JANUS NEUTRON IRRADIATION OF A MOUSE CELL LINE
CONTAINING A SHUTTLE VECTOR PLASMID

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INTRODUCTION

Recent reports indicate that high LET neutrons are significantly more toxic to biological systems than low LET radiation.¹ On a unit dose basis neutrons are much more effective in transforming cultured cells than are X or gamma rays.² Also, they are 2.7 times more effective in producing chromosomal aberrations³ and 10 times more efficient in inducing mutations in mammalian cells than are gamma rays.⁴ In contrast, certain sources of neutrons are no more effective than gamma rays at producing double strand DNA breaks (DSB) and are actually less effective in producing single strand DNA breaks (SSB).⁵ For example, the relative biological effect (RBE) for JANUS neutron-induced DSBs is 1, while the RBEs for chromosomal aberrations, mutations, cellular transformation, and carcinogenesis are in the range of 4 to 20.

One of the possibilities that may account for the significantly more toxic effect of neutrons to biological systems is the difference in the quality of the lesions produced. Results from investigations in which chemical modifiers of radiation damage were used support this hypothesis. In particular, aminothiol compounds can protect against radiation induced mutagenesis,⁶⁻⁸ transformation⁹ and carcinogenesis.¹⁰⁻¹² Whereas these radioprotectors have been found to protect significantly against both chromosomal aberrations and mutations, they were ineffective in protecting against DSBs induced by neutrons.¹³

The study presented here represents the initial steps of our attempt to characterize JANUS neutron induced mutagenesis in mammalian cells. The approach which we are taking is to use a mammalian cell system which allows one to determine the actual changes in DNA base sequence which occur when a gene mutates. Recently, several systems have been described which make possible the rapid and unambiguous determination of DNA base sequence changes

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in genes of eukaryotic cells.¹⁴ In some of these systems, a target gene is introduced into the mammalian cells as part of a shuttle vector which is capable of replication in both mammalian cells and bacteria. In this study we have used such a system for the analysis of neutron-induced mutations in the presence and absence of the radioprotector N-(2-mercaptoethyl)-1,3-diaminopropane, WR1065.

METHODOLOGY

Cell Culture

The basic cell culture medium was alpha minimal essential medium supplemented with 10% fetal calf serum. HAT medium contains 0.1 mM hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine. G418 medium contains 1 mg of the antibiotic G418 per ml. Selection for 6-thioguanine (TG) resistance was carried out in medium supplemented with 36 μ M TG.

The construction of the A9I2 cell line has been described in detail.^{15,16} Briefly, this line was constructed by the introduction of the retroviral shuttle vector pZip-GptNeo into a mouse fibroblast cell line. As a target gene, this vector contains the *E. coli* gpt gene which codes for the enzyme xanthine (guanine)phosphoribosyltransferase (XPRTase). In addition, this vector contains a 232 bp fragment containing the origin of replication of SV40. The presence of this origin is required for the rapid recovery of integrated vector sequences from mammalian cells. The vector was introduced by infection into the mouse A9 cell line,¹⁷ which is deficient in hypoxanthine (guanine) phosphoribosyltransferase (HPRTase). A stable cell line (A9I2), which contains a single copy of the vector integrated into chromosomal DNA was isolated. Expression of the gpt gene and the neo gene from transposon Tn5 enables the cells to grow in both HAT and G418 medium.

JANUS Neutron Mutagenesis

A9I2 cells were routinely maintained in HAT medium to reduce the number of preexisting mutants at the gpt locus. On the day of irradiation the cells were harvested, counted, and resuspended in 15 ml centrifuge tubes at a density of 1×10^6 cells/ml. Cells were irradiated in the JANUS research reactor at Argonne National Laboratory which provides fission-spectrum neutrons with a mean energy of 0.85 MeV. A dose rate of 24 cGy/min was used in these experiments. Radioprotector WR1065 was present at a concentration of 1 mM only during irradiation. Following irradiation, cells were washed twice with phosphate-buffered saline, recounted, and plated. Cell survival was determined by plating appropriate numbers of cells to give 80-200 surviving colonies. Two weeks after plating, the dishes were stained with 0.5% methylene blue and colonies were counted.

