ANNUAL REPORT
U.S. Department of Energy

TITLE: Adaptive response against spontaneous neoplastic transformation induced by low dose ionizing radiation.

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Executive Summary

This research project addresses the key question "Do low doses of ionizing radiation protect against spontaneous neoplastic transformation?". The experimental system employed to address this question is the HeLa x skin fibroblast human hybrid cell system with which the investigators have over 12 years of experience and, importantly, with which they have made several observations which can be expanded upon to address the question posed above. The endpoint employed is neoplastic transformation frequency. Two radiation sources will be used. These are Cs-137 gamma rays (0.66 MeV gamma energy) and 80 kVp X-rays used in fluoroscopy. While these two energies of photons are in what is normally considered the low LET range (0.4 to 5.0 keV/u), they do have a difference in biological effectiveness of perhaps a factor of 2. Notice 99-14 specifically noted the need to quantify the generality and extent of the apparent adaptive response in cells irradiated with small doses of radiation. This is the focus of the proposed effort. Total doses to be used, whether single or fractionated, will be no more than 10 cGy. Appropriate attention is given to the necessity for accurate dosimetry. The experimental protocol is designed such that the data obtained will be amenable to the rigorous statistical analysis so essential to evaluate low dose effects. The investigators have already demonstrated that irradiation with a single dose of 1 cGy results in a neoplastic transformation frequency that is a factor of two LOWER than that of sham-irradiated cells (Radiation Research, 149, 517-520, 1998). It is proposed to expand on this observation by establishing a dose-response relationship (e.g. 0.1, 1.0 and 10.0 cGy) as well for repeated low dose exposures (e.g. 10 x 0.1 cGy and 10 x 1 cGy). These proposed experiments directly address the question posed in Notice 99-14. Transformation frequencies will be compared to those predicted by linear extrapolation of high dose data (2 and 4 Gy) through the spontaneous background frequency. This will provide an estimate of the factor by which such linear extrapolations can overestimate low dose data. It is also proposed to take advantage of another observation in the investigators laboratory to address, albeit indirectly, the question of the role of repair in this response. The investigators have previously shown that post-irradiation holding at room temperature prior to plating for focus formation results in enhanced cell kill and enhanced neoplastic transformation, most likely through the promotion of misrepair. It is now hypothesized that if the adaptive response involves the induction of a repair process then the reduction in transformation frequency following exposure to 1 cGy would be abrogated by post-irradiation holding at room temperature.

The research design for this project described three phases (IA & IB, II and III). Phase IA was designed to be the first to be tackled and consisted of expanding our initial observation using a dose of 1 cGy to include studies with 0.1 and 10.0 cGy. It was estimated that this phase would take 6 to 9 months to complete. At the time of writing this report we are at the end of the first 9 months. The initial step was to screen serum lots for use over the whole proposed funding period. This component of the study took longer than expected and we ended up screening 15 lots of serum between October 1999 and March 2000. The experimental design for phase IA required six repeats of a protocol which included eight arms: 0, 0.1, 1.0, and 10.0 cGy with both immediate and delayed post-irradiation plating. This number of repeats were considered necessary for rigorous statistical analysis. Three of these repeats were accomplished over the period November through January using the best of the initial five serum lots screened. Unfortunately, the spontaneous (0 cGy) transformation frequencies were lower than had been obtained in the initial serum screen, and too low to hope to be able to obtain decent statistics for the experiment. After some trouble shooting regarding optimizing incubator pH it was decided to screen further serum lots before proceeding any further. This was done and a new serum lot was selected for the remainder of the study. Since then we have completed one experiment.

Due to problems with serum lots we are approximately 3 months behind our anticipated schedule. It is too early to conclude anything from the data gathered to date.

Research Objectives

This project is being conducted to ascertain the shape of the dose response curve for neoplastic transformation in vitro over the dose range 0.0 to 10 cGy, and how this depends on radiation quality and dose fractionation. Preliminary data already have indicated that at a dose of 1 cGy the induced transformation frequency is less than the spontaneous transformation frequency. The results will be compared with animal and human epidemiological data on the induction of cancer by low doses of radiation. This will hopefully allow for a more informed estimation of the risk of cancer induction at low doses (see Pollycove, 1998; Rossi, 1999).

Methods and Results

Cell system

The non-tumorigenic HeLa x skin fibroblast cell line CGL1 (Stanbridge et al., 1981) is maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 5% calf serum, 2mM glutamine, non-essential amino acids and 100 IU/ml penicillin (growth medium). Cultures are incubated in 5% CO₂ in air at 37°C and routinely tested for mycoplasma contamination by the 4,6-diamidino-2-phenylindole (DAPI) assay (Russell et al., 1975). Since it is well known that serum lot number can effect both spontaneous and induced transformation frequencies, the same serum lot number will be used for all the experiments reported. An initial phase of the study has been to screen several (15) lots of serum for spontaneous and high radiation-dose (4 Gy) induced transformation frequency. A serum lot will be selected which gives an intermediate level of spontaneous transformation (around 5×10^{-5}). This will make the experiments logistically reasonable. Cells in the population doubling (cell doubling) number range 43 to 45 will be used.

Gamma Irradiation conditions

The cells as adherent subconfluent cultures in T-75 flasks are irradiated in a Shepherd and Associates Mark I self-shielded ¹³⁷Cs gamma irradiator. The dose-rate can be adjusted from 200 cGy/min to 0.33 cGy/min by the use of attenuators. For the proposed studies a dose-rate of 0.33 cGy/min will be used for all low dose studies (0.1, 1.0, 10.0 cGy). For high dose studies (2 and 4 Gy) a dose-rate of 1.5 Gy/min will be used. For sham irradiated cell the flasks will be put in the irradiator with the source in the off position for the same period of time as the cells to be irradiated.

Dosimetry

¹³⁷Cs gamma dosimetry was performed using a Victoreen dosimeter cross calibrated on a Varian Clinac 600C. The dosimeter was placed in the irradiation position of the flask. Flatness of field will be checked to ensure uniformity across the irradiated area.

Protocol for quantitative assay of neoplastic transformation

Cells are seeded into T-75 flasks such that 2 to 3 days later they are approaching confluence (10^6 cells/flask) at which point they are irradiated. Identical control cultures are sham-irradiated, i.e. taken to and placed inside the irradiator with the source in the "off" position. Following completion of treatment, the cells are subcultured and plated into T-25 flasks for assay of clonogenic survival at 10 days post-irradiation, and into T-75 flasks for assay for foci expressing cell surface intestinal alkaline phosphatase (IAP) at 21 days post-irradiation using the Western Blue staining method which we developed (Mendonca et al., 1992). IAP is a marker for neoplastic transformation in this system (Mendonca et al., 1991). Our previous studies have shown that the transformation frequency is dependent upon the density of viable cells plated post-irradiation (Sun et al., 1988) and we attempt to control this parameter as carefully as possible. We aim to plate at a viable cell density of 50 cells/cm². However, if necessary, we are able to correct for variations in viable cell density as described previously (Sun et al., 1988). In the past related study (Redpath and Antoniono, 1998), minimal corrections for cell density (+/- 4%) were needed. The scoring of IAP positive foci is done by two independent scorers under blinded conditions as to the treatment. Cells are refed at days 5, 10, 14, 17 and 20 post-irradiation.

Calculation of transformation frequency

Transformation frequency can be expressed in three different ways from the same set of raw data. These are fraction of flasks containing foci, foci per flask, and foci per surviving cell. We propose to analyze our data sets using each these definitions.

Statistical Analysis

The null method (Han and Elkind, 1979; Balcer-Kubiczek et al. 1987; Redpath and Antoniono, 1998) is used to calculate transformation frequencies expressed as foci per surviving cell. This method is used to avoid possible errors from satellite colony formation as a consequence of the multiple refeedings which cultures must receive during the 21 day post irradiation expression period. The null method compares the average number of transformed foci per dish ($\bar{x}$) with the fraction of flasks without foci ($f = n/N$) and assumes that the potential number of foci follows a Poisson distribution ($\bar{x} = -\ln f = -\ln (n/N)$). The null method also allows for calculation of the standard error and 95% confidence intervals. Data is also presented as fraction of flasks containing foci and number of foci per flask.

Thus, for all proposed experiments, there are three dependent measures to be ascertained from each flask: 1) number of foci per surviving cell, 2) number of foci per flask, and 3) the fraction of flasks with foci. Statistically, the first two measures are count data. Therefore we propose Poisson regression as the general framework to evaluate these data (Cameron and Trivedi, 1998). The presence or absence of foci in each flask will be modeled with logistic regression (Hosmer & Lemeshow, 1989). For each of the three dependent variables, the statistical goal is to estimate the change in the dependent measure as a function of dose of radiation, relative to sham irradiation. This estimation will include the specification of confidence bounds.

