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Radioprotection of Intestinal Stem Cells and Whole Body Radiation Lethality from Photons and Neutrons by Prostaglandins Alone or in Combination with WR-2721

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Technical Report

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WR-2721. These results suggest that a high degree of protection might be achieved by the combination of protectors with different mechanisms. The use of this approach to radiation protection may, in turn, reduce the toxic effects of any single protective agent. Further evidence that the mechanism(s) of radioprotection by WR-2721 and PGs are different came from results showing that WR-2721 decreased the number of DNA single strand breaks by radiation whereas PGs did not affect radiation-induced strand breaks. These data are not conclusive proof that PGs do not protect DNA in some way. However, they suggest that other targets may be protected by PGs and that these targets may be important for cell survival following irradiation.

Future studies will focus on structure-activity relationships for PG-induced protection. New analogues will be tested to search for more protective agents. These agents will be added to WR-2721 to investigate the most protective combination with the least toxicity. In addition, studies into the mechanism of PG-induced protection will be done to explore how these potent bio-active compounds protect cells from a wide variety of injurious agents including photon and neutron irradiation.

These studies should add significant new information about radiation lesions and how protective agents reduce these lesions. The investigations outlined in this report added knowledge to the practical aspects of radiation protection and those planned for the future should continue to add to this knowledge.

CONVERSION TABLE

Conversion factors for U.S. Customary to metric (SI) units of measurement.

MULTIPLY \longrightarrow BY \longrightarrow TO GET
 TO GET \longleftarrow BY \longleftarrow DIVIDE

| | | |
|---|------------------------------|---|
| angstrom | 1.000 000 X E -10 | meters (m) |
| atmosphere (normal) | 1.013 25 X E +2 | kilo pascal (kPa) |
| bar | 1.000 000 X E +2 | kilo pascal (kPa) |
| barn | 1.000 000 X E -28 | meter ² (m ²) |
| British thermal unit (thermochemical) | 1.054 350 X E +3 | joule (J) |
| calorie (thermochemical) | 4.184 000 | joule (J) |
| cal (thermochemical)/cm ² | 4.184 000 X E -2 | mega joule/m ² (MJ/m ²) |
| curie | 3.700 000 X E +1 | giga becquerel (GBq)* |
| degree (angle) | 1.745 329 X E -2 | radian (rad) |
| degree Fahrenheit | $T_K = (T_F + 459.67) / 1.8$ | degree kelvin (K) |
| electron volt | 1.602 19 X E -19 | joule (J) |
| erg | 1.000 000 X E -7 | joule (J) |
| erg/second | 1.000 000 X E -7 | watt (W) |
| foot | 3.048 000 X E -1 | meter (m) |
| foot-pound-force | 1.355 818 | joule (J) |
| gallon (U.S. liquid) | 3.785 412 X E -3 | meter ³ (m ³) |
| inch | 2.540 000 X E -2 | meter (m) |
| jerk | 1.000 000 X E +9 | joule (J) |
| joule/kilogram (J/kg) (radiation dose absorbed) | 1.000 000 | Gray (Gy)** |
| kilotons | 4.183 | terajoules |
| kip (1000 lbf) | 4.448 222 X E +3 | newton (N) |
| kip/inch ² (ksi) | 6.894 757 X E +3 | kilo pascal (kPa) |
| ktap | 1.000 000 X E +2 | newton-second/m ² (N-s/m ²) |
| micron | 1.000 000 X E -6 | meter (m) |
| mil | 2.540 000 X E -5 | meter (m) |
| mile (international) | 1.609 344 X E +3 | meter (m) |
| ounce | 2.834 952 X E -2 | kilogram (kg) |
| pound-force (lbf avoirdupois) | 4.448 222 | newton (N) |
| pound-force inch | 1.129 848 X E -1 | newton-meter (N·m) |
| pound-force/inch | 1.751 268 X E +2 | newton/meter (N/m) |
| pound-force/foot ² | 4.788 026 X E -2 | kilo pascal (kPa) |
| pound-force/inch ² (psi) | 6.894 757 | kilo pascal (kPa) |
| pound-mass (lbm avoirdupois) | 4.535 924 X E -1 | kilogram (kg) |
| pound-mass-foot ² (moment of inertia) | 4.214 011 X E -2 | kilogram-meter ² (kg·m ²) |
| pound-mass/foot ³ | 1.601 846 X E +1 | kilogram/meter ³ (kg/m ³) |
| rad (radiation dose absorbed) | 1.000 000 X E -2 | Gray (Gy)** |
| roentgen | 2.579 760 X E -4 | coulomb/kilogram (C/kg) |
| shake | 1.000 000 X E -8 | second (s) |
| slug | 1.459 390 X E +1 | kilogram (kg) |
| corr (mm Hg, 0°C) | 1.333 22 X E -1 | kilo pascal (kPa) |