For assays of mutation induction, cells were plated in medium containing hypoxanthine, thymidine, and G418. Cells were grown in this medium for 10 days following irradiation to allow for the expression of mutations. Mutation frequencies were determined by plating cells in 100 mm culture dishes containing medium with TG at a density of 2×10^5 cells/dish. Twenty replicate dishes were set up in each experiment. To determine the plating efficiency in the absence of selection, parallel dishes containing

nonselective medium were inoculated with 200 cells/dish. After 10 days, dishes were stained with 0.5% methylene blue. Mutation frequency is expressed as mutants per 10^6 cells following a correction for plating efficiency at the time of selection.

Recovery of Vector Sequences

Prior to staining, mutant colonies were isolated and expanded for further analysis. To recover vector sequences, each mutant line was fused with monkey COS cells¹⁸ as previously described.¹⁶ Two days later, low molecular weight DNA was extracted by the Hirt procedure¹⁹ and used to transform *E. coli*. Transformants were selected on plates containing kanamycin. To determine the structure of the recovered plasmids, small scale plasmid preparations were made from 2 ml overnight cultures. Restriction digest analysis of recovered plasmids was carried out using KpnI and Xho I digestion and agarose gel electrophoresis. DNA sequencing of plasmid DNA by the dideoxy method was carried out as previously described.¹⁶

RESULTS

Using the A9I2 cell line, we investigated JANUS fission-spectrum neutron-induced cytotoxicity and mutagenicity in the presence and absence of radioprotector WR1065. Cell killing was measured using a colony forming assay, while the mutation frequency was measured at the gpt locus by determining the frequency of TG-resistant mutants. Typical exponential survival curves were obtained with or without WR1065 (Figure 1). The D_{10} values were 67 cGy and 50 cGy in the presence and absence of WR1065. Thus, the radioprotector reduced neutron-induced cytotoxicity in A9I2 cells by a dose modification factor (DMF) of 1.34 (i.e., a ratio of terminal slopes).

The results of the mutation frequency assay are presented in Table 1. A large increase in mutation frequency was observed following irradiation with 50 and 100 cGy. Mutation frequencies of about 100 and 360 mutants per 1×10^6 survivors were observed over a background of about 4 per 10^6 . WR1065 provided substantial protection against the induction of mutations by JANUS neutrons in agreement with previous results.⁴

The protocol for recovery of vector sequences from mutant cell lines uses the COS cell fusion technique.¹⁶ Following fusion, the SV40 origin of replication is apparently activated and the vector sequences are excised from chromosomal DNA by intrastrand recombination between the viral LTRs. The resulting circular molecules have the properties of bacterial plasmid due to the presence of the pBR322 origin of replication and can be recovered from the heterokaryons by the Hirt method¹⁹ and subsequent transformation into *E. coli*.

Restriction digest analysis of the recovered plasmid DNAs was carried out by means of KpnI digestion and agarose gel electrophoresis. Digestion of recovered plasmids having intact gpt genes with KpnI should produce three fragments, approximately 3.5, 0.9, and 0.7 kb long. The plasmid molecules recovered from mutant cell lines yielded a variety of restriction patterns (Figure 2). In many of the mutants recovered from irradiated cultures, the recovered plasmids had altered the KpnI restriction patterns.

Because of the limited amount of data obtained thus far no conclusion can be reached as of yet concerning the effect of WR1065 on neutron irradiated cells. Only two mutants were analyzed, and one of them exhibited a normal restriction enzyme pattern (Figure 3). Profiles of additional, selected mutants are shown in Figures 4 and 5.

While the number of mutants characterized is limited and thus precludes any specific conclusions, examples of the mutations analyzed are listed in Table 2. Some mutants (e.g., 15S1 and 12A2) had small three-base deletions within the gpt sequence. Base substitutions were observed in some mutants (e.g., 13A3, 17A2, and 17W3) involving G:C to T:A transversions and G:C to A:T transitions within the gpt coding sequence. All of the gpt gene was missing from plasmid recovered from many mutants. These plasmids all contain vector DNA joined to nonvector DNA, presumably host chromosomal DNA. In the mutant designated 16S7, a duplication 66 base pairs occurred near vector-nonvector DNA junction. The vector-nonvector junction in this mutant was identical to that determined in 15A2, 17S1 and 15WA3. One of the two mutants obtained by neutron irradiation in the presence of the radioprotector WR1065 also exhibited this same mutation while the other had a base substitution of G:C to T:A at base 304 of the gpt gene. Plasmid recovered from the mutant designated 17A4 had a different vector-nonvector junction. We have not yet determined how these mutations arose in A9I2 cells. Possible mechanisms include deletion, insertion, gene amplification, inversion, or translocation.

