PRELIMINARY CHARACTERIZATION OF THE P3 HUMAN EPITHELIAL CELL LINE FOR RADIOBIOLOGICAL RESEARCH

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John M. Holland
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This thesis has been approved for graduation with honors from the Department of Biology.

Joseph D. Harrington

Fr. Joseph D. Harrington, Advisor

Dr. James J. Manion

Dr. James J. Manion

Rev. Robt. N. Butko

Fr. Robert N. Butko

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Date

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Abstract

Preliminary experimentation was performed to characterize the P3 human epithelial cell line for radiobiological research. Specifically, work was done to determine if the cell line should be used to evaluate mechanisms of radiation damage and repair as revealed by survival response experimentation and via the 6-thioguanine mutation assay.

Growth analysis was performed as well as a 6-thioguanine toxicity study and a 6-thioguanine mutation expression time curve. Preliminary results were obtained from survival experiments following P3 cell irradiation with 50 KvP X-rays, ^{60}Co gamma rays, and Janus Reactor fission spectrum neutrons with a mean energy of 0.86 MeV. These and preliminary results of 6-thioguanine resistance mutation frequency experiments utilizing Janus Reactor fission spectrum neutrons will be discussed.

INTRODUCTION AND LITERATURE REVIEW

Most biological research involving tissue culture has been performed utilizing non-human, rodent fibroblast cell lines. However, problems arise when conclusions concerning carcinogenesis are drawn from such research. In fact, experimentation utilizing fibroblast cells may not be particularly relevant to the understanding of the induction and growth of human carcinomas. Further, since most human cancers are of epithelial origin, radiobiological work with a human epithelial cell line becomes all the more important.[1]

Problems arise in trying to establish a definite correlation between studies involving rodent cell lines and human cell lines. Most work has been performed utilizing rodent cell lines, yet their relationship to human cells grown *in vitro* remains questionable. For example, it has been observed that rodent fibroblast lines display a significantly different survival curve than human fibroblast cell lines following irradiation.[2] This seems to indicate that rodent cell lines have a different survival response than human cell lines after treatment with radiation. Further, it has been noted that the incidence of transformation in human cells appears to be significantly lower than that observed in rodent cell lines following the same dose of radiation. Other differences between transformed human cell lines and transformed rodent cell lines concern contact inhibition, serum and calcium requirements, and surface topography.[3] From such observations, there appears to exist a substantial difference between human cells and rodent cells in their response to radiation treatment. Therefore, radiobiological research utilizing human cell lines acquires further significance.

A human epithelial cell line has been derived from an ovarian teratocarcinoma by J. Zeuthen.[4] The Mammalian Cell Biology Group at Argonne National Laboratory received cells derived from this teratocarcinoma, designated "P3," from C.A. Jones who is currently utilizing these cells for chemical-induced mutogenesis assays.[5]

The aim of initial work with P3 cells was to begin preliminary characterization of the cell line for potential use in radiobiological research. Specifically, the goal of this study was to analyze the P3 human epithelial cell line for possible use in radiation induced 6-thioguanine mutogenesis assays.

The 6-thioguanine mutation assay works on the principle that a mutagen has damaged the HGPRT locus on the long arm of the X chromosome. This locus is responsible for the formation of the enzyme, hypoxanthine guanine phosphoribosyl transferase (HGPRT). HGPRT is used to convert hypoxanthine to guanine in a salvage process much more efficient than "de novo" synthesis of purine bases. 6-thioguanine is a cytotoxic purine base imitator. Wild-type cells with HGPRT will take up 6-thioguanine and die. Mutant cells which lack the ability to produce HGPRT will survive in the presence of 6-thioguanine by utilizing "de novo" purine base synthesis. Then, mutants with damaged HGPRT loci can be effectively distinguished from normal, wild-type cells. It has been observed that mutagens including radiation may be involved in the process of activating previously quiescent oncogenes resulting in the formation of cancerous cells.[6,7,8]

Of course, it must be realized that studies concerning mutation and mutation frequencies performed in vitro with human cells cannot reproduce all the conditions of mutation within the human body. Still, several advantages exist in favor of using cultured human cells for such work. First, since survival as well as mutation induction can be measured, changes in observed mutation frequency due to potential changes in survival may be distinguished from changes in the mutation frequency due to the mutation induction process itself. As such, survival factors are considered for more accurate quantitative analysis. Secondly, the effects of radiation can be measured much more directly upon cultured cells due to the absence of complex tissue and organ relations found in the living organism. Thirdly, in vitro experiments utilizing human cultured cells are much quicker and less expensive than comparable *in vivo* studies.[9]

Further, it should be noted that most cell lines used in radiobiological research are "established" cell lines. An established cell line is one that has been transformed to have an infinite life span. The P3 human epithelial cell line is also transformed. Eventually, it will be important to compare the P3 cells with "normal" cells of both fibroblasts and epithelial origin.

Using rodent and human cell lines the Mammalian Cell Biology Group at Argonne National Laboratory is searching for the mechanisms whereby radiation damage and repair are mediated and how these mechanisms may be relevant to carcinogenesis in humans.

MATERIALS AND METHODS

Cell Culture

P3 cells supplied by C.A. Jones were grown attached to plastic Petri dishes in Eagle's minimum essential medium as altered by Stanners et al[10] supplemented with 10% fetal calf serum. Ribosides and deoxyribosides were excluded from the medium, and the amount of sodium bicarbonate was reduced to one-third that of normal Eagle's medium with Earle's saline. 0.02 M HEPES buffer (pH = 7.2) was added. Cells were grown at 37° C in a humidified atmosphere of 2% CO_2 in air.