* The becquerel (Bq) is the SI unit of radioactivity; 1 Bq = 1 event/s.

**The Gray (Gy) is the SI unit of absorbed radiation.

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SECTION 1

INTRODUCTION

The mammalian intestine is one of the most radiosensitive and radiation dose limiting organs of the body. Photon doses in the range between 10 and 100 Gray and fission neutron doses in the range between 4 and 40 Gray cause a complicated gastrointestinal (G1) radiation syndrome associated with diarrhea, malabsorption, bacterial sepsis and the loss of intestinal stem cell reproductive integrity that leads to mucosal collapse. The actual cause of death is not associated with any one specific effect and is most likely a response to the perturbation of tissue function associated with the collapse of structural integrity. The return of structural integrity of the intestine following radiation is dependent on the survival and the proliferation of intestinal stem cells; those cells that reproduce their population and give rise eventually to the differentiated absorptive villus columnar epithelium.

There are several factors that influence the radiosensitivity of the G1 system. Some endogenous factors relate to the number, the proliferative state, and the cell cycle position of the stem cells within the intestinal crypts (1-5). In addition, there are several exogenous agents that influence radiosensitivity (6). Of the radioprotectants, WR-2721 [S-2-(3-aminopropylamino) ethylphosphorothioic acid] is one of the most effective and widely studied (7-9).

Historically, most of the exogenous radioprotectants have been thiol containing agents that are thought to protect by "scavenging" free radicals produced by ionizing radiation. Of the naturally occurring sulfur-containing amino acids, cystamine and cysteamine are the most potent protectors, thus the S-H bond within both endogenous and exogenous radioprotective molecules appears to be one critical factor. The mechanisms of

radiation protection by thiol compounds are thought to occur through hydrogen atom donation, competition of sulfhydryl groups with free radicals (free radical scavenging) that would otherwise react with oxygen to form damaging oxygen radicals (10), or by the induction of hypoxia (11). These mechanisms are attributed, in general, to radiation protection by compounds containing thiols both exogenously administered or endogenous thiol compounds such as glutathione (12,13). In apparent contrast to this general axiom, an experimental observation was made in 1982 and reported in 1983 (14) that showed that the non-sulfur containing prostaglandins (PGs), the naturally occurring and potent bioactive cyclooxygenase products of the arachidonic acid cascade, protected the murine intestine from gamma irradiation.

PGs were discovered years ago through their potent bioactivity. The smooth-muscle-stimulating activity of seminal fluid was first described by Goldblatt (15) and Von Euler (16) in the early 1930s and the bioactive component was named prostaglandin (PG) by Von Euler. Further research on the nature of the bioactive component of seminal fluid was delayed until 1957 when Bergstrom described and later characterized two PGs from sheep vesicular glands and named one PGF for its solubility in phosphate (fosfate in Swedish) and the other, PGE for its solubility in ether (17).