DISCUSSION

We have demonstrated the potential of a shuttle vector system to study neutron induced mutagenesis in the presence or absence of chemical modifiers such as the radioprotector WR1065. The vector, pZipGptNeo was stably maintained in cells grown under selective conditions, and exhibited a low spontaneous mutation rate. The exposure of plasmid-bearing mouse cells to JANUS fission-spectrum neutrons resulted in a dose-dependent increase in the mutation frequency for the plasmid gpt gene. Many of the TG^R mutants analyzed by DNA sequencing were shown to carry rearrangements of vector sequence (Table 2). However, due to the small number of mutants analyzed, these results should be evaluated in the context of preliminary data that demonstrate the utility of the system for the assessment of radiation induced molecular damage.

The reduction in the frequency of mutation induction in the presence of radioprotector WR1065 in this study suggests that similar protection is afforded to the integrated gpt gene as has been reported for cellular genes.⁶ It has been proposed that thiols such as cysteamine stabilize the chromatin and thus reduce recombination events.²⁰ Evidence of this reduction has been demonstrated for chromosomal aberration-formation in neutron irradiated cells.³

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REFERENCES

1. C. K. Hill, F. M. Buonaguro, C. P. Myers, A. Han and M. M. Elkind, Fission-spectrum neutrons at reduced dose rates enhance neoplastic transformation. Nature **289**, 67-69 (1982).
2. C. K. Hill, B. A. Carnes, A. Han and M. M. Elkind, Neoplastic transformation is enhanced by multiple low dose of fission-spectrum neutrons. Radiat. Res. **102**, 404-410 (1985).
3. J. L. Schwartz, S. M. Giovanazzi, T. Karrison, C. Jones and D. J. Grdina, 2-[(Aminopropyl)amino] ethanethiol (WR1065)-mediated reductions in ^{60}Co gamma ray and fission-spectrum neutron-induced chromosome damage in V79 cells. Radiat. Res. **113**, 145-154 (1988).
4. D. J. Grdina, B. Nagy, and C. P. Sigdestad, Radioprotectors in treatment therapy to reduce risk in secondary tumor induction. Pharmacology and Therapeutics **37**, 21-25 (1988).
5. K. M. Prise, S. Davies and B. D. Michael, The relationship between radiation-induced DNA double-strand breaks and cell killing in hamster V79 fibroblasts irradiated with 250 kVp X-rays, 2.3 MeV neutrons or ^{238}Pu α -particles. Int. J. Radiat. Biol. **52**, 893-902 (1987).
6. D. J. Grdina, B. Nagy, C. K. Hill, R. L. Wells and C. Peraino, The radioprotector WR1065 reduces radiation-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in V79 cells. Carcinogenesis **6**, 929-931 (1985).
7. B. Nagy, P. J. Dale and D. J. Grdina, Protection against cis-diamminedichloroplatinum cytotoxicity and mutagenicity in V79 cells by 2-[(aminopropyl)amino] ethanethiol. Cancer Res. **46**, 1132-1135 (1986).
8. B. Nagy and D. J. Grdina, Protective effects of 2-[(aminopropyl)amino] ethanethiol against bleomycin and nitrogen mustard-induced mutagenicity in V79 cells. Int. J. Radiat. Oncol. Biol. Phys. **12**, 1475-1478 (1986).
9. C. K. Hill, B. Nagy, C. Peraino and D. J. Grdina, 2-[(aminopropyl)amino] ethanethiol (WR1065) is anti-neoplastic and anti-mutagenic when given during ^{60}Co γ -ray irradiation. Carcinogenesis **7**, 665-668 (1986).
10. L. Milas, N. Hunter, C. L. Stephens and L. J. Peters, Inhibition of radiation carcinogenesis by S-2(3-aminopropylamino)-ethylphorothioic acid. Cancer Res. **44**, 5567-5569 (1984).
11. C. A. Apffel, J. E. Walker and S. Issarescu, Tumor rejection in experimental animals treated with radioprotective thiols. Cancer Res. **35**, 429-437 (1975).
12. D. J. Grdina, C. Peraino, B. A. Carnes and C. K. Hill, Protective effect of WR2721 against induction of altered hepatocyte foci in rats treated once with gamma radiation within one day after birth. Cancer Res. **45**, 5379-5381 (1985).
13. D. J. Grdina, C. P. Sigdestad, P. J. Dale, and J. M. Perrin, the effect of 2-[(aminopropyl)amino] ethanethiol on fission-neutron-induced DNA damage and repair. Br. J. Cancer **59**, 17-21 (1989).
14. R. B. DuBridge and M. P. Calos, Recombinant shuttle vectors for the study of mutation in mammalian cells. Mutagenesis **3**, 1-9 (1988).
15. C. R. Ashman, Retroviral shuttle vectors as a tool for the study of mutational specificity. Mutation Res. **220**, 142-149 (1989).

