6, 46/81 mutants from *tk*+/- lines, and 20/46 mutants from *tk*-/+ lines had lost heterozygosity at the *tk* locus (Amundson and Liber, 1992). Among mutants from WI-L2-NS heterozygotes, 53/53 from *tk*+/- lines and 53/53 from *tk*-/+ lines had lost heterozygosity at *tk*. Fluorescence *in situ* hybridization of interphase nuclei showed that 23/23 X-ray induced LOH mutants of TK6 retained only one copy of the *tk* gene, while 8/8 mutants of WI-L2-NS heterozygotes had lost heterozygosity, but retained two copies of the tk gene.

FIGURE 4 X-ray dose response for mutation at at the *tk* locus in *tk* heterozygotes derived from WI-L2-NS and from TK6. Each point is the average of 15 (TK6 derived *tk*+/-), 10 (TK6 derived *tk*-/+), 3 (WI-L2-NS derived *tk*+/-), or 3-6 (WI-L2-NS derived *tk*-/+) experiments. Error bars are standard error of the mean for all experiments. Background mutant fractions were approximately 4 x 10^{-6}, 1 x 10^{-6}, 130 x 10^{-6}, and 30 x 10^{-6} for TK6 derived *tk*+/-, TK6 derived *tk*-/+, WI-L2-NS derived *tk*+/-, and WI-L2-NS derived *tk*-/+ respectively.

4. DISCUSSION

In studies of *tk* heterozygotes derived from TK6 we found that one allele (*tk*+ -) was 6-10 fold more mutable by X-rays than the other (*tk*- +). The difference in mutability of these two alleles could be explained by a lack of appearance of a specific class of *tk* mutants, the slow growth mutants (Amundson and Liber, 1991). Slow growth mutants previously had been associated with loss of heterozygosity (LOH). RFLP analysis of mutants from *tk*+ - and *tk*- + heterozygotes revealed more LOH at the *tk* locus in mutants of *tk*+ - lines than in those of *tk*- + lines. Even among mutants which had lost heterozygosity at TK1, LOH was of a greater extent in mutants of *tk*+ - lines. FISH analysis has indicated that the majority of X-ray-induced LOH in TK6 occurred via deletion, as all mutants examined retained only one copy of the *tk* sequence. Densitometric studies of *tk*- mutants of TK6 have implicated recombination in spontaneous mutation, but not X-ray-induced mutation (Li *et al.*, 1992).

We previously suggested a model involving a heterozygous growth locus ("G") to explain the yields of TK-SG and TK-NG mutants recovered from the two alleles of TK6 (Amundson and Liber, 1992). Of the mutants analyzed, all X-ray-induced TK6 mutants which had lost heterozygosity had done so due to deletion of the active allele, suggesting that deletion was the major mechanism for LOH, as predicted by this model.

While deletions presumably occur in WI-L2-NS derived heterozygotes also, another mechanism is evidently producing a far greater yield of mutants in these lines. We have suggested that this increased mutational yield may be due to a recombinational mechanism active in these cells. In WI-L2-NS derived heterozygotes, the *tk+ -* lines were only about 3 fold more mutable than the *tk- +*, but each allele was 25-50 fold more mutable than the corresponding allele in TK6 derived heterozygotes. The yield of late-appearing mutants following 150 cGy X-rays was 81% from WTK-1 and 76% from WTK-B, approximately that seen from TK6. All early mutants analyzed had normal doubling times (TK-NG). However, only about 90% of the late mutants had longer than normal doubling times (TK-SG). Furthermore, after several weeks in culture about half these mutants grew with normal doubling times. This is similar to the behavior of the σ (small) colony *tk-/-* mutants of L5178Y mouse lymphoma cells. After five days in culture, 17-83% of the σ colony mutants had regained normal growth rates. This was attributed to spontaneously arising variants with normal doubling times (Moore *et al.*, 1985). Such a mechanism could explain the transiently slowly-growing mutants observed in WTK-1 and WTK-B, or these mutants could result from the genomic instability that reportedly affects cell division for many generations after X-irradiation (Chang and Little, 1992). If some cells die at each division, this would be observed as a slowing of growth in the culture. If the effect were transient in nature, such a clone could regain a normal growth rate. Currently, it seems that about 40% of the *tk-* mutants from either allele of the WI-L2-NS lines may be true slow growth mutants. The late-appearing mutants in WTK lines do not share all the characteristics of those in the TK6-derived lines, and so may arise via an unrelated mechanism.

Every mutant analyzed from WTK-1 or WTK-B had lost heterozygosity at the *tk* locus, but retained two copies of the gene as evidenced by FISH analysis. This, and evidence from a plasmid based recombination assay (manuscript in preparation) strongly suggests that differential capacities for recombination may explain the increased survival and mutation observed in cell lines derived from WI-L2-NS. The two alleles are still differentially mutable in the WI-L2-NS derived lines, but this difference cannot be explained by recovery of late mutants. This may suggest that there are limitations on the extent of recombinational events which can be recovered from the two homologous chromosomes. These restrictions would appear to be different from those controling deletions in the same system.