Research Design

All of the experiments involve immediate and delayed post-irradiation plating. The delayed plating arm is included because of the documented necessity to allow time for the induction of the adaptive response in irradiated cells (Joiner, 1994). We have elected for a post-irradiation holding time of 24 hours at 37°C since that has been previously shown to demonstrate an adaptive response in transformation studies (Azzam et al., 1996; Redpath and Antoniono, 1998).

Serum Screening

We screened 15 lots of serum over the period October 1999 through March 2000. The results of those serum tests are shown in Table 1. We began our radiation studies in December using serum lot JRH8K2236. Since we were unhappy with the spontaneous transformation frequencies we were seeing in the early radiation studies, which were lower than that seen in the serum screen, we initiated additional serum screens as well as making an adjustment in the incubator pH since pH is known to influence transformation frequencies in this system (Mendonca et. al., 1991). At the end of March 2000 we completed the last serum screen and selected a new serum lot to be used for the remainder of the study.

Phase IA

Apart from the initial phase of screening serum lots, the first phase of the proposed study is to determine if there is a dose-response relationship in the induction of an adaptive response. The research design consists of four different radiation treatments (0, 0.1, 1.0, 10.0 cGy) and two different post-irradiation manipulations (immediate plating, and hold at 37°C for 24h and plate). Each combination of radiation treatment and post-irradiation manipulation will be run at the same time and it is planned to be repeated six times. This design is perhaps best envisaged in the matrix below. The original design stipulated 30 T-75 flasks per treatment condition for each of the six repeats. In view of the lower than desired spontaneous transformation frequency we have increased this number to 48 T-75 flasks per treatment condition.

Dose (cGy)	immediate plating	37°C delayed plating
0**	48*	48
0.1	48	48
1.0	48	48
10.0	48	48

* = number of T-75 flasks

** sham irradiation

It was anticipated that these experiments would take 6 to 9 months to complete. To date we have done three experiments with the suboptimal serum lot (JRH8K2236). We did these while we rescreened additional lots of serum. The results from these experiments are summarized in Table 2. We have also run one experiment with a new, more optimal serum lot (JRH7C2059). The results from this experiment are summarized in Table 3. It may be noted from the data in Table 2 that the experimental variation in spontaneous transformation frequency within experiments with a given serum lot is almost as large as that that is seen between individual serum lots (Table 1). This is unfortunate but is a common finding in all in vitro transformation assays. It is our hope that the experimental design of many experiment repeats with large numbers of flasks (and therefore cells at risk) will allow for the minimization of this potentially confounding effect on the experimental results.

It is too early yet to draw any firm conclusions from the data, although the presence of an adaptive response has not been detected to date.

Relevance, Impact and Technology Transfer

The proposed experiments will provide quantitative data on the existence and magnitude of a possible (see preliminary data) adaptive response against neoplastic transformation by ionizing radiation. The doses used will be 10 cGy or less and including fractionated exposures (e.g. 10 x 0.1 cGy). Radiation sources of covering the low LET range of 0.5 to 5.0 keV/u will be employed. This will provide information as to possible RBE effects in the low LET range of relevance to environmental cleanup. Information will be generated regarding the factor by which linear extrapolation of high dose data may overestimate low dose responses.

Project Productivity

The project is running about three months behind schedule due to unforeseen difficulties in screening and selecting for a suitable serum lot with which to carry out the project.

Personnel Supported

J. Leslie Redpath, Ph.D.
Eugene Elmore, Ph.D.
Lily Mechetner, B.S.

Publications

None so far.

Interactions

None so far.

Transitions

None so far.

Patents

None so far.

Future Work

The majority of the originally proposed work remains to be done. We should be finished the single dose studies within the next 4 months and then we will move to the fractionated dose experiments. Following that we will carry out the comparison of 80 kVp x-rays and Cs-137 gamma rays at a single dose. Finally, we will carry out the proposed repair inhibition studies to determine if the adaptive response is indeed a repair related phenomenon.

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Feedback

NA

Appendices

NA

Quantities/Packaging

NA

PROJECT TITLE

Adaptive response against spontaneous neoplastic transformation in vitro induced by ionizing radiation

Principal Investigator: J. Leslie Redpath, Ph.D., Radiation Oncology, UC Irvine.

Co-Investigators: E. Elmore, Ph.D., Radiation Oncology, UC Irvine.
T. Taylor, Ph.D., Epidemiology, UC Irvine.

PROJECT ABSTRACT

The following paragraphs outline a project which addresses the area "Understanding biological responses to radiation and endogeneous damage". The proposed research addresses the following key questions: (a) "How much do low doses of radiation protect against subsequent low doses of ionizing radiation?". The experimental system to be employed to address these questions is the HeLa x skin fibroblast human hybrid cell system with which the investigators have over 12 years of experience and, importantly, with which they have made several observations which can be expended upon to address the question posed above. The endpoint to be employed is neoplastic transformation frequency. Two radiation sources will be used. These are Cs-137 gamma rays (0.66 MeV gamma energy) and 80 kVp X-rays used in fluoroscopy. While these two energies of photons are in what is normally considered the low LET range (0.4 to 5.0 keV/u), they do have a difference in biological effectiveness of perhaps a factor of 2. Notice 99-14 specifically notes the need to quantify the generality and extent of the apparent adaptive response in cells irradiated with small doses of radiation. This is the focus of the proposed effort. Total doses to be used, whether single or fractionated, will be no more than 10 cGy. Appropriate attention is given to the necessity for accurate dosimetry. The experimental protocol is designed such that the data obtained will be amenable to the rigorous statistical analysis so essential to evaluate low dose effects. **The investigators have already demonstrated that irradiation with a single dose of 1cGy results in a neoplastic transformation frequency that is a factor of two LOWER than that of sham-irradiated cells (Radiation Research, 149, 517-520, 1998).** It is proposed to expand on this observation by establishing a dose-response relationship (e.g. 0.1, 1.0 and 10.0 cGy) as well for repeated low dose exposures (e.g. 10 x 0.1 cGy and 10 x 1 cGy). These proposed experiments directly address the question posed in Notice 99-14. Transformation frequencies will be compared to those predicted by linear extrapolation of high dose data (2 and 4 Gy) through the spontaneous background frequency. This will provide an estimate of the factor by which such linear extrapolations can overestimate low dose data. It is also proposed to take advantage of another observation in the investigators laboratory to address, albeit indirectly, the question of the role of repair in this response. The investigators have previously shown that post-irradiation holding at room temperature prior to plating for focus formation results in enhanced cell kill and enhanced neoplastic transformation, most likely through the promotion of misrepair. It is now hypothesized that if the adaptive response involves the induction of a repair process then the reduction in transformation frequency following exposure to 1 cGy would be abrogated by post-irradiation holding at room temperature.

PROGRESS REPORT – 3/10/00

The research design for this project described three phases (IA & IB, II and III). Phase IA was designed to be the first to be tackled and consisted of expanding our initial observation using a dose of 1 cGy to include studies with 0.1 and 10.0 cGy. It was estimated that this phase would take 6 to 9 months to complete. The initial step was to screen serum lots for use over the whole proposed funding period. This was done and a serum lot was chosen (September and October). The experimental design for phase IA required six repeats of a protocol which included eight arms: 0, 0.1, 1.0, and 10.0 cGy with both immediate and delayed post-irradiation plating. This number of repeats were considered necessary for rigorous statistical analysis. Three of these repeats were accomplished over the period November through January. Unfortunately, the spontaneous (0 cGy) transformation frequencies were lower than had been obtained in the initial serum screen, and too low to hope to be able to obtain decent statistics for the experiment. After some trouble shooting regarding optimizing incubator pH it has been decided to screen further serum lots before proceeding any further. We are in the middle of that now.

Face Page (DOE F 4650.2 (10-91)

Adaptive response against spontaneous neoplastic transformation induced by low dose ionizing radiation.