Subsequent investigations revealed a family of naturally occurring PGs (18,19), Figure 1. It is now recognized that all mammalian tissue (other than mature erythrocytes) can synthesize PGs through the cyclooxygenase pathway of the arachidonic acid (AA) cascade. Later, a lipoxygenase pathway of AA was found along with the lipoxygenase products, the leukotrienes (LTs) or slow reacting substances. The LTs have been implicated in several physiological and pathological processes such as neutrophil function and anaphylaxis (20).

Under normal physiological conditions, PGs are synthesized "on demand" and then rapidly catabolized with variable but short half-lives measured in seconds to a few minutes, therefore, PG concentrations in tissues and plasma are normally low. PGs appear to mediate several-biological phenomena under normal conditions, but excessive and chronically high PG concentrations are implicated in the pathogenesis of a variety of diseases including hypertension, asthma, calcium metabolic defects, angina pectoris and peptic ulcer. As a result of the array and sometimes antagonistic biologic effects of the family of PGs and LTs, the metabolism, pharmacology, mode of action, stimulation and inhibition of AA have important implication in cell biology and clinical medicine.

In contrast to the reports of a pathological influence of PGs, Robert et al. (21) showed that exogenous PGs protected the gastric and intestinal mucosa when given before ulcerogenic treatments such as indomethacin or absolute ethanol. Robert et al. (22) further showed that this effect was true cytoprotection rather than an antiulcer effect observed with compounds similar to cimetidine. These intriguing studies on PG-induced cytoprotection of the gastrointestinal mucosa from a variety of injurious agents prompted investigations of the role that PGs may play in the expression of intestinal injury and subsequent recovery after ionizing radiation.

Based on the cytoprotective effects of PGs reported by Robert et al. (21), pilot studies were done to measure a possible radioprotective effect of PGs on normal tissue. In our initial studies, five 350 g rats were given 50 ug 16-16 dm PGE₂ subcutaneously (sc) in the dorsal neck region, 1 hour before a whole body dose of 13.5.0 Gy ¹³⁷Cs gamma rays. Another 5 rats were given 0.5 ml of 5% ethanol solution, the solvent for the PG, 1 hour before 13.5.0 Gy. Three days later, tritiated thymidine (³HTdR, 6.7 Ci/mM, 1 uCi/g body weight) was given intraperitoneally (IP) to both groups, and the animals were killed 1 hour

later. Autoradiographs of 5 μ cross-sections of the ileum showed an increased number of crypt cells that incorporated $^3\text{HTdR}$ in PG treated compared with control rats. The number of ^3H radiolabeled structures per ileal circumference in the control animals was 34 ± 4 compared with 135 ± 5 in PG treated rats [microcolony assay (23)]. A detailed study of PG-induced radioprotection of intestinal clonogenic cells in mice showed that both the shoulder and the slope of the radiation survival curve was increased compared with the control curve (14).

The radioprotective effect was also PG-dose dependent at low doses. There was a rapid increase in survival at low PG doses followed by a plateau region at higher PG concentrations. PG-induced radioprotection was seen within 5 minutes after PG administration and reached the maximum at 1 hour. No protective effect occurred if the time between a single dose of PG and irradiation was 4 hours or more. No PG-induced radioprotection was seen if the PG was given after irradiation.

Radioprotection of the intestine was also reflected in an increase in the $\text{LD}_{50/6}$. The 16-16 dm PGE_2 increased the $\text{LD}_{50/6}$ from a control value of 16.3 ± 0.14 Gy (95% confidence limit) to 20.25 ± 0.55 Gy; an increase of 24% for a dose modification factor (DMF) of 1.24.

The studies outlined above were all done with the 16-16 dm PGE_2 synthetic analogue of PGE_2 , however, a similar radioprotective effect has now been seen with a number of both natural and synthetic PGs (Figure 3).

with Dr. E. John Ainsworth at the Lawrence Berkeley Laboratory, the possibility of PG-induced radioprotection of the bone marrow stem cells (CFU-S) was investigated. The 16,16 dm PGE₂ was given sc to donor mice 1 hour before ⁶⁰Co irradiation. After exposure, dilutions of marrow cell suspensions were made from donor femurs and injected into the tail veins of lethally irradiated mice. [method of McCulloch and Till, (24) as modified by Ainsworth et al.(25)].