Many factors influence the mutant fraction which can be recovered at a particular target locus. Broad differences between hemizygous and heterozygous loci have been well recognized in the past, as well as differences between cell lines derived from different species. Cell lines with repair defects have been shown to have different mutabilities from "normal" cell lines. We have demonstrated differential mutability of the two alleles of the autosomal thymidine kinase locus, and suggested it may be explained by the presence of linked heterozygous essential genes. We have also suggested that recombination may be responsible for the increased mutation at *tk* in more radioresistant related cell lines. As LOH is now held to be an important event in the progression of many cancers, issues involving heterozygous essential genes and recombinational mechanisms should be considered in the interpretation of mutation studies and their extrapolation to human risk estimates. A better understanding of these systems will also aid in the elucidation of the mechanisms which produce mutations relevant to human disease.

ACKNOWLEDGMENTS

This work was supported in part by ACS grant # CN-68 and NIH grant CA-49696. SAA was supported in part by NIH training grant # CA 09078.

5. REFERENCES

Amundson, S.A. and H.L. Liber (1991) *Mutat. Res.*, **247**: 19-27

Amundson, S.A. and H.L. Liber (1992) *Mutat. Res.*, **267**: 89-95.

Amundson, S.A., K.B. Wolfson, F. Xia and H.L. Liber (1993) *Mutat. Res.*,

Benjamin, M.B., H. Potter, D.W. Yandell and J.B. Little (1991) *PNAS (USA)* **88**: 6652-6656.

Bradshaw, H.D. and P.L. Deininger (1984) *Molec. Cell. Biol.*, **4**(11), 2316-2320

Darroudi, F. and A.T. Natarajan (1989) *Mutat. Res.*, **212**: 123-135.

Evans, HH., J. Mencl, MF Horng, M. Ricanati, C. Sanchez and J. Hozier (1986) *PNAS*(USA) **83**:4379-3

Evans, H.H., M. Ricanati, M-F Horng and J. Mencl (1989) *Mutat. Res.*, **217**: 53-63.

Furth, E.E., W.G. Thilly, B.W. Penmann, H.L. Liber and W.M. Rand (1981) *Anal. Biochem.*, **110**: 1-8.

Glaser, T., W.H. Lewis, G.A.P. Bruns, P.C. Watkins, C.E. Rogler, T.B. Shows, V.E. Powers, H.F. Willard, J.M. Goguen, K.O.J. Simola and D.E. Housman (1986) *Nature* **321**:882-887.

Hamilton, A.A. and J. Thacker (1987) *Molec. Cell. Biol.*, **7(4)**: 1409-1414.

Kemp, L.M., S.G. Sedgwick and P.A. Jeggo (1984) *Mutat. Res.*, **132**, 189-196.

Levy, J.L., M. Virolainen and V. Defendi (1968) *Cancer* **22**, 517-524.

Liber, H.L., D.W. Yandell and J.B. Little (1989) *Mutat. Res.*, **216**, 9-17.

Lichter,P., T. Cremer, J. Borden, L. Manuelidis, D.C. Ward (1988) Hum. Genet. 80: 224-

Maniatis, T., E.F. Fritsch, J. Sambrook (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Moore, P.D., K.Y. Song, I. Chekuri, L. Wallace and R.S. Kucherlapati (1986) *Mutat. Res.* **160**: 149-155.

Mussa, T.A.K., B. Singh and P.E. Bryant (1990) *Mutat. Res.*, **231**: 187-193

Shiomi,T., N. HiedaShiomi, K. Sato, H. Tsuji, E. Takahashi and I. Tobari (1981) *Mutat. Res.* **83**:107-16.

Skopek, T.R., H.L. Liber, B.W. Penman and W.G. Thilly (1978) *Biochem. Biophys. Res. Comm.*, **84**, 411-416.

Tatsumi, K. and H. Takebe (1984) *Gann*, **75**: 1040-1043.

Taylor, A.M.R., D.C. Harnden, C.F. Arlett, S. Harcourt, S. Stevens and B.A. Bridges (1975) *Nature (Lond.)* **258**, 427-429.

Waldren, C., C. Jones and T.T. Puck (1979) *PNAS(USA)*, **76(3)**: 1358-1362.

Waldren, C., L. Correll, M.A. Sognier and T.T. Puck (1986) *PNAS (USA)*, **83**: 4839-4843.

Yandell, D.W., T.P. Dryja and J.B. Little (1986) *Somat. Cell Molec. Genet.* **12**: 255-263.