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PROJECT ABSTRACT

The following paragraphs outline a project which addresses the area "Understanding biological responses to radiation and endogeneous damage". The proposed research addresses the following key questions: (a) "How much do low doses of radiation protect against subsequent low doses of ionizing radiation?". The experimental system to be employed to address these questions is the HeLa x skin fibroblast human hybrid cell system with which the investigators have over 12 years of experience and, importantly, with which they have made several observations which can be expended upon to address the question posed above. The endpoint to be employed is neoplastic transformation frequency. Two radiation sources will be used. These are Cs -137 gamma rays (0.66 MeV gamma energy) and 80 kVp X-rays used in fluoroscopy. While these two energies of photons are in what is normally considered the low LET range (0.4 to 5.0 keV/u), they do have a difference in biological effectiveness of perhaps a factor of 2. Notice 99-14 specifically notes the need to quantify the generality and extent of the apparent adaptive response in cells irradiated with small doses of radiation. This is the focus of the proposed effort. Total doses to be used, whether single or fractionated, will be no more than 10 cGy. Appropriate attention is given to the necessity for accurate dosimetry. The experimental protocol is designed such that the data obtained will be amenable to the rigorous statistical analysis so essential to evaluate low dose effects. The investigators have already demonstrated that irradiation with a single dose of 1cGy results in a neoplastic transformation frequency that is a factor of two LOWER than that of sham-irradiated cells. It is proposed to expand on this observation by establishing a dose-response relationship (e.g. 0.1, 1.0 and 10.0 cGy) as well for repeated low dose exposures (e.g. 10 x 0.1 cGy and 10 x 1 cGy). These proposed experiments directly address the question posed in Notice 99-14. Transformation frequencies will be compared to those predicted by linear extrapolation of high dose data (2 and 4 Gy) through the spontaneous background frequency. This will provide an estimate of the factor by which such linear extrapolations can overestimate low dose data. It is also proposed to take advantage of another observation in the investigators laboratory to address, albeit indirectly, the question of the role of repair in this response. The investigators have previously shown that post-irradiation holding at room temperature prior to plating for focus formation results in enhanced cell kill and enhanced neoplastic transformation, most likely through the promotion of misrepair. It is now hypothesized that if the adaptive response involves the induction of a repair process then the reduction in transformation frequency following exposure to 1 cGy would be abrogated by post-irradiation holding at room temperature.

RELEVANCE TO ENVIRONMENTAL MANAGEMENT

Diagnostic radiologic examinations are a major source of low dose radiation exposure to the general public. However, over the next 100 years a new major population cohort is projected to be exposed to low doses and dose-rates through the process of clean-up of materials associated with nuclear weapons and nuclear power production. The projected costs associated with protection of workers and the public against such low doses of radiation are enormous. If it could be demonstrated that the risks associated with such exposures are lower than currently estimated then significant savings could be realized and applied to more critical issues in health care. The experiments outlined in this proposal specifically address the need stated in Notice 99-14 to quantify the generality and extent of the apparent adaptive response in cells irradiated with small doses of radiation. Moreover, the endpoint to be employed is neoplastic transformation in vitro which is highly relevant to the major human risk associated with low dose radiation exposure, namely cancer induction. While it is well recognized that quantifying the effects of low doses presents a considerable challenge, arguments are presented that with sufficient care given to experimental design such that the data will be amenable to the most rigorous of statistical analysis, the necessary quantitation can be achieved.

BUDGET EXPLANATION - YEAR 1

A. Senior Personnel

J. Leslie Redpath Ph.D., Principal Investigator, 20% effort. Dr. Redpath will be responsible for the overall management of the project. Together with the consultant statistician he will decide on the experimental design and oversee the final data analysis, present the data at necessary meetings, prepare any required reports, and write manuscripts for publication in the peer-reviewed literature.

E. Elmore, Ph.D., Co-investigator, 30% effort. Dr. Elmore is a cell biologist with over 25 years experience in studies of cellular transformation and mutagenesis. He has recently gained experience in the hybrid cell transformation assay to be employed in the proposed experiments. He will be responsible for the day to day supervision of the technical staff on the project and will report to Dr. Redpath weekly on the status of the project. He will also assist in the performance of the experiments.

Thomas H Taylor, PhD, Co-investigator, 15% effort. Dr. Taylor is a Senior Biostatistician with the Epidemiology Division, Department of Medicine, and the Chao Family Comprehensive Cancer Center, at the University of California, Irvine. Dr Taylor will supervise the computerization of data, including at least 10-percent double entry for quality assurance. Dr Taylor will be responsible for the Poisson regressions, the bootstrapping operations, the construction of statistical tests and communication of statistical results.

B. Other Personnel

M. Wakabayashi, B.S., Laboratory Assistant, 100% effort. Ms. Wakabayshi is a full time technician who will carry out the transformation assays. She has been in Dr. Redpath's laboratory for over one year and is well trained in tissue culture.

C. Fringe Benefits

Fringe benefits are calculated with actual rates at 27% for the senior personnel and 23% for the other personnel

D. Equipment

None

E. Travel

It is planned that the P.I. will attend two meetings per year related to this project. These would be the annual meeting of the Radiation Research Society and either a DOE meeting called specifically with respect to this project or, in the absence of such a meeting, the NCRP annual meeting.

One RRS meeting, 5 days (\$200 reg., \$500 transportation, \$600 hotel, \$300 meals)=\$1,600

One DOE/NCRP meeting, 2-3 days (\$500 transportation, \$300 hotel, \$120 meals)=\$920

Total = \$2,520

F. Trainee/Participant Costs

None

G. Other Direct Costs

1. Materials and Supplies

This is a tissue culture intensive study. Based on the experiments outlined in the experimental design section, and our past experience, we have estimated the following needs on an annual basis:

Media (Autopow MEM), 200 litres at \$14.13/10L	\$282.60
Calf Serum , 10 litres at \$28.00/500ml	\$560.00
T-75 flasks, 5000 T-75 flasks at \$67.90/100	\$3395.00
T-25 flasks, 500 T-25 flasks at \$150/500	\$150.00
1 ml disposable pipettes, 1 case at \$180.00/case	\$180.00
5 ml disposable pipettes, 30 boxes at \$60.00/box	\$1,800.00
10 ml disposable pipettes, 15 boxes at \$63.00/box	\$945.00
Pipettor tips, 10 pkgs. at \$125.00/pkg.	\$1,250
Western Blue, 22 litres at \$33.00/100ml	\$7260.00
Miscellaneous chemicals	\$1,000.00
<hr/> Total	<hr/> \$16,822.60

2. Publication/Documentation/Dissemination Costs

Estimated costs are \$500.00/year \$500.00

3. *Consultant Services*
None

4. *Computer Services*
None

5. *Subcontracts*
None

6. *Other*
None

BUDGET EXPLANATION - YEARS 2 and 3

A. Senior Personnel

Same as Year 1. Salaries have been calculated to include any projected merit increases as well a 4% cost of living increase per year.

J. Leslie Redpath Ph.D., Principal Investigator, 20% effort.

E. Elmore, Ph.D., Co-investigator, 30% effort.

T. H. Taylor, Ph.D., Co-investigator 15% effort.

B. Other Personnel

Same as Year 1. Salaries have been calculated to include any projected merit increases as well a 2% annual range adjustment.

M. Wakabayashi, B.S., Laboratory Assistant, 100% effort.

C. Fringe Benefits

Fringe benefits are calculated with actual rates at 27% for the senior personnel and 23% for the other personnel

D. Equipment

None

E. Travel

Same as Year 1 adjusted at 4%/year for inflation.

Year 2 Total = \$2,621

Year 3 Total = \$2,726

F. Trainee/Participant Costs

None

G. Other Direct Costs

1. Materials and Supplies

Same as Year 1 adjusted at 4%/year for inflation.

Year 2 Total = \$17,496

Year 3 Total = \$18,195

2. Publication/Documentation/Dissemination Costs

Same as Year 1 adjusted at 4%/year for inflation.

Year 2 Total = \$520

Year 3 Total = \$541

3. Consultant Services

None

4. Computer Services

None

5. Subcontracts

None

6. Other

None

PROJECT DESCRIPTION

A. GOAL

The goal of this proposal is to provide quantitative data on the neoplastic transformation frequency of human hybrid cells in vitro irradiated with low doses (up to 10 cGy) of Cs-137 gamma rays and 80 kVp x-rays for comparison with the transformation frequency in sham irradiated cells. The experiments will be designed such that the data are amenable to rigorous statistical analysis. Care will be taken to minimize the influence of variables which can influence the outcome of such experiments. For example by using the same serum lot for all of the proposed experiments. Transformation frequencies will be compared to those predicted by linear extrapolation of high dose data (2 and 4 Gy) through the spontaneous background frequency. This will provide an estimate of the factor by which such linear extrapolations can overestimate low dose data.