Both the shoulder and the D₀ of the CFU-S survival curve from PG treated animals were increased compared to the controls that were qualitatively similar to the results found in the intestinal cell renewal system (26). The increase in the shoulder portion of the curve is particularly interesting since marrow stem cells normally do not exhibit a survival curve shoulder. These results show that PG-induced radioprotection is not unique to the intestine and may occur in a variety of tissues.

The results showing PG-induced protection at microgram quantities versus the milligram amounts of WR-2721 needed for protection suggested that the mechanism of PG radioprotection was different from the mechanism of protection by the WR compounds. To explore the possibility that different mechanisms may result in additivity of radioprotection by PGs and the WR compounds, the combination of increasing doses of radioprotection by PGs and the WR compounds, the combination of increasing doses of WR-2721 and 16-16 dm PGE₂ (10 ug/mouse) was compared to increasing doses of WR-2721 alone before a single dose of 13.5 Gy ¹³⁷Cs (Figure 4).

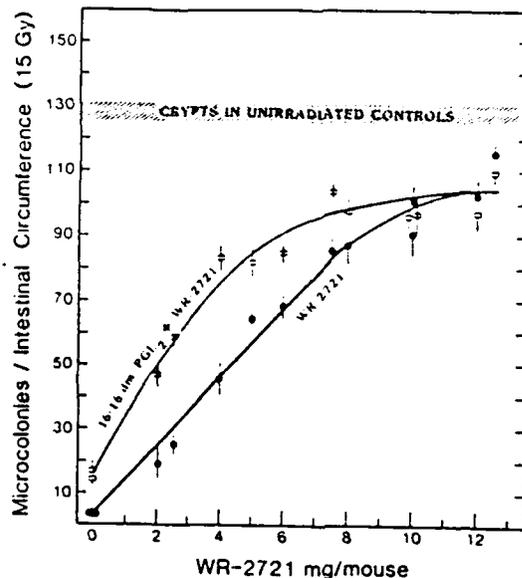


Figure 4. Microcolonies per intestinal circumference in control animals and in mice given 10 ug 16,16 dm PGE₂ 1 hour before 13.5.0 Gy ¹³⁷Cs gamma radiation irradiation alone or in combination with increasing doses of WR-2721.

The radioprotective effect of 10 ug 16-16 dm PGE₂ was additive to the protective effect of WR-2721 about 10 mg of WR-2721. Above that level there was no additional effect of this PG with regard to cell survival.

The results of the studies briefly outlined above suggested that PGs should be explored as a unique and novel class of protectors from many injurious agents including radiation. The experiments summarized above led to the following set of objectives:

Investigate the efficiency of several prostaglandins (PGs) as a radioprotectants of the intestinal cell renewal system in mice.

- 1) by measuring PG-induced radioprotection of intestinal stem cells using the microcolony assay when the PGs were given before:
 - a. ¹³⁷Cs gamma irradiation
 - b. 0.85 MeV (average energy) fission spectrum neutrons from the JANUS reactor at Argonne National Laboratory
 - c. 25 MeV (average energy) fast neutrons generated at the cancer treatment facility (CTF) at Fermilab

- 2) by measuring the $LD50/6$ in controls and in mice treated with the most effective PG radioprotectors found in part 1 before;
 - a. ^{137}Cs gamma radiation
 - b. JANUS neutrons
 - c. Fermilab neutrons

Investigate intestinal stem cell radioprotection and the shift in $LD50/6$ in mice given the most radioprotective PGs found in part 1 in combination with WR-2721 before gamma or neutron irradiation.

Measure the number of initial single strand breaks in DNA and measure the repair rate of these breaks in intestinal cells given the most protective PGs alone or in combination with WR-2721.