Perhaps due to their epithelial morphology, the P3 cells display a great deal of clumping during trypsinization. This problem becomes especially significant in two areas: 1) Determining the actual number of cells counted by the 100 micron aperture Coulter Counter. A clump of two or three cells potentially can be counted as one very large cell by the Coulter Counter. Further, these clumps may later break down into their constituent two or three cells resulting in an incalculable increase in the actual number of cells involved in the treatment. Therefore, it becomes vital to attain a single-cell suspension before counting to accurately determine the number of cells involved; 2) Metabolic cooperation between clumped cells may affect the quantification of 6-thioguanine mutation frequency due to the ability of mutant cells to obtain hypoxanthine guanine phosphoribosyl transferase from wild-type cells through gap junctions.[11]

To aid in rectifying this clumping problem, calcium and magnesium free saline and trypsin solutions were utilized during the trypsination process as suggested by J. Paul.[12] The dissociation process is as follows: 1) aspirate media; 2) rinse twice with saline; 3) apply magnesium and calcium free trypsin (0.02% solution); 4) incubate for approximately 8 minutes; 5) mix thoroughly via pipetting. Single cell suspension is checked via microscopic observation and by looking at the size distribution on a multichannel analyzer.

Radiation Types and Treatments

Three different types of radiation were used in this study: 1) X-rays were produced at 50 KVP and 20 ma, filtered with 0.18 mm aluminum, and delivered at a dose-rate of 25.03 rads/sec at 23 cm;[13] 2) Fission-spectrum neutrons were produced by the Janus Reactor at the Division of Biological and Medical Research, Argonne National Laboratory. The average energy of this beam was 0.85 MeV with less than a 4% gamma-ray contamination.[14] Neutrons were delivered at a nominal dose-rate of 12 rads/min. The actual neutron-absorbed dose-rate was arrived at by multiplying by a factor of 0.861 to account for the polystyrene surface of the T flasks traversed by incident neutrons and the growth medium traversed by back-scattered neutrons;[13] 3) Cobalt 60 gamma radiation was delivered at a rate of 50 rads/min by a Gammabeam-650 irradiator (Atomic Energy of Canada, Ltd., Ottowa, Ontario, Canada), which consisted of 12 Cobalt 60 sources varying from about 18 to 1800 Ci in strength. Dose-rates were measured with a Shonka-type air wall ionization chamber (Caradin, Lisle, Illinois), which was calibrated at the National Bureau of Standards against a standard chamber of the International Commission on Radiobiological Units. The maximum variation in Cobalt 60 gamma radiation dose from sample to sample was less than 4%.[15]

After the media was aspirated, X-rays were delivered to P3 cells attached to the surface of 100 mm Petri dishes. P3 cells were subjected to neutron irradiation while attached in media filled T_{25} flasks with the surface having the cells attached placed nearest the neutron source. Cobalt 60 gamma radiation was also administered to P3 cells attached in T25 flasks filled with media in a similar manner as neutron irradiation.

Growth Analysis

Twenty 100 mm Petri dishes were plated each with 20,000 P3 cells and incubated at 37° C. Two plates were counted at intervals over a seven day period to determine growth characteristics of the P3 cells.

Another preliminary study was performed to determine if 50 cells is an appropriate lower limit to distinguish between abortive colonies and slow-growing survivor colonies. Based upon similar work of Puck and Marcus,[16] P3 cells were irradiated with 50 Rad 50 KvP X-rays and plated at a concentration of approximately 800 cells/plate into 16 large Petri dishes. Plates were then stained and colonies over 50 cells in size were counted periodically over a 15 day period.

6-Thioguanine Toxicity Study

To determine the proper concentration of 6-thioguanine to be utilized in mutation experiments, P3 cells were plated into Petri dishes with untreated media. After a few hours to allow for attachment, the untreated media was aspirated and replaced with media containing increasing concentrations of 6-TG. These plates were then incubated for 15 days and stained with methylene blue. Surviving colonies were counted, and the surviving fraction for each 6-thiaguanine concentration calculated.

6-Thioguanine Mutation Expression Time

An experiment was performed to determine the time needed after irradiation for maximal number of 6-thioguanine mutations to be expressed.

On Day 0, two samples of P3 cells were irradiated with 100 rads and 300 rads 50 KvP X-rays respectively.

A portion of these cells were placed at a concentration of approximately 80,000 cells per Petri dish in 5.0 microgram/ml 6-TG media. Another portion of P3 cells were plated out to determine plating efficiencies. Remaining cells of both samples were then placed in 850 cm^2 roller bottles. Periodically, over a twelve day period, cells from both samples were again plated directly from roller bottles into 5.0 microgram/ml 6-TG media and into untreated media to determine plating efficiencies. 6-thioguanine mutation plates were incubated for 15 days and stained. Plating efficiency plates were incubated and stained after 12 days.

Radiation Survival Determination

P3 cells have been studied for survival response following irradiation with 50 KvP X-rays, ^{60}Co gamma rays, and Janus Reactor fission spectrum neutrons. Following irradiation, cells were incubated in Petri dishes for 12 days and stained. An untreated sample was also incubated and stained after 12 days to determine plating efficiency.