B. SIGNIFICANCE TO ENVIRONMENTAL MANAGEMENT NEEDS.

The proposed experiments will provide quantitative data on the existence and magnitude of a possible (see preliminary data) adaptive response against neoplastic transformation by ionizing radiation. The doses used will be 10 cGy or less and including fractionated exposures (e.g. 10 x 0.1 cGy). Radiation sources of covering the low LET range of 0.5 to 5.0 keV/u will be employed. This will provide information as to possible RBE effects in the low LET range of relevance to environmental cleanup. Information will be generated regarding the factor by which linear extrapolation of high dose data may overestimate low dose responses.

C. BACKGROUND

Quantitative assays of radiation-induced neoplastic transformation using *in vitro* cell systems can provide information on the effect of various physical and chemical factors on the yield of transformed cells. Such information is of potential value to the estimation of the risk of cancer induction following exposure to ionizing radiation, as well as to the development of approaches to protect against such risks. Since our interests are ultimately in issues related to human cancer, it is desirable to make such studies with human-derived cell lines.

There is a long history of quantitative studies of radiation-induced neoplastic transformation *in vitro*. However, until relatively recently, such studies have almost exclusively been carried out with rodent-derived cell lines. The reason behind this is that normal human cells *in vitro* are extremely refractory to the induction of neoplastic transformation by ionizing radiation, or other carcinogens. There are human-derived epithelial cell systems which, once immortalized (e.g. by viral infection or oncogene transfection), can be neoplastically transformed by ionizing radiation (or review see Rhim and Dritschillo, 1991), but these are generally not suitable for quantitative studies since the induced frequencies are very low and there are no reliable markers of neoplastic transformation. Since it is well recognized that the transition from a normal to a malignant state is a multistep process involving the passage through a preneoplastic state, it would seem reasonable to examine the effects of radiation at different points along this pathway. Preneoplastic cells are by definition non-tumorigenic, yet they are more susceptible to neoplastic transformation than normal cells. Furthermore, a significant number of human beings are carrying cell populations which are in a preneoplastic state and thus are at risk for the development of cancer. Thus, preneoplastic cell systems are attractive to study for both pragmatic and practical reasons. With the above points in mind, we have, over the past 12 years, developed and applied a human-derived preneoplastic cell system to the study of radiation-induced neoplastic transformation *in vitro*. This system is attractive in that it allows for quantitative studies, as well as having a molecular marker for the tumorigenic phenotype, and a molecular target, damage to which is associated with the onset of tumorigenicity. This system is the HeLa x skin fibroblast human hybrid cell system.

When HeLa cells are fused with normal diploid human skin fibroblasts the resulting hybrids, while transformed in culture, are non-tumorigenic and are remarkably cytogenetically stable (Stanbridge et al., 1982). Furthermore, the cell surface expression of the HeLa tumor-associated antigen, now identified as intestinal alkaline phosphatase, IAP (Latham and Stanbridge, 1990), is suppressed in the non-tumorigenic hybrids and is re-expressed in the rare spontaneously arising tumorigenic segregants that have been isolated from late passage cultures (Stanbridge et al., 1982). In addition, onset of tumorigenicity is associated with the loss of one copy each of chromosomes 11 and 14 (Stanbridge et al., 1981), and microcell transfer of a single copy of chromosome 11 back into HeLa cells, or the non-tumorigenic hybrid, results in suppression of expression of IAP and loss of the tumorigenic phenotype (Srivatsan et al., 1986; Saxon et al., 1986; Kaebling and Klinger 1986). Our molecular and cellular studies of radiation-induced tumorigenic cell lines have supported the earlier observation with spontaneously arising tumorigenic segregants that IAP expression is uniformly associated with tumorigenicity (Mendonca et al., 1991), and that loss of fibroblast chromosome 11 is an important event associated with the onset of tumorigenicity (Mendonca et al., 1995). Interestingly, a wide range of IAP activity is associated with tumorigenicity in this system (Mendonca et al., 1991; Sun et al., 1996) and the data are suggestive of a possible tumor suppressor gene dosage effect. This notion is further strengthened by the observation that loss of only one of the fibroblast chromosome 11's is necessary for neoplastic transformation (Mendonca et al., 1995). During this past funding period we have made the very important new observation that loss of 11 may be necessary but not sufficient for neoplastic transformation, and we have found strong evidence for a role for damage to chromosome 14 in the onset of the tumorigenic phenotype.

In terms of radiation effects on this system, we are able to induce IAP expression in these cells and this forms the basis of the quantitative assay system we have developed (Redpath et al. 1987; Sun et al., 1988) and applied in many different quantitative studies. The assay gives results which are often qualitatively and quantitatively in good agreement with those seen using the C3H10T1/2 mouse embryo-derived cell system. However, the assay offers several significant practical advantages over the C3H10T1/2 system, apart from the fact that it uses human-derived cells. These are (a) a specific marker for neoplastic transformation (IAP expression), (b) a less stringent cell density effect which translates into being able to plate larger numbers of surviving cells per flask and thus cutting down on total flask number and hence incubator space, and (c) the expression time is only 21 days as opposed to 40-60 days for the C3H10T1/2 system. In our opinion these advantages are critical in terms of the very large tissue culture experiments that have to be carried out in the context of the present proposal.

In terms of highlighting some past quantitative work with this system several studies come to mind. First, we were among the first to publish the differences in cell cycle dependence of radiation-induced neoplastic transformation by low- and high-LET radiations (Redpath and Sun, 1990; Redpath et al., 1995). Second, we were able to demonstrate in a quantitative fashion the delayed expression of potentially transforming lesions and the role of epigenetic factors in this process (Mendonca et al., 1993). Third, we have shown that post-irradiation holding at room temperature resulted in enhanced cell kill and neoplastic transformation, most likely through the promotion of misrepair of DNA damage (Redpath et al., 1994; 1995).

The induction of an adaptive response in cells and tissues following exposure to low doses of ionizing radiation and other DNA damaging agents has been documented for a variety of endpoints including cell kill, micronucleus formation, chromosome aberration induction, mutation induction and neoplastic transformation (Olivieri et al. 1984; Sanderson and Morley, 1986; Cai and Liu, 1990; Kelsey et al., 1991; Shadley and Dai, 1992; Wolff, 1992; Joiner, 1994; Azzam et al., 1994; Marples and Skov, 1996; Joiner et al., 1996; Boothman et al., 1996; Azzam et al., 1996). Four recent reviews on adaptive response to DNA damaging agents, including radiation, have discussed mechanisms as well as implications for radiation risk assessment (Polycove, 1998; Stecca and Gerber, 1998; Trosko, 1998; Wolff, 1998). Adaptive responses have typically been detected by exposing cells to a low radiation dose (in the range 1-10 cGy) and then challenging the cells to a higher dose of radiation and comparing the outcome to that seen with the challenge dose only. For an adaptive response to be seen the challenge dose must be delivered within 24 hours of the inducing dose (Joiner, 1994 and references therein). This adaptive response is almost certainly mediated through the transient induction of a protein(s) involved in DNA repair since the induction of such a response is inhibited by prevention of protein synthesis (Wolff, 1992; Boothman et al., 1996), as well as by inhibition of poly (ADP-ribose) polymerase (Wolff, 1992), an enzyme involved in DNA strand-break rejoining.

There are only three published studies which have examined adaptive response using the end-point of neoplastic transformation including one from the P.I.'s laboratory (Azzam et al. 1994; 1996; Redpath and Antoniono, 1998). The first reported that exposure to low doses of radiation reduces the effectiveness of a subsequent challenge dose in inducing neoplastic transformation of C3H10T1/2 cells (Azzam et al., 1994). In a subsequent study, the same research group demonstrated that the exposure to low doses of radiation (0.1 to 10 cGy) resulted in a suppression of the transformation frequency of C3H 10T1/2 cells to levels significantly below (factor of 3 to 4) that seen for spontaneous transformation of unirradiated cells (Azzam et al., 1996). In view of the potential importance of this latter observation to the carcinogenic risk of exposure to low doses of ionizing radiation, we decided to look for adaptive response induced by a low dose (1 cGy) of ionizing radiation (Cs-137 gamma rays) against spontaneous neoplastic transformation using the HeLa x skin fibroblast human hybrid cell system to test for the generality of the observation (Redpath and Antoniono, 1998). From the pooled data the reduction factor was 2 to 3 and was statistically significant. These observations will be discussed in more detail in the preliminary results section.