SECTION 2

MATERIALS AND METHODS

2.1 THE EFFICIENCY OF DIFFERENT PROSTAGLANDINS (PGs) AS RADIOPROTECTANTS OF THE GI SYSTEM FROM PHOTON AND NEUTRON RADIATION INJURY

These experiments investigated the dose response of several PGs to establish the most protective PGs and the time course for the maximum degree of PG-induced radioprotection. The results of these studies identified those PGs that were used in the other studies. To investigate the dose response of PG-induced radioprotection, the following PGs were obtained from several sources: E₂, F_{2α}, I₂, TBX, 16-16 dm PGE₂, PGI₂ and I₂ analogues were obtained from Upjohn and from Schering AG, Berlin; misoprostol and several other analogues were obtained from G.D Searle and Co. The PGs were diluted with the appropriate solvent for that particular PG such that the same volume of 0.2 cc solution per mouse was delivered subcutaneously (sc). Control animals were injected with the same solvent without the PGs. B6D2F₁ male mice from Jackson Laboratories were used for these studies. For the photon studies, a single dose of 13.5 Gy ¹³⁷Cs gamma radiation was given to groups of controls receiving no treatment, controls that received only the solvent, and PG treated animals (5 mice per group). A dose of 13.5 Gy was chosen because the resulting survival of intestinal stem cells was about 5 microcolonies per circumference that was toward the lower end of the exponential portion of the survival curve; therefore, radioprotection of the stem cells was reflected by an increase in survival without interference of the shoulder region of the curve. The animals were irradiated 2 hours after PG or solvent administration which was the time found to provide the maximum degree of protection. The PG doses were 0, 1, 5, 10, 25, 50, and 100 ug/mouse.

Four days after irradiation, the mice were killed by cervical fracture. The jejunum was removed and fixed in alcohol, formalin and acetic acid (AFA, 20:2:1), embedded in paraffin, and 5 μ cross-sections were placed on slides. After staining with Harris hematoxylin, the regenerative foci or microcolonies of intestinal epithelial cells were counted in 18 cross-sections per mouse. The average number of microcolonies per cross-section for each group of PG-treated mice ($N = 5$) was plotted versus dose of drug. The degree of radioprotection was measured by the increase in the number of colonies per intestinal circumference compared to control values.

Selected doses of the most protective PGs that were chemically stable were given orally to mice before 13.5 Gy ^{137}Cs . Four days later, the animals were killed by cervical fracture and the survival of intestinal stem cells was evaluated by the microcolony assay as described. The rationale for this route of PG administration comes from the observations of Robert et al. (21) that cytoprotection from ulcerogenic treatment was greatest when the PGs were given orally. Although cytoprotection and radioprotection may occur through different mechanisms, the number of similarities are so many that the mechanisms must be related; therefore, oral administration may be an effective method of PG-induced radioprotection.

The PGs that protect the gut most from ^{137}Cs radiation injury were used to investigate PG-induced radioprotection of intestine from neutron injury. Male B6D2F₁ mice (90-130 days old) were taken to the Fermilab CTF and given the PG before graded doses of 9.5 Gy Fermilab neutrons to produce a cell survival curve. The irradiation was accomplished by placing ten mice, 1 at a time, into perforated plastic tubes that, in turn, were placed between two A-150 tissue equivalent disks for build-up and backscatter. The disks holding the mice were placed perpendicular to the neutron beam at a distance of

153 cm from the Be target. The dose rate was about 50 Gy per minute (see reference 27 for details of the irradiation procedure). Following irradiation, the animals were taken back to Rush and killed 4 days later for the microcolony assay as described.

Similar studies were done using the fission spectrum neutrons of the JANUS reactor at Argonne National Laboratory. Mice were sent directly to the animal facilities at Argonne from Jackson Laboratories and housed there until the experiments. This was necessary to prevent any possibility of cross infection between the animal colonies of Rush and Argonne. This precaution was not necessary for the Fermilab experiments since Fermilab does not have an animal facility. At four days after irradiation, the animals were killed for the microcolony assay as described.