6-Thioguanine Mutation Assay

Following irradiation, P3 cells were placed in 850 cm^2 tissue culture roller bottles at a concentration of approximately 10^6 cells per roller bottle. Such a high concentration of cells was necessary for statistical reasons due to the low frequency of 6-thioguanine resistant mutants induced during this assay. Allowing for exponential growth, the P3 cells were then reseeded into new roller bottles on day 5 to avoid confluency at time of plating. On day 8, samples were then plated into 5.0 microgram/ml 6-thioguanine media and incubated for 15 days when the Petri dishes were stained with methylene blue.

RESULTS

Growth Analyses

In order to better evaluate the growth characteristics of the P3 cells, a growth curve was plotted over a seven day period after plating. (fig. 1). For the P3 cells, after plating, there appears to be approximately a 36 hour lag period due to the trauma of the plating procedure upon the cells (PE=31%±9.5%). After this initial lag, the P3 cells attain exponential growth with an average doubling time of 19.0 hours.

Appropriate colony size for counting was also evaluated. Fifty cells per colony does appear to be a sufficient lower limit to distinguish slow growing colonies from abortive colonies in determining cell survival. (fig. 2). However, for the 6-thioguanine mutation assay, a greater cut-off number may prove more appropriate in discriminating mutant colonies from abortive colonies. From evaluating these colonies, counting appears to be best performed after allowing an incubation time of between 12-14 days for P3 cells grown in untreated media and 16-17 days for cells grown in 6-thioguanine treated media.

6-Thioguanine Toxicity

After treating P3 cells with increasing concentrations

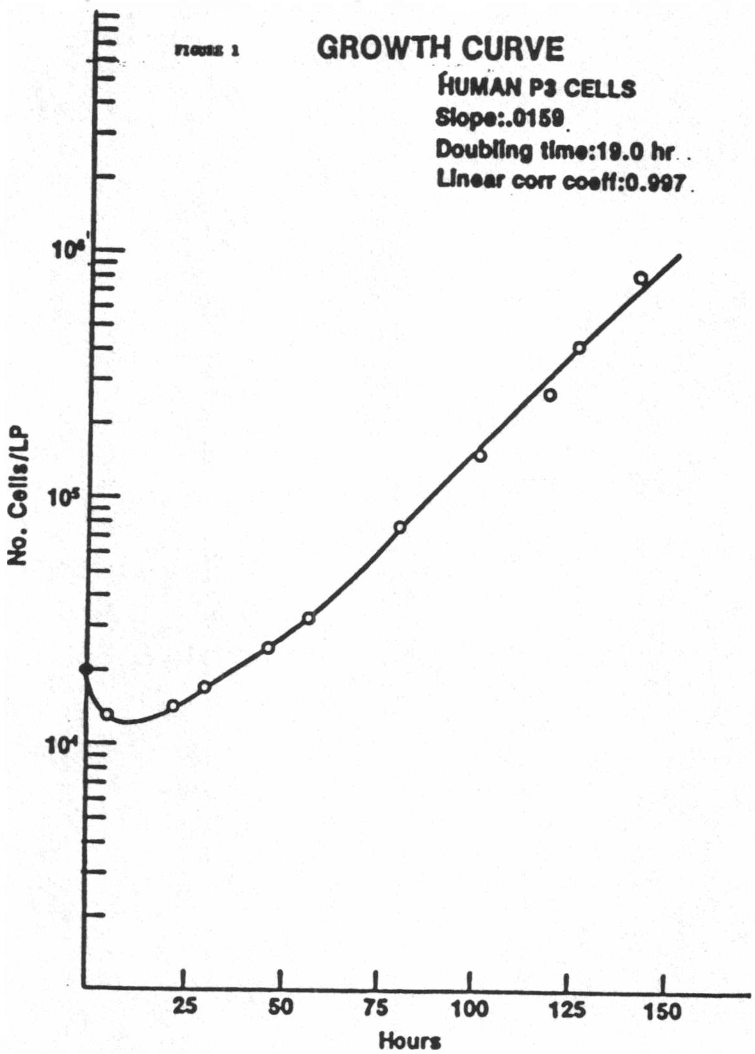

Fig. 1. Growth Curve of Human P3 Cells. Initial plating was 2 x 10^4 cells/Petri dish. Slope and linear correlation coefficient values were determined by linear regression of the last six points on the curve.

of 6-thioguanine media and allowing for a 15 day incubation time, colonies were counted and surviving fraction was plotted against 6-TG concentration on a log vs. log scale. (fig. 3).