D. RESEARCH PLAN.

1. Preliminary Studies

We have performed a study which demonstrated an adaptive response against spontaneous neoplastic transformation induced by 1 cGy of gamma radiation and which was published last year (Redpath and Antoniono, 1998). Details of

this study can be found in the attached reprint. Briefly, there were three arms to the study: Arm A - sham irradiation, Arm B - 1 cGy followed by immediate plating for the transformation assay, Arm C - 1 cGy followed by 24 hours holding at 37°C before plating for the transformation assay. The experiment was repeated four times over a period of 6 months. Arm B of the third repeat was lost due to contamination. The data from this study are shown in Tables I, II and III. The data from individual experiments as well as the pooled data were analyzed using the null method. Arm C demonstrated a reduced transformation frequency (factor of 2) compared to arms A and B and this was statistically significant when the data from all four experiments were pooled. Only in the case of the first experiment was a statistically significant difference seen on an individual experiment basis. Statistical analysis of the transformation frequency data was also performed using a weighted two-way ANOVA of the transformation frequency estimates where the two factors were experiment and treatment and where the weights were determined by the estimated standard errors. This method allowed for the estimate of treatment differences corrected for experimental effects. This analysis also revealed a statistically significant difference only between Arms A and C. These observations point out the importance of sample size when one is looking for differences of the order of inherent variation of the parameter under study, in this case spontaneous transformation frequency. These preliminary studies are very useful in planning the proposed experiments (see Section D.2.1.).

It is of interest to compare our findings with those of Azzam *et al.* (1996) who used a similar treatment protocol with C3H10T1/2 cells. In that study three different priming doses (0.1, 1.0 and 10.0 cGy) were used and it was found that for all radiation doses the transformation frequency following delayed (24 h) plating of irradiated cells was significantly reduced compared to that for immediately plated cells or for unirradiated cells. From the pooled data the reduction factor was a factor of 3 to 4 depending on the method of analysis. This is a little larger, although most likely not significantly so, than the factor of 2 to 3 seen in the present study.

Azzam *et al.* (1996) chose to use a clone of C3H10T1/2 cells in which the spontaneous transformation frequency was significantly higher (about five-fold) than normal. Furthermore, the clone of C3H10T1/2 cells used by Azzam *et al.* (1996) had a low plating efficiency of 0.2. The rationale for using this clone was presumably logistic. The size of the experiment in terms of number of cells at risk and hence number of flasks to be processed through the assay would be proportionately reduced with such a clone. This clone of C3H10T1/2 cells had a spontaneous transformation frequency three-fold greater, and a plating efficiency three-fold less, than that of the hybrid cells used in the present study, indicative perhaps that this clone of C3H10T1/2 cells is more genetically unstable than the hybrid cells. In terms of the pooled data, it would appear that these differences did not have much impact on the induction of an adaptive response. Our analysis of the the C3H10T1/2 data on an individual experiment basis revealed a significant difference between the control and delayed plating arms in all three experiments. Thus, it may be that the induction of an adaptive response is more readily discernable in cells which are more predisposed to spontaneous neoplastic transformation, i.e. more genetically unstable. Such cells could well be more affected by the induction of DNA repair systems, or other systems which confer cellular radioresistance. In this regard it has been shown that not all human cells exhibit an adaptive radiation survival response as determined by increased resistance to a high dose challenge following low dose priming (Boothman *et al.*, 1996). In the experiments reported in ref. 11, only the human tumor cells showed an adaptive response with none being seen in normal skin fibroblasts or cancer-prone human cells (xeroderma pigmentosum fibroblasts). Since cancer cells tend to be more genetically unstable than normal cells this would fit the paradigm proposed above for the difference between the human hybrid cell and C3H10T1/2 data.

TABLE I

Comparison of Spontaneous Transformation Frequency expressed as Transformants per Surviving Cell with that for Cells Gamma Irradiated with 1 cGy and plated Immediately or after 24 h Holding.

Treatment Arm	Expt. #	# Surv. Cells S (x 10 ⁻⁵)	# Foci	# Flasks N	# Flasks without foci n	T.F. +/- s.e. (x 10 ⁻⁵)	95% conf. int. (x 10 ⁻⁵)
A	1	4.2	24	101	83	4.86+/-1.15	2.56-7.16
	2	3.72	16	100	85	4.37+/-1.13	2.11-6.63
	3	2.44	23	75	57	8.11+/-1.92	4.30-11.9
	4	5.32	10	100	90	1.98+/-0.63	0.72-3.24
	sum	15.82	73	376	315	4.21+/-0.54	3.13-5.29
B	1	3.33	18	102	85	5.36+/-1.30	2.76-7.96
	2	4.31	9	100	92	2.01+/-0.81	0.58-3.43
	4	3.08	25	100	77	8.49+/-1.77	4.95-12.03
	sum	10.72	52	302	254	4.88+/-0.71	3.46-6.30
C	1	3.4	4	102	100	1.16+/-0.58	0.0-2.32
	2	3.96	9	100	91	2.43+/-0.81	0.81-4.05
	3	2.79	20	75	60	5.99+/-1.55	2.89-9.09
	4	5.23	3	100	97	0.58+/-0.34	0.01-1.26
	sum	15.38	36	377	348	1.96+/-0.36	1.24-2.68

T.F. = transformation frequency = transformants per surviving cell = $\bar{x} \pm N/S$ where $\bar{x} = -\ln n/N \pm 1/n - 1/N$

TABLE II

Comparison of Spontaneous Transformation Frequency expressed as Foci per Flask and Fraction of Flasks with Foci with that for Cells Gamma Irradiated with 1 cGy and plated Immediately or after 24 h Holding.

Treatment Arm	Exp. #	Total # Foci/Total # Flasks	# Flasks with Foci/Total # Flasks
A	1	24/101	18/101
	2	16/100	15/100
	3	23/75	18/75
	4	10/100	10/100
	sum	73/376	61/376
B	1	18/102	17/102
	2	9/100	8/100
	4	25/100	23/100
	sum	52/302	48/302
C	1	4/102	2/102
	2	9/100	9/100
	3	20/75	15/75
	4	3/100	3/100
	sum	36/377	29/377

TABLE III**P-values for Fisher's Exact Test to compare transformation frequencies.**

	ARM A vs. ARM C		ARM A vs. ARM B		ARM B vs. ARM C	
	Foci/Flask	Fraction of Flasks with Foci	Foci/Flask	Fraction of Flasks with Foci	Foci/Flask	Fraction of Flasks with Foci
Exp. 1	0.000038	0.000105	0.303	0.855	0.00260	0.000410
Exp. 2	0.199	0.276	0.199	0.183	1.000	1.000
Exp. 3	0.718	0.694	N/A	N/A	N/A	N/A
Exp. 4	0.082	0.082	0.00850	0.021	0.0000071	0.0000292
Sum	0.000119	0.000308	0.487	0.917	0.00390	0.000954

2. Research Design and Methodologies

Notice 99-14 stresses the need to produce quantitative rather descriptive data. To do quantitative studies on radiation-induced neoplastic transformation in vitro, very large tissue culture experiments cannot be avoided. Furthermore, they have to be repeated to determine reproducibility. Through our earlier study, we have demonstrated that we are capable of performing such large scale experiments. We also believe that we have a transformation assay system that is more efficient than the widely used C3H10T1/2 assay in generating data, if only in terms of time to complete an experiment. Because of the shorter assay time, it also has the advantage of less opportunity for culture contamination and loss of the experiment. In the proposed experiments we have incorporated an experimental design which will lend itself to rigorous statistical analysis. We have also been careful to ensure accurate radiation dosimetry and dose delivery. In view of the practical importance of repeated exposure to low radiation doses we have incorporated such a component into our experimental design.

2.1 Research Design

All of the experiments described below involve immediate and delayed post-irradiation plating. The delayed plating arm is included because of the documented necessity to allow time for the induction of the adaptive response in irradiated cells (Joiner, 1994). We have elected for a post-irradiation holding time of 24 hours at 37°C since that has been previously shown to demonstrate an adaptive response in transformation studies (Azzam et al., 1996; Redpath and Antoniono, 1998).