16. C. R. Ashman, P. Jagadesswaran and R. L. Davidson, Efficient recovery and sequencing of mutant genes from mammalian chromosomal DNA. Proc. Natl. Acad. Sci. USA 83, 3356-3360 (1986).
17. J. W. Littlefield, Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145, 709-710 (1964).
18. Y. Gluzman, SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23, 175-182 (1981).
19. B. Hirt, Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26, 365-369 (1967).
20. P. E. Brown, Mechanism of action of aminothiol radioprotectors. Nature 213, 363-365 (1967).

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LEGENDS TO FIGURES

- Figure 1. Toxicity of JANUS neutrons to A9I2 cells in the absence of (o) or presence (●) of WR1065. Radioprotector WR1065 at 1 mM was present only during irradiation. The surviving fractions were determined by a colony forming assay 10 days after treatment. Lines were fitted by a least-squares analysis. Error bars are the standard error of the mean of three independent determinations.
- Figure 2. Digestion with KpnI of mutant plasmids isolated from mutants obtained following exposure to JANUS fission-spectrum neutrons designated 17A1 and 17S3.
- Figure 3. Digestion with KpnI of a plasmid isolated from a mutant designated 17S1 (without WR1065), and from a plasmid designated, 17WA3 with (WR1065) following exposure to JANUS fission-spectrum neutrons.
- Figure 4. Gel electrophoresis of plasmids isolated from mutants designated, 17A4, 17S4, 17S5, following exposure to JANUS fission-spectrum neutrons and from a plasmid isolated from a spontaneous mutant designated, 1701.
- Figure 5. Gel electrophoresis of plasmids isolated from mutants designated 17S1-2 and 17WA3, obtained following exposure to JANUS fission-spectrum neutrons, and a spontaneous mutant designated, 1703.

Table 1. JANUS Neutron-Induced Mutation Frequencies

Dose (cGy)	WR1065	TG ^R Mutants/10 ⁶ Cells
50	-	106
50	+	6
100	-	361
100	+	11

Table 2. Sequence Analysis of TG^R Mutants

Mutant	Dose (cGy)	WR1065	Alteration
1301	0	-	G:C to A:T at 145
1701	0	-	G:C to C:G at 139
15A2	50	-	Vector-nonvector junction in <u>neo</u> fragment
17A2	50	-	G:C to T:A at 304
17A4	50	-	Vector-nonvector junction in MoMLV sequence
15WA3	50	+	Same as 15A2
17WA3	50	+	Same as 17A2
15S1	50	-	3 bp deletion at 367-369
16S7	50	-	Same as 15A2 with 66 bp duplication near junction
17S1	50	-	Same as 15A2
13A3	100	-	Same as 1301
12A2	200	-	Same as 15S1

3.7
1.9
1.3
0.7

17S1 (KpnI)

1 2 3 4 5 6

17WA3 (KpnI)

1 2 3 4 5 6

λ

3.7

1.9

1.3

0.7

λ

17A4-1

17A4-3

17S4-1

17S4-2

pSV₂-gpt

17O1-1

17O1-3

17S5-1

17S5-2

λ

17WA3-2

17WA3-4

1703-3

1703-6

pSV₂-gpt

17S2-1

17S2-2

17S1-1

17S1-2

2