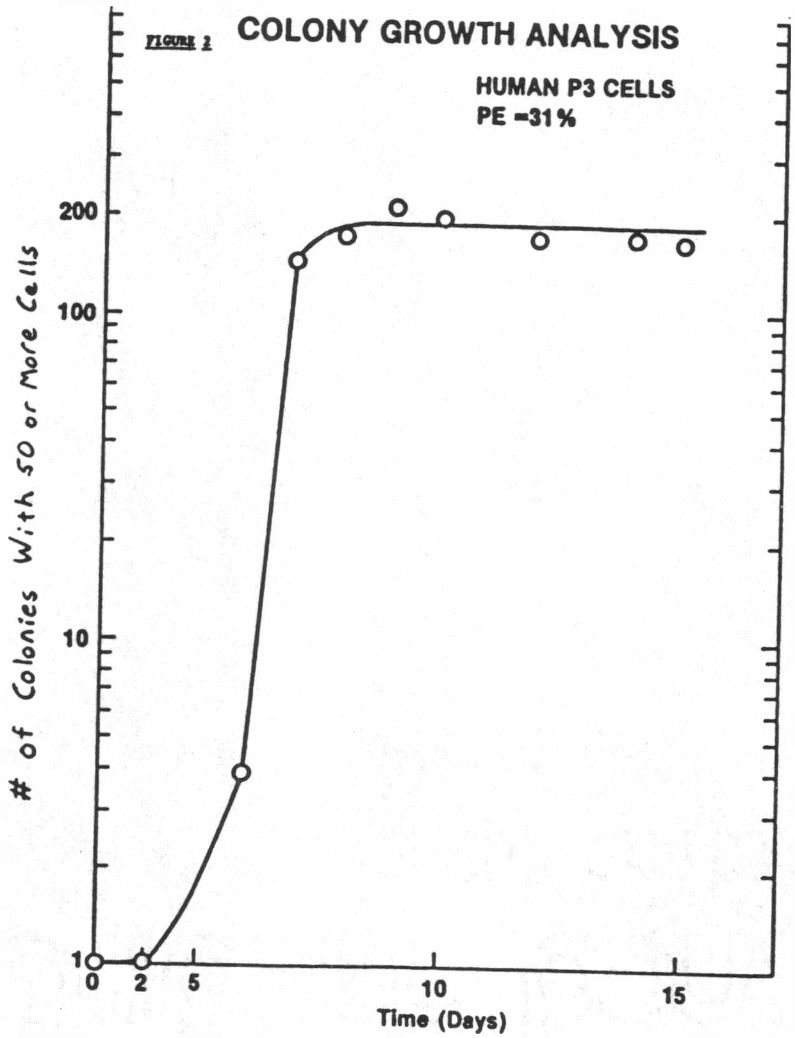

Fig. 2. Colony Growth Analysis of Human P3 Cells. Following irradiation with 50 rads 50 KvP X-rays, cells were plated at approximately 800 cells/dish.

The resulting curve has a sigmoid shape and reveals that at 5.0 microgram/ml 6-TG media, one should expect less than 2.56 x 10^6 surviving fraction of wild-type P3 cells. When the toxicity curve plateaus one is seeing spontaneous occurring mutants otherwise known as background frequency.

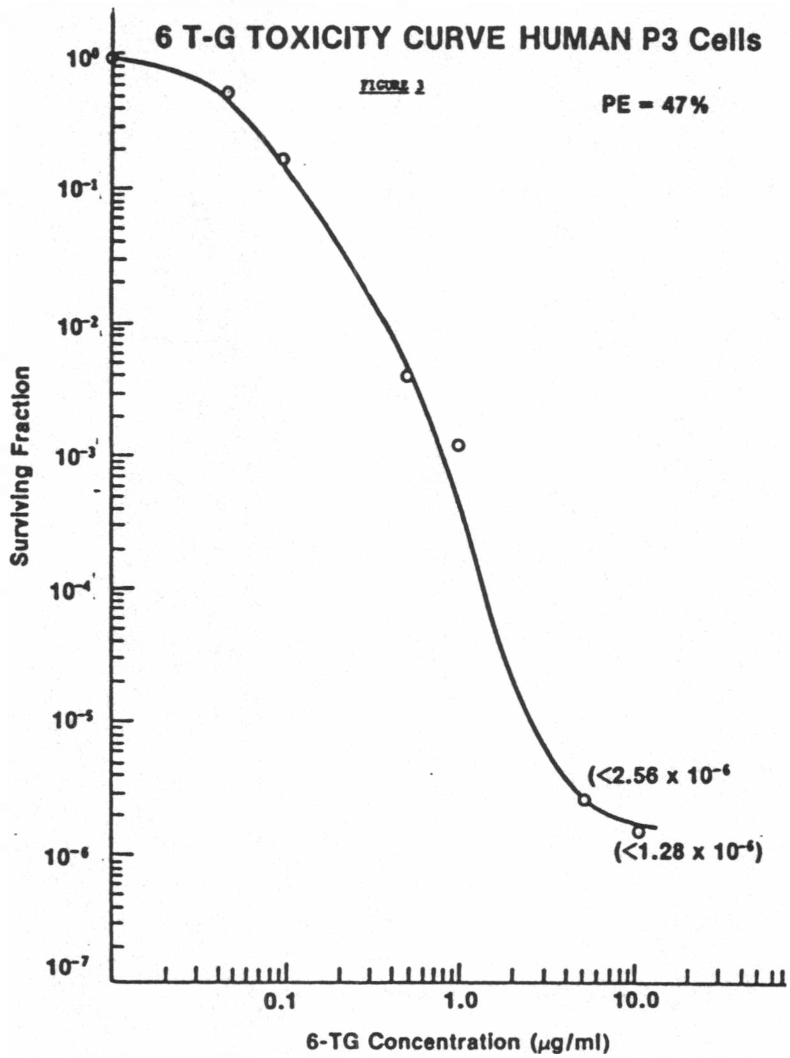

Fig. 3. 6-Thioguanine Toxicity Curve of Human P3 Cells. Cells were plated at an initial density of 8×10^4 cells/Petri dish.

6-Thioguanine Mutation Expression Time

To determine appropriate cell density, a small, preliminary experiment was performed. On expression day 13 after 100 rad irradiation, P3 cells were plated into 100 mm Petri dishes at three densities: 9.6×10^4, 4.8×10^4 and 2.4×10^4 cells/dish. Following incubation time, the mutation frequencies were analyzed for the three samples. At 9.6 x 10^4 cells/Petri dish, 125 (±19.4) mutants/10^6 survivors were observed. At 4.8 x 10^4 cells/Petri dish, 145 (±24.5) mutants/10^6 survivors were observed, and at 2.4 x 10^4 cells/Petri dish, 133 (±25.9) mutants/10^6 survivors were noted. These preliminary results reveal that there does not seem to exist a significant difference in mutation frequency due to cell density at these concentrations at the 100 rad dose level. Accordingly, an 8 x 10^4 cells/Petri dish density should result in optimal mutation expression without suffering the effects of metabolic cooperation.[5]

To determine the optimal time after irradiation to allow for the P3 cells to express their mutant phenotype, a semi-logarithmic curve was plotted with number of 6-TG resistant mutants vs. time in days. (fig. 4).