Phase IA

The first phase of the proposed study will be to determine if there is a dose-response relationship in the induction of an adaptive response. The research design consists of four different radiation treatments (0, 0.1, 1.0, 10.0 cGy) and two different post-irradiation manipulations (immediate plating, and hold at 37°C for 24h and plate). Each combination of radiation treatment and post-irradiation manipulation will be run at the same time and will also be repeated six times. It is anticipated that these experiments will take 6 to 9 months to complete. This design is perhaps best envisaged in the matrix below:

Dose (cGy)	immediate plating	37°C delayed
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		plating
0**	30*	30
0.1	30	30
1.0	30	30
10.0	30	30

* = number of T-75 flasks

** sham irradiation

Phase IB

The second part of the first phase will be to compare single exposures with fractionated exposures. We will choose a total dose based on the results from Phase IA. for purpose of illustration we will assume that to be 1.0 cGy. The research design consists of three different radiation treatments (0, 10 x 0.1cGy, and 1 x 1.0 cGy plus 9 x 0 cGy) and two different post-irradiation manipulations (immediate plating, and hold at 37°C for 24h and plate). Each combination of radiation treatment and post-irradiation manipulation will be run at the same time and will also be repeated six times. It is anticipated that these experiments will take 6 to 9 months to complete. This design is perhaps best envisaged in the matrix below:

Dose (cGy)	immediate plating	37°C delayed plating
10 x 0 cGy**	30*	30
10 x 0.1 cGy	30	30
1 x 1.0 cGy + 9 x 0 cGy**	30	30

* = number of T-75 flasks

** sham irradiation

Phase II

The second phase of the study will be to choose a radiation treatment which demonstrates the best adaptive response (assume 1 cGy for the purpose of presentation) and repeat the first study with an additional post-irradiation manipulation (hold at 22°C for 24h and plate). The rationale behind this phase is based upon our previous observation an enhance misrepair of radiation damage under these conditions (Redpath et al. 1994, Redpath and Antoniono, 1996). Each of combination of radiation treatment and post-irradiation manipulation will be repeated six times. It is anticipated that these experiments will take 6 to 9 months to complete. Again the design is illustrated by the matrix below.

Dose (cGy)	immediate plating	37°C delayed plating	22°C delayed plating

0**	30*	30	30
1.0	30	30	30

* = number of T-75 flasks

** sham irradiation

Phase III

A recent analysis of the apparently similar incidences of radiation-induced breast cancer in acutely exposed A-bomb survivors on the one hand and TB patients who received multiple fluoroscopies on the other, invoked the likelihood that the risk estimates from the fractionated X-ray fluoroscopy cohorts are increased due to the lower energy x-rays (Brenner, 1999). In view of this possible influence of radiation quality (even within the low LET range) on the incidence of radiation-induced cancer, it is proposed to compare gamma radiation with 80 kVp X-rays from a diagnostic fluoroscopic unit which is housed in the adjacent laboratory to that of the P.I. for the induction of neoplastic transformation *in vitro*. The radiation dose which demonstrated the best adaptive response in the first phase of the study will be chosen (assume 1 cGy for presentation purposes). Each of combination of radiation treatment and post-irradiation manipulation will be repeated six times. It is anticipated that these experiments will take 6 to 9 months to complete. Again the design is illustrated by the matrix below.

Dose (cGy)	immediate plating	37°C delayed plating
0**	30*	30
1.0 (gamma)	30	30
1.0 (80 kVp)	30	30

* = number of T-75 flasks

** sham irradiation

2.2. Methodologies

Cell system

The non-tumorigenic HeLa x skin fibroblast cell line CGL1 (Stanbridge et al., 1981) is maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 5% calf serum, 2mM glutamine, non-essential amino acids and 100 IU/ml penicillin (growth medium). Cultures are incubated in 5% CO₂ in air at 37°C and routinely tested for mycoplasma contamination by the 4,6-diamidino-2-phenylindole (DAPI) assay (Russell et al., 1975). Since it is well known that serum lot number can effect both spontaneous and induced transformation frequencies, the same serum lot number will be used for all the experiments reported. An initial phase of the study will be to screen several (up to 4) lots of serum for spontaneous and high radiation-dose (4 Gy) induced transformation frequency. A serum lot will be selected which gives an intermediate level of spontaneous transformation (around 5×10^{-5}). This will make the experiments logistically reasonable. Cells in the population doubling (cell doubling) number range 43 to 45 will be used.

Gamma Irradiation conditions

The cells as adherent subconfluent cultures in T-75 flasks will be irradiated in a Shepherd and Associates Mark I self-shielded ¹³⁷Cs gamma irradiator. The dose-rate can be adjusted from 200 cGy/min to 0.33 cGy/min by the use of attenuators. For the proposed studies a dose-rate of 0.33 cGy/min will be used for all low dose studies (0.1, 1.0, 10.0 cGy). For high dose studies (2 and 4 Gy) a dose-rate of 1.5 Gy/min will be used. For sham irradiated cell

the flasks will be put in the irradiator with the source in the off position for the same period of time as the cells to be irradiated. For fractionated irradiations a 4h interval between the 10 fractions will be used for a total elapsed time of 32h from beginning to end of treatment. This corresponds to about 1.5 doublings of these cells after which the cells will still be subconfluent. The flasks containing the cells will be returned to a 37°C incubator between fractions. Sham irradiated cells will be treated as above, i.e. with fractionated sham irradiations.

80 kVp X-irradiation conditions

The cells as adherent subconfluent cultures in T-75 flasks will be irradiated using a Phillips OPTIMUS 2000 x-ray generator. The T-25 flasks containing cells will be placed on a 2 mm thick lucite sheet in a 10 x 10 cm field. The x-rays will be delivered from below, i.e. through the sheet. The x-ray generator will be operated at 80 kVp with a source to target distance of 60 cm and a resulting exposure rate of approximately 20 mR/mAs. For sham irradiated cell the flasks will be put on the lucite sheet in the x-ray suite with no beam for the same period of time as the cells to be irradiated. For fractionated irradiations a 4h interval between the 10 fractions will be used for a total elapsed time of 32h from beginning to end of treatment. This corresponds to about 1.5 doublings of these cells after which the cells will still be subconfluent. The flasks containing the cells will be returned to a 37°C incubator between fractions. Sham irradiated cells will be treated as above, i.e. with fractionated sham irradiations.

Dosimetry

¹³⁷Cs gamma dosimetry will be performed using a Victoreen dosimeter cross calibrated on a Varian Clinac 600C. The dosimeter will be placed in the the irradiation position of the flask. Flatness of field will be checked to ensure uniformity across the irradiated area. The 80 kVp x-ray exposure will be measured using a calibrated MDH 1015C x-ray monitor placed in the irradiation position of the flask.

Protocol for quantitative assay of neoplastic transformation

Cells are seeded into T-75 flasks such that 2 to 3 days later they are approaching confluence (10^6 cells/flask) at which point they are irradiated. Identical control cultures are sham-irradiated, i.e. taken to and placed inside the irradiator with the source in the "off" position. Following completion of treatment, the cells are subcultured and plated into T-25 flasks for assay of clonogenic survival at 10 days post-irradiation, and into T-75 flasks for assay for foci expressing cell surface intestinal alkaline phosphatase (IAP) at 21 days post-irradiation using the Western Blue staining method which we developed (Mendonca et al., 1992). IAP is a marker for neoplastic transformation in this system (Mendonca et al., 1991). Our previous studies have shown that the transformation frequency is dependent upon the density of viable cells plated post-irradiation (Sun et al., 1988) and we will attempt to control this parameter as carefully as possible. We will aim to plate at a viable cell density of 50 cells/cm². However, if necessary, we are able to correct for variations in viable cell density as described previously (Sun et al., 1988). In the past related study (Redpath and Antoniono, 1998), minimal corrections for cell density (+/- 4%) were needed. The scoring of IAP positive foci will be done by two independent scorers under blinded conditions as to the treatment. Cells are refed at days 5, 10, 14, 17 and 20 post-irradiation.

Calculation of transformation frequency

Transformation frequency can be expressed in three different ways from the same set of raw data. These are fraction of flasks containing foci, foci per flask, and foci per surviving cell. We propose to analyze our data sets using each these definitions.

Statistical Analysis

The null method (Han and Elkind, 1979; Balcer-Kubiczek et al. 1987; Redpath and Antoniono, 1998) will used to calculate transformation frequencies expressed as foci per surviving cell. This method is used to avoid possible errors from satellite colony formation as a consequence of the multiple refeedings which cultures must receive during the 21 day post irradiation expression period. The null method compares the average number of transformed foci per dish ($\bar{f}$) with the fraction of flasks without foci ($f = n/N$) and assumes that the potential number of foci follows a Poisson distribution ($\chi^2 = -\ln f = -\ln (n/N)$). The null method also allows for calculation of the standard error and 95% confidence intervals. Data will also be presented as fraction of flasks containing foci and number of foci per flask.

Thus, for all proposed experiments, there are three dependent measures to be ascertained from each flask: 1) number of foci per surviving cell, 2) number of foci per flask, and 3) the fraction of flasks with foci. Statistically, the first two measures are count data. Therefore we propose Poisson regression as the general framework to evaluate these data (Cameron and Trivedi, 1998). The presence or absence of foci in each flask will be modeled with logistic regression (Hosmer & Lemeshow, 1989). For each of the three dependent variables, the statistical goal is to estimate the change in the dependent measure as a function of dose of radiation, relative to sham irradiation. This estimation will include the specification of confidence bounds.