The same most protective PGs used above were used to measure an increase in the LD50/6. Each PG was given to groups of animals at the optimum time before increasing doses of ¹³⁷Cs. Control animals were given the solvent for each PG. About 20 animals were irradiated at each of 6 graded doses. Deaths were recorded twice daily for estimates of the LD50/6.

2.2 INTESTINAL STEM CELL SURVIVAL AND LD50/6 MEASUREMENTS OF ANIMALS GIVEN THE MOST RADIOPROTECTIVE PGs IN COMBINATION WITH WR-2721.

The rationale for these studies came from the possibility that the mechanism of PG radioprotection was different comparing PGs and the WR compounds. This possibility was suggested by the evidence that PG-induced radioprotection was additive to the S-H free radical scavenging mechanism associated with radioprotection by WR-2721. To investigate this possibility, male B6D2F₁ mice were given the most protective PGs 2 hours before and WR-2721 (400 mg/Kg) a half hour before irradiation with ¹³⁷Cs. Selected studies were also

done with JANUS neutrons. Intestinal stem cell survival curves were assayed as described above as well as the LD50/6 measurements. These values were compared to those for controls and for WR-2721 treated animals and to the PG treated animals in part 1.

2.3 THE NUMBER OF INITIAL SINGLE STRAND BREAKS AND THE BREAK REPAIR RATE OF DNA OF INTESTINAL CELLS FROM CONTROL IRRADIATED ANIMALS OR MICE GIVEN WR-2721, THE MOST PROTECTIVE PGs, OR THE COMBINATION OF THE TWO.

In the presence of a strong alkaline solution, isolated DNA will unwind from its normal helical structure. If a DNA damaging agent has been administered before DNA isolation, the alkaline treatment will fragment the DNA where the damage has occurred. Radiation produces both single and double strand DNA breaks in a dose dependent manor (28). When DNA was isolated from irradiated tissue and filtered or eluted, a dose dependent degree of damage was seen. WR-2721 decreased the number of initial single strand breaks in several tissues including the intestine. Dr. David Grdina at Argonne National Laboratory has kindly collaborated on a study to measure the number of single strand breaks in PG treated animals. To measure the number of initial strand breaks, mice treated with the most protective PG were given the following doses of gamma irradiation 1 hour later: 0, 5, 10, and 15 Gy. Separate groups of mice were given 16,16 dm PGE₂. The mice were killed immediately after irradiation and the intestines were flushed and submerged in cold EDTA-ringers-phosphate solution. The intestines were cut open and cells were gently scraped off using a microscope slide. Alkaline elution was done as described in detail (27) and the eluted DNA and filter retained DNA was measured using a Perkins-Elmer fluorometer. To measure the rate of repair of the DNA, a single dose of 15 Gy was given to animals treated with PGs or WR-2721. Mice were killed

immediately or at 13.5, 30, 45, 60, 120, and 180 minutes after irradiation. The rate of repair of DNA in controls or in radioprotected mice were reflected in the reduced number of strand breaks measured with increasing time after radiation.

2.4 STATISTICAL ANALYSIS OF DATA.

Data which generated survival curves were analyzed with a computer program designed to apply a linear quadratic formula including a least square analysis of the linear terminal portion of the curve. This analysis provided a D_0 value with 95% confidence limits. The LD50 data were analyzed with a probit program (Statistical Analysis Systems Inc.) that provided the sigmoid and transformed lines of best fit to the data along with the radiation dose that causes 50% mortality within 6 days in the experimental population. Differences in the single dose measurements were analyzed by an analysis of variance comparing treatment with time.

SECTION 3

RESULTS

The dose responses of essentially all the PGs tested to date were similar (Figure 5). There was a rapid increase in protection at low PG doses followed by a plateau at higher doses that confirms our previous results. Of the PG tested currently, three analogues stand out as the best candidates for further studies; 16,16 dm PGE₂ (29), misoprostol (30) a PGE₁ analogue, and iloprost (31), a PGI₂ analogue. The dose response of iloprost is shown in Figure 6.