Results of this study seem to indicate an optimal time of 12 days after treatment in both the 100 rad and 300 rad treatment samples. This time corresponds to the plateau of the curve. This would seem to contradict the 6-7 optimal expression time obtained by C.A. Jones utilizing chemical mutagens on the P3 cells.[3] However, in this current study's curve, plotted points at days 8, 9, and 11 are suspect due to a significant variance in plating efficiencies noted for these days (approximate average of 0.45 for these days versus the approximate average of 0.25 for other days). The import of consistent plating

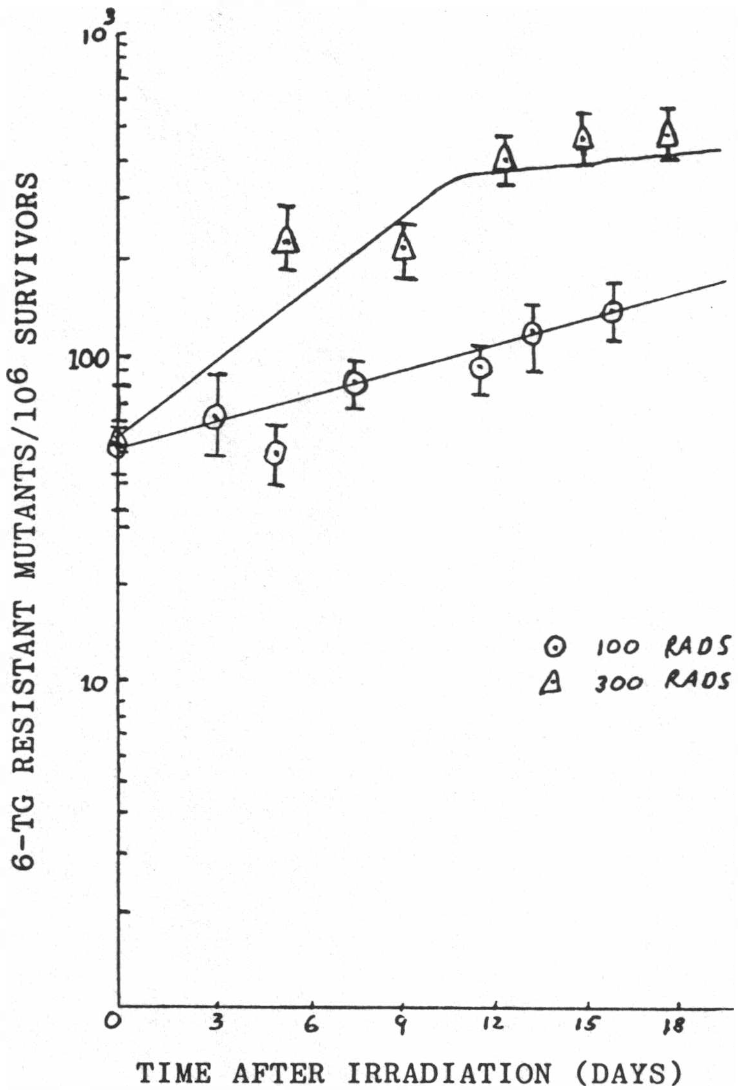

Fig. 4. 6-Thioguanine Resistance Expression Time. Control plating efficiency values from roller bottles varied from .206 to .514.

efficiencies can best be expressed in evaluating an equation by which mutation frequencies are determined; that is:

$$\text{Mutation Frequency}/10^6 \text{ Survivers} = \frac{\#\text{countable colonies} \times 10^6}{\#\text{plated cells} \times \text{PE}}$$

These preliminary results suggest a need for repetition in order to better determine the initial day of optimal expression.

Survival Analysis

Curves were plotted following experiments measuring the survival response of P3 cells to three types of ionizing radiation. All curves were plotted in a semi-logarithmic fashion with surviving fraction vs. dose in rads. Basic surviving fraction can be determined by using the following equation:

$$\text{Surviving Fraction} = \frac{\text{\# countable colonies}}{\text{\# plated cells x PE}}$$ [17]

Using a compilation of data from three experiments, the survival response of P3 cells to 50 KvP X-rays was evaluated. (fig. 5). It is interesting to note the fairly large shoulder uncharacteristic of most human fibroblast cell lines. Human fibroblast cells usually have exponential shoulders surviving fraction curves.[18] In contrast to human fibroblasts, the P3 epithelial cells appear to have a substantial shoulder. Such a shoulder represents the observation that damage must be accumulated before killing. If there is no such accumulation necessary before killing, one would expect a linear relationship between killing and dose. A Dq value of 156 rads appropriately reflects the presence of a substantial shoulder upon the curve.[19]