PHASE IA

The experiment in the first part of Phase I consists of four levels of radiation (0, 0.1, 1, and 10 cGy), and two levels of plating delay (none vs 24 hours). The experiment is performed in six replicates in order to balance across the cells of the design influences from changes in circumstances over time (e.g., technical skill, nutrient fluid, etc.).

For the Poisson regressions, separate dose-response curves will be estimated for each level of plating delay, as shown, with dose transformed to natural log. Because the log of zero is undefined, the sham radiation dose will be represented by an approximation of ambient radiation levels in the irradiator with the source in the off position: 3×10^{-5} cGy, with corresponding natural log of about -10.4.

Data from each level of plating delay will be modeled, separately, as follows:

$$E[y|x] = \exp(B_d(\ln(\text{dose})) + B_r(\text{replicate}) + B_{dr}(\ln(\text{dose}) \times \text{replicate}))$$

where x is the vector of explanatory variables (viz., dose, replicate). In this instance, the coefficient B_d indicates the percent change in the conditional mean response for a one-percent increase in dose. However, a meaningful interpretation of B_d in this situation depends upon the lack of an interaction of dose with replicate. A significant interaction of dose with replicate will be evidence that one or more replicates of the experiment are flawed. Should this occur, we will strive to identify the flawed replicate(s) and the reasons for the flaw, and eliminate the offending replicate(s) from analysis if such may be done on a rational basis.

To assess statistical significance of the plating variable, data will be analyzed in the full model, which specifies three main effects (viz., dose, plating, replicate), three pairwise interactions (dose x plating, dose x replicate, and plating x replicate) and the three-way (dose x plating x replicate). Again, terms involving replicates will be interpreted as quality-control measures, and any flawed replicates will be removed from the final analyses if this can be justified in a rational way. Of greatest interest is the coefficient for the interaction of dose and plating delay, which, if significant, will indicate there are quantitative differences between the dose-response curve at each of the two plating-delay levels.

Confidence intervals will be produced about the coefficients associated with each term in the Poisson-regression models in two ways: The statistical software used to perform the regression (SAS) produces standard errors based upon Poisson Maximum Likelihood Estimates (SAS Institute, 1997). However, in the likely case that the Poisson assumption of equidispersion is untenable (ie., conditional variances differ materially from their associated conditional means), MLE estimates may be quite inaccurate. Therefore, we will estimate the standard errors about model coefficients by bootstrap (Efron, 1979; Horowitz, 1997). Bootstrap estimates require no specification of the relation between means and variances, and are well accepted in this area of statistics (Cameron & Trivedi, 1998).

The analysis of presence or absence of foci in each flask will proceed by logistic regression. In this case, the log of the odds of foci being observed is modeled, separately for each plating delay, as

$$B_{d1}(\text{dummy1}) + B_{d2}(\text{dummy2}) + B_{d3}(\text{dummy3}) + B_r(\text{replicate}) + B_{dr}(\text{dose} \times \text{replicate})$$

where the four levels of dose (0, 0.1, 1, 10 cGy) are represented by three dummy variables. The sham dose is represented by the value zero in each of the dummy variables, and serves as baseline for contrasting the remaining three levels of dose, each of which is coded with a one in the dummy with associated subscript, and zero in the other two dummy variables. The coefficients of the dummy variables are interpreted as the log of the odds of foci

formation at each non-sham dose, relative to the sham dose. There is no need to bootstrap values of the standard error of the coefficients for any logistic regression in this proposal.

PHASE-IB

A second part of the Phase I experiment concerns effects of repeated exposures to radiation. There are three levels of dose, including a sham, each repeated 10 times (0 cGy x 10, 0.1 cGy x 10, and 1.0 cGy x 1 plus 0 cGy x 9), and two levels of plating delay (none vs 24 hours). The experiment is replicated six times, with justification as before. Analyses of these data is similar to that already described. Effects of replicate will be taken as quality-control measures. Separate dose-response curves will be estimated for each level of plating delay separately, and the effect of plating delay will be assessed with a full model, with special interest on the interaction of dose and plating delay.

PHASE II

The Phase II experiment has a single dose level, but retains the plating-delay variable (none vs 24 hours) and introduces a temperature variable (22°C vs 37°C). Again, the experiment will be completed in six replicates, and analysed using Poisson regression, with standard errors on the coefficients determined by the bootstrap. The initial model is

$$E[y|x] = \exp(B_p(\text{plating}) + B_t(\text{temperature}) + B_r(\text{replicate}) + B_{pr}(\text{plating} \times \text{temperature}) + B_{pr}(\text{plating} \times \text{replicate}) + b_{tr}(\text{temperature} \times \text{replicate}) + b_{ptr}(\text{plating} \times \text{temperature} \times \text{replicate}))$$

Again, terms involving replicate will be assessed as quality-control measures, as described above. Given that the levels of plating and temperature are coded as 0,1, then the interpretation of the coefficients B_p and B_t is straightforward. The coefficient B_p will estimate the proportionate change in the conditional mean (viz., number of transformants per surviving cell, or number of flasks containing foci, or number of foci per flask) at the 24-hour plating delay, relative to immediate plating. The coefficient B_t will estimate the proportionate change in the conditional mean as temperature moves from 22°C to 37°C. In the likely event that equidispersion does not obtain, then standard errors for the coefficients will be estimated by bootstrap, as described above.

PHASE III

The purpose of the phase-III experiment is to compare the effects of 137Cs gamma radiation to those of 80 kVp X-rays, at the dose level which evidenced best adaptive response in the phase-I experiment. Because a sham-irradiated group will be included for each radiation type, the experiment has three factors: two levels of dose (sham vs irradiated), two levels of radiation type (gamma vs x-ray), and two levels of plating delay (none vs 24hr delay). The experiment will be performed in six replicates. As before, significant replicate effects (as main effect or in interactions) will be an indication of flaws in one or more replications of the experiment. Flawed replicates will be identified and removed from consideration, if this may be done on a rational basis.

Of chief interest here is the two-way interaction of dose and type of radiation, and the three-way interaction of dose, radiation type, and plating delay. As above, we will construct significance tests for these coefficients based upon standard errors from the bootstrap, as the Poisson assumption of equidispersion is unlikely to obtain. Given an indication that the dose-response effect is different between the two radiation types, we will proceed to estimate the percent change in the conditional means of each dependent variable for a one-percent change in dose, as described for the Phase-I experiment. Briefly, we will analyze data from each radiation type separately, encoding the natural log of the dose, recoding the sham level of radiation as the natural log of ambient radiation levels in the irradiator with the source in the off position (about 3×10^{-5} cGy). Confidence intervals may be constructed about these estimates using the bootstrap to determine standard errors of the coefficients.

Data will be analyzed using the computer facilities of the Epidemiology Division / Chao Family Comprehensive Cancer Center, at the University of California, Irvine. The specific platform to be used is a Digital Equipment Corporation Alpha 2100 running SAS version 6.12 under Open VMS. The Poisson regressions and bootstrapping procedures will be completed using SAS Proc Genmod (set up for random resampling and capture of results for the bootstrap operations).

AMOUNT OF DATA

The table below shows the number of levels of the various factors involved in each proposed experiment, the number of flasks per cell of the design, and the total number of flasks required for each experiment and for the overall proposal.

	levels of dose	levels of plating delay	levels of temp	levels of radiation type	levels of replicates	flasks per cell of design	total number flasks
Experiment							
Phase-Ia	4	2	1	1	6	30	1440
Phase-Ib	3	2	1	1	6	30	1080
Phase-II	1	2	2	1	6	30	720
Phase-III	2	2	1	2	6	30	1440
TOTAL							4680

As may be seen in the table, the total number of flasks for the overall proposal is 4680. Three dependent measures will be calculated by laboratory personnel for each flask (viz., transformants per surviving cell, presence or absence of foci, number of foci per flask). Thus the number of data records to be computerized is 14040 (4680 x 3). Data entry will proceed in batches corresponding to each cell of each experiment, allowing codes for independent variables to be supplied by the computer. The three dependent measures for each flask in each cell of each experiment will be double entered, with entries compared programatically. While discrepancies in the double-entry will be resolved on a case by case basis, a 10-percent sample of data from each replicate of each experiment will be verified against original records as quality control. Any data-entry error rate exceeding one percent will precipitate re-entry of the data from that replicate.

Timetable

The research design is split into four phases. The estimated time to complete each phase is given below.

Phase IA - 9 months

Phase IB - 9 months

Phase II - 9 months

Phase III - 9 months

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COLLABORATIVE ARRANGEMENTS.

None

BIOGRAPHICAL SKETCHES.

See attached pages

FACILITIES AND RESOURCES.

See attached pages

CURRENT AND PENDING SUPPORT.

See attached pages

ASSURANCES AND CERTIFICATIONS.

Assurance of Compliance

Certifications regarding lobbying; debarment, suspension and other responsibility matters;
and drug-free workplace requirements

APPENDIX.

Reprints of each of the following papers are provided.

REDPATH, J.L., ANTONIONO, R.J., MENDONCA, M.S. and SUN, C., 1994, The effect of post-irradiation holding at 22°C on the repair of sublethal, potentially lethal and potentially neoplastically transforming damage in gamma-irradiated HeLa x skin fibroblast human hybrid cells, *Radiat. Res.*, **137**, 323-329.

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Office of Science, Notice 99-14, Low Dose Radiation Research Program, Agency: U.S. D.O.E.
Pre-application response from:
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The following paragraphs outline a project which would address the area "Understanding biological responses to radiation and oxidative damage". The proposed research would focus on the following key question: (a) "How much do low doses of radiation protect against subsequent low doses of ionizing radiation?". The experimental system which would be employed to address this question is the HeLa x skin fibroblast human hybrid cell system with which we have over 12 years of experience and, more importantly, with which we have made several observations which could be expanded on to address the question posed above. We have employed this system extensively in the study of radiation-induced neoplastic transformation in vitro. While similar results have been obtained in comparable settings with the more widely used C3H10T1/2 cell assay, in our opinion the hybrid cells possess certain advantages, not the least of which is that an experiment can be completed in 21 days as opposed to 40 to 60 days [1]. The other positive features of the hybrid assay are that it uses human-derived cells, tumorigenicity correlates with the expression of a cell surface marker [2], and neoplastic transformation is associated with damage to chromosomes 11 and 14 [3].

(a) "How much do low doses of radiation protect against subsequent low doses of ionizing radiation?". Notice 99-14 specifically notes the need to quantify the generality and extent of the apparent adaptive response in cells irradiated with small doses of radiation. This would be a major focus of our proposed effort. We have already demonstrated that irradiation with a single dose of 1cGy results in a neoplastic transformation frequency that is a factor of two LOWER than that of sham-irradiated cells [4]. We would propose to expand on this observation by performing the studies to determine the single-dose response relationship of this adaptive response (e.g. doses of 0.1 cGy and 10 cGy. We would also examine repeated low dose treatments (e.g. 10 x 0.1 cGy, 10 x 1 cGy). Such experiments would directly test the question posed in Notice 99-14. We would also propose to take advantage of another observation in our laboratory to address, albeit indirectly, the question of the role of repair in this adaptive response. We have previously shown that post-irradiation holding at room temperature prior to plating for focus formation results in enhanced cell kill and enhanced neoplastic transformation, most likely through the promotion of misrepair [5]. We would hypothesize that if the adaptive response involves the induction of a repair process then the observed reduction in transformation frequency following exposure to 1 cGy would be abrogated by post-irradiation holding at room temperature. This hypothesis could readily be tested.

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Table 1: Comparison of Spontaneous Transformation Frequencies Using Different Serum Lots

Experiment Number	Serum Lot #	Number of Surviving Cells S($\times 10^5$)	Number of Foci	Number of Flasks, N	Number of Flasks without Foci, n	Transformation Frequency ($\times 10^{-5}$)	+/-SE ($\times 10^{-5}$)
1	Gibco 1026098	3.240	14	75	62	4.41	1.23
	JRH #8K2236	3.150	28	75	20	7.39	1.66
	Hyclone AJG10659	3.210	14	75	62	4.44	1.23
2	JRH #8K2236	1.240	3	30	27	2.54	1.47
	JRH #8N2272	1.090	2	30	28	1.90	1.34
	Hyclone AJH10175	1.100	0	30	30	NA	
	Hyclone AJD10710	0.768	1	30	29	1.32	1.32
	Omega 24342	1.120	2	30	28	1.85	1.31
3	JRH CS #7C2059	1.620	5	36	31	3.33	1.49
	JRH NBCS #8N2272	0.944	2	36	34	2.18	1.54
	Hyclone AJD10202	1.500	2	36	34	1.37	0.97
	Hyclone AJG10636	1.440	2	36	34	1.43	1.01
	Omega 04001	1.430	2	36	34	1.44	1.02
	Omega 31120	1.460	2	36	34	1.41	1.00
4	JRH #8K2236	2.020	2	48	46	1.01	0.71
	JRH CS #7C2059	1.860	7	48	41	4.07	1.54

Table 2: Comparison of Spontaneous Transformation Frequencies to that Induced by Low Dose Gamma Radiation:
Effect of Immediate vs. Delayed Plating.

Serum Lot 1

Treatment Arm	Experiment Number	Number of Surviving Cells S($\times 10^5$)	Number of Foci	Number of Flasks, N	Number of Flasks without Foci, n	Transformation Frequency ($\times 10^{-5}$)	+/-SE ($\times 10^{-5}$)	95% Confidence Intervals ($\times 10^{-5}$)	
Control, Immediate	1	1.070	2	30	28	1.93	1.36	0.91	5.31
	2	0.969	3	30	27	3.26	1.88		
	3	0.654	3	30	27	4.83	2.79		
	sum	2.693	8	90	82	3.11	1.10		
0.1 cGy Immediate	1	1.040	1	30	29	0.98	0.98	0.21	3.72
	2	0.731	2	30	28	2.83	2.00		
	3	0.847	2	30	28	2.44	1.73		
	sum	2.618	5	90	85	1.96	0.88		
1 cGy Immediate	1	1.060	4	30	27	2.98	1.72	0.20	3.60
	2	0.893	2	30	28	2.32	1.64		
	3	0.754	0	30	30	0.00	0.00		
	sum	2.707	6	90	85	1.90	0.85		
10 cGy Immediate	1	1.040	1	30	29	0.98	0.98	0.00	2.79
	2	0.921	2	30	28	2.25	2.59		
	3	0.974	1	30	29	1.04	1.04		
	sum	2.935	4	90	86	1.39	0.70		
Control 24 Hr Delay	1	1.020	1	30	29	0.99	0.99	-0.19	2.62
	2	0.833	0	30	30	0.00	0.00		
	3	0.654	2	30	28	2.37	1.67		
	sum	2.507	3	90	87	1.22	0.70		
0.1 cGy 24 Hr Delay	1	1.200	1	30	29	0.84	0.84	-0.34	1.01
	2	0.930	0	30	30	0.00	0.00		
	3	0.847	0	30	30	0.00	0.00		
	sum	2.977	1	90	89	0.34	0.34		
1 cGy 24 Hr Delay	1	1.210	2	30	28	1.71	1.21	0.19	3.42
	2	0.883	0	30	30	0.00	0.00		
	3	0.754	3	30	27	3.00	1.73		
	sum	2.847	5	90	85	1.81	0.81		
10 cGy 24 Hr Delay	1	1.160	1	30	29	0.88	0.88	0.00	2.79
	2	0.801	2	30	28	2.58	1.83		
	3	0.974	1	30	29	0.99	0.99		
	sum	2.935	4	90	86	1.39	0.70		

Table 3: Comparison of Spontaneous Transformation Frequencies to that Induced by Low Dose Gamma Radiation:
Effect of Immediate vs. Delayed Plating.

Serum Lot 2

Treatment Arm	Experiment Number	Number of Surviving Cells S($\times 10^5$)	Number of Foci	Number of Flasks, N	Number of Flasks without Foci, n	Transformation Frequency ($\times 10^{-5}$)	+/-SE ($\times 10^{-5}$)
Control Immediate	1	1.700	4	48	44	2.46	1.27
0.1 cGy Immediate	1	1.650	4	48	44	2.53	1.27
1 cGy Immediate	1	1.650	4	48	44	2.52	1.26
10 cGy Immediate	1	2.170	4	48	44	1.92	0.961
Control 24 Hr Delay	1	1.580	4	48	44	2.65	1.32
0.1 cGy 24 Hr Delay	1	0.722	4	48	44	5.79	2.89
1 cGy 24 Hr Delay	1	1.670	5	48	44	2.51	1.25
10 cGy 24 Hr Delay	1	1.690	4	48	44	2.47	1.24