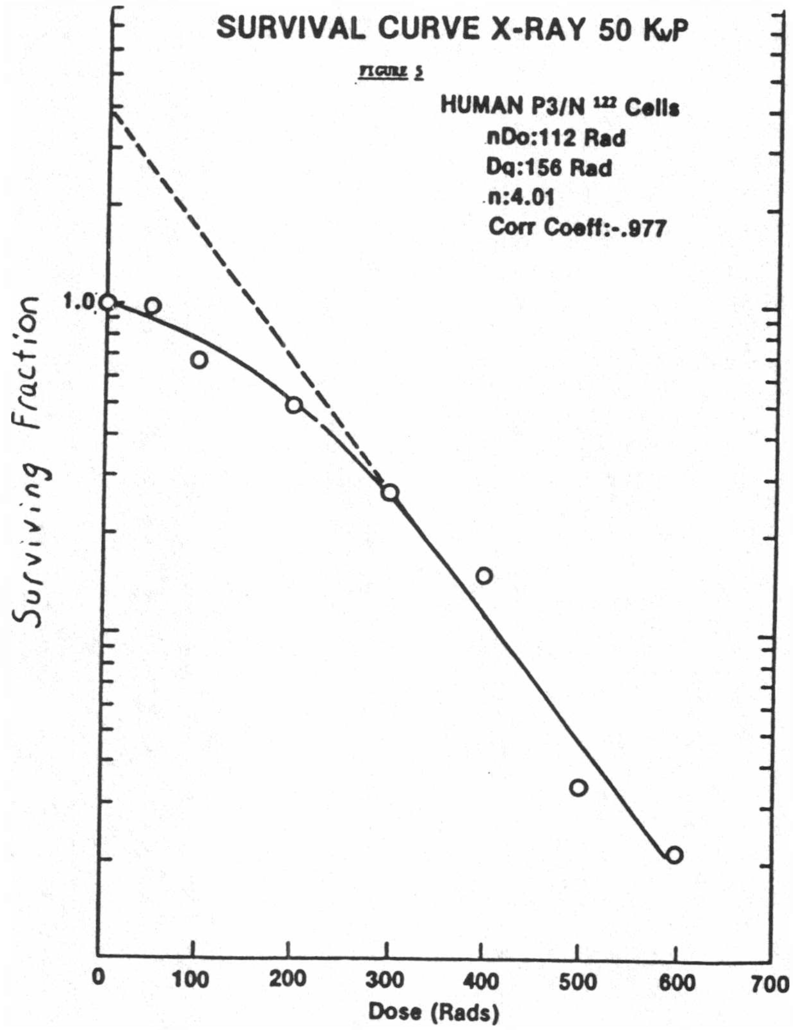

Fig. 5. Human P3 Cell Survival Curve following irradiation with 50 KvP X-rays. The line is fitted via linear regression of the last four points on the curve. Data points represent a compilation of three separate experiments.

Such a shoulder suggests some form of sublethal damage repair mechanism ongoing in the P3 cells.[20] Via further evaluation of this survival curve, one notes a D_0 of 112 rads which reflects the linear relationship between killing and dose after the initial shoulder.[21] These values are determined utilizing linear regression from the last four values and from equations from Hall.[22]

Evaluating another preliminary curve utilizing data obtained from a single experiment with ^{60}Co gamma radiation as a source, one notes the absence of such a large initial shoulder when compared to the results obtained from X-irradiation. (fig. 6).

A final survival curve was plotted for averaged results from two experiments from P3 cell irradiation with Janus Reactor fission spectrum neutrons. (fig. 7). From these preliminary results, there appears to be a slight shoulder upon the curve reflected by the small Dq value of 14.3 rads. The strong killing effect of Janus Reactor fission spectrum neutrons upon P3 cells is reflected by a D_0 of 62 rads.

<u>6-Thioguanine Mutation Assay</u>

6-Thioguanine mutation induction following irradiation of P3 cells with Janus Reactor fission spectrum neutrons was analyzed via taking the average of mutation frequency values obtained via two separate experiments. These average values were then plotted against actual dose along with results from similar experiments with the rodent fibroblast (V79) cell line. (Personal communication, Dr. C.K. Hill). (fig. 8). Interestingly, it appears that Janus Reactor fission spectrum neutrons have a slightly greater effect upon the frequency of 6-TG resistant mutants in P3 cells than in V79 cells.

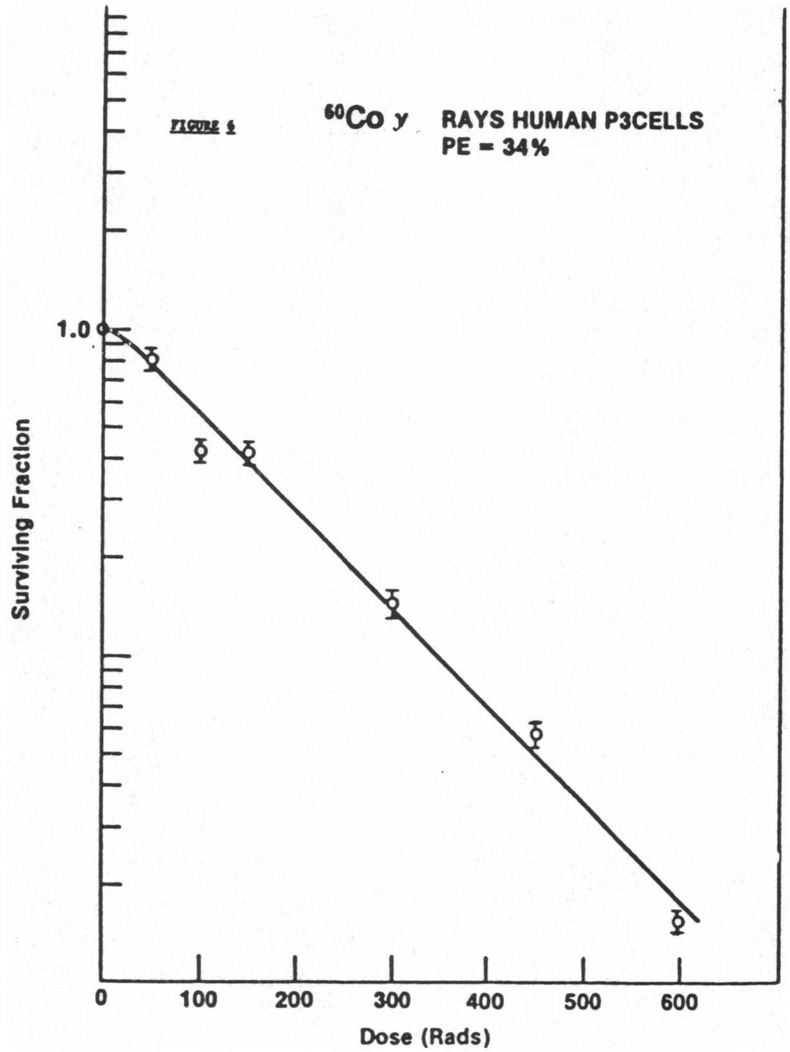

Fig. 6. Human P3 Cell Survival Curve following irradiation with ^{60}Co gamma rays. The line is fitted by linear regression of the last six data points. Error bars reflect standard error of the mean and range from ±0.14% to ±4.2%.

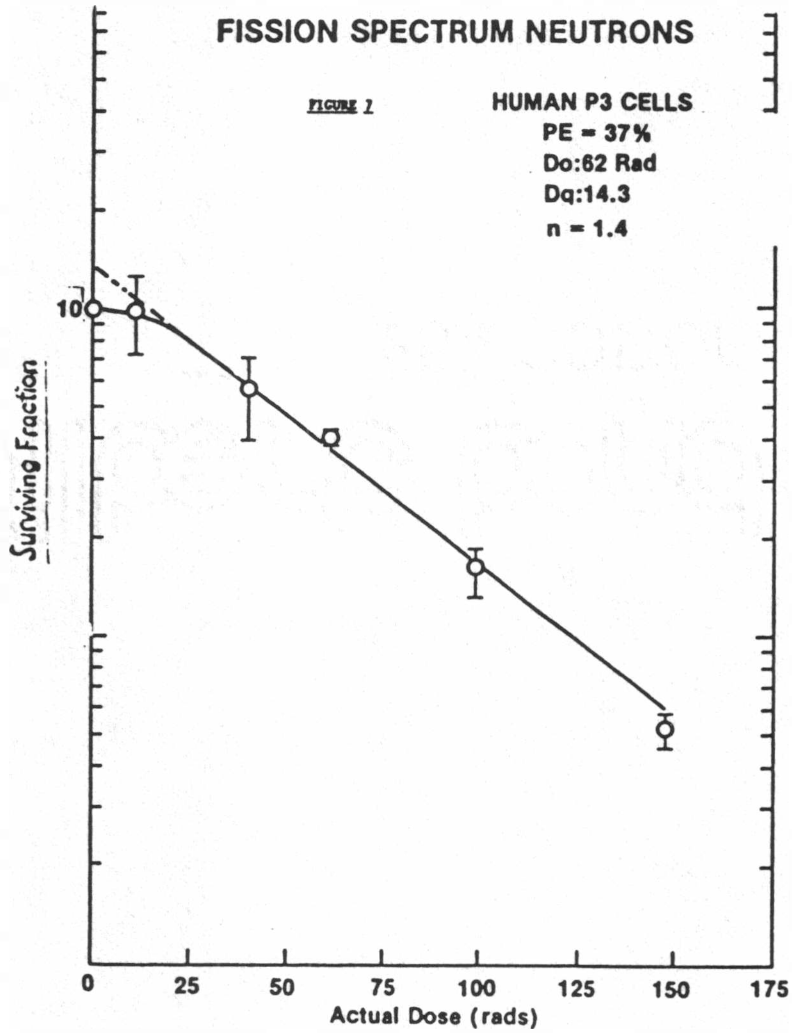

Fig. 7. Human P3 Cell Survival Curve following irradiation with Janus Reactor fission spectrum neutrons. The line is fitted by linear regression of the curve's last four data points. Data points represent an average of two separate experiments. Error bars reflect the range of the results of these two experiments.

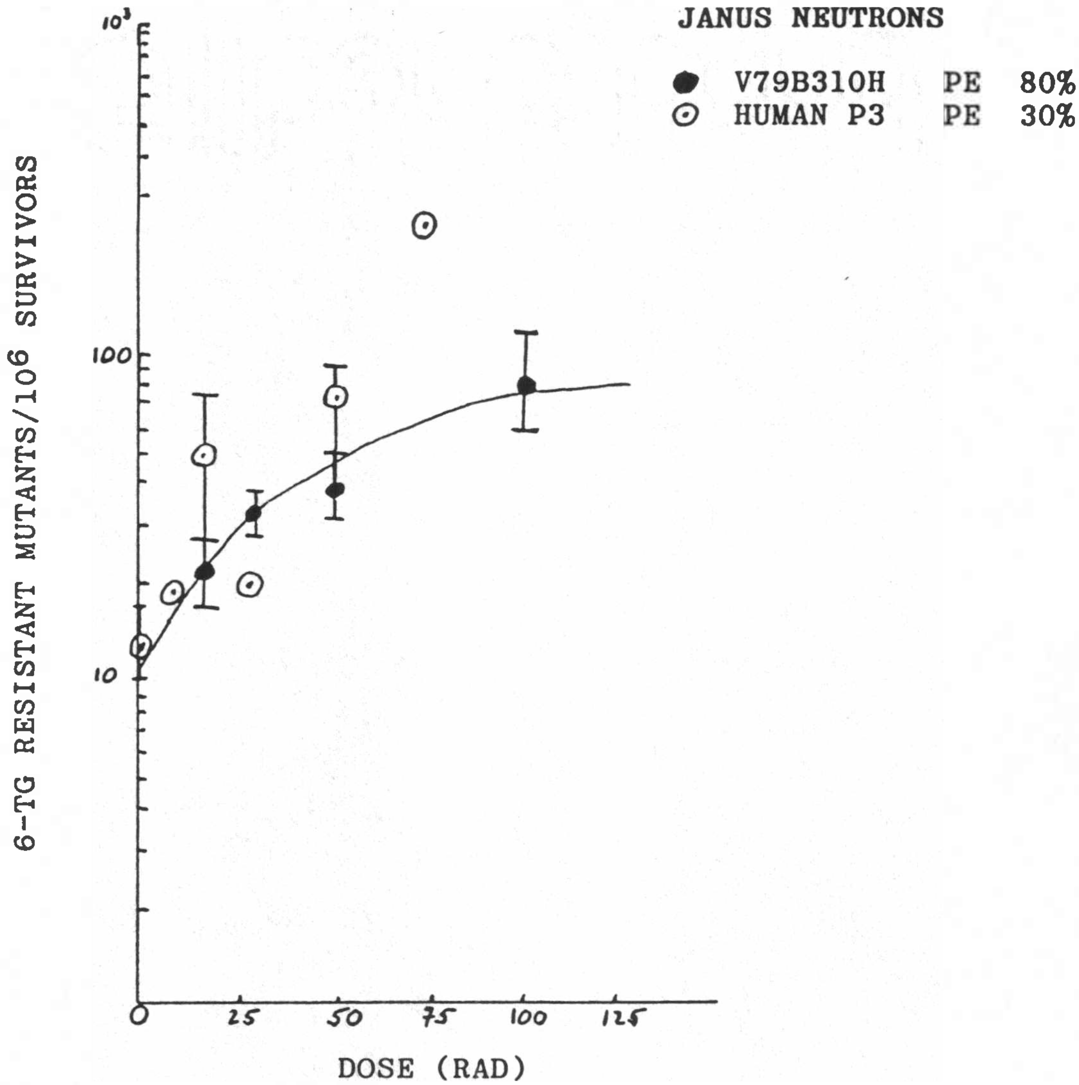

Fig. 8. 6-Thioguanine Resistant Mutant Frequency following irradiation with Janus Reactor fission spectrum neutrons of V79 rodent fibroblast cells and P3 human epithelial cells. Error bars on V79 points represent a reflection of the standard error of the mean. Error bars on P3 points reflect the range of results of two separate experiments.

DISCUSSION AND CONCLUSION

In evaluating the effects of the three types of radiation upon the survival response of the P3 cells, the term "relative biological effectiveness (RBE)" is often used.[23] As defined by the International Commission on Radiobiological Units and Measurements, the relative biological effectiveness (RBE) is the ratio of absorbed doses of two radiations required to produce the same biological effect.[24] In preliminary comparison of 50 KvP X-rays to ^{60}Co gamma rays, the RBE of gamma rays relative to X-rays is 1.09 at the 5% survival level. Further, the RBE of Janus Reactor fission spectrum neutrons relative to X-rays at the 5% survival level is 3.15. Finally, to compare Janus Reactor fission spectrum neutrons with ^{60}Co gamma rays at the 5% survival level, the RBE is 2.88. This RBE value is high when compared to the V79 RBE value of 2.3, perhaps suggesting that P3 epithelial cells might be more sensitive to Janus Reactor fission spectrum neutron irradiation than fibroblast cells. (Personal communication, Dr. C.K. Hill).

In evaluating growth curves and in actually counting stained colonies, certain steps might be taken to improve the ability to distinguish small legitimate P3 cell colonies from abortive colonies. In mutation assays, the 6-thioguanine level in media might be increased to 10.0 micrograms/ml to further reduce background survivors. Also, the P3 cells might be treated with HAT medium periodically before such experiments to rid the population of any background 6-thioguanine resistant mutants.[25] HAT medium contains hypoxanthine, aminopterin and thymidine. Hypoxanthine can be converted into vital purine bases via the hypoxanthine guanine phosphorbosyl transferase enzyme. Thymidine can be converted into needed phrimidine bases via the thymidine kinase salvage enzyme. Aminopterin acts to prevent the "de novo" synthesis of purine and pyrimidine bases that would normally occur in the absence of hypoxanthine, thymidine or their respective salvage enzymes. Thus, any mutants lacking the HGPRT bases will not be able to survive in HAT medium. Finally, incubation time of samples being treated with 6-TG media might be increased to 16 or 17 days to better distinguish mutant colonies from abortive colonies.

In conclusion, the P3 human epithelial cell line seems to work fairly well in radiation survival response experiments. Perhaps more importantly, however, preliminary results seem to suggests a strong potential for this human epithelial cell line in quantitative radiation induced 6-thioguanine resistance mutation assays. Current work involves further radiobiological characterization of the P3 cell line to evaluate the 6-thioguanine resistance mutation response following irradiation with 50 KvP X-rays and ^{60}Co gamma rays.

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