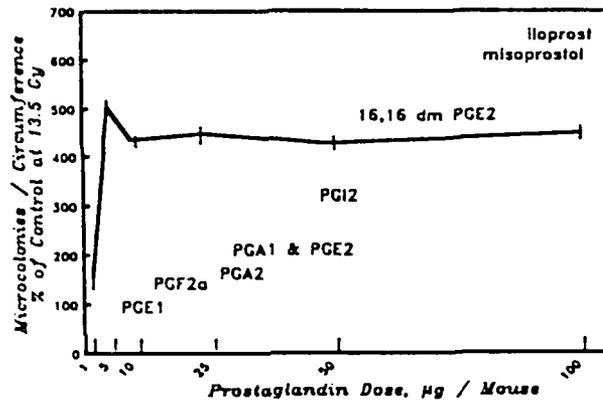


Figure 5. Intestinal microcolonies that reflects crypt stem cell survival at a single radiation dose of 13.5 Gy versus dose of several PGs.

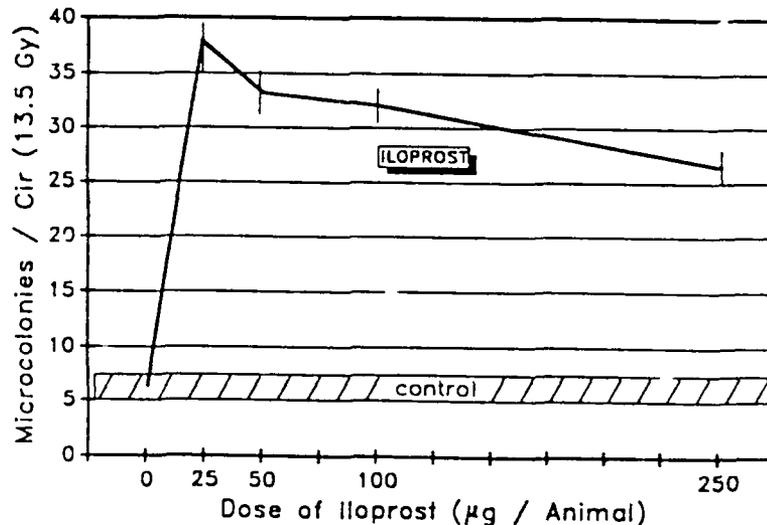


Figure 6. Microcolonies per intestinal circumference versus dose of iloprost given sc before 13.5 Gy ¹³⁷Cs gamma radiation. Data are presented as means of 5 mice \pm 1 SEM.

These three analogues were used for the other studies reported here. To investigate the oral activity of these analogues, they were given orally to mice as described in the methods section. Of the most protective PGs, iloprost protected mice the most effectively when given orally (Figure 7).

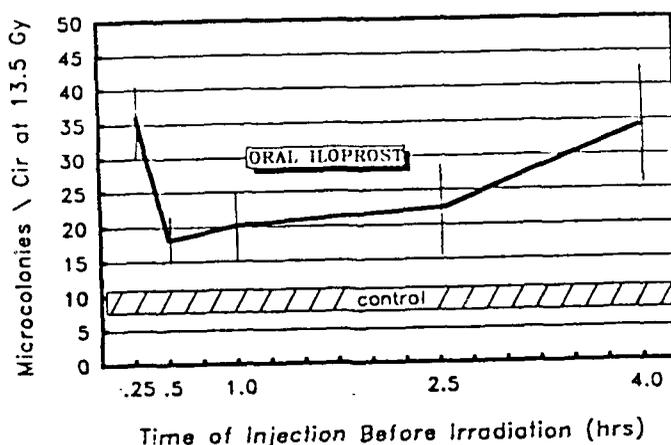


Figure 7. Microcolonies per intestinal circumference versus time of oral iloprost administration (25 ug/0.2 cc) before 13.5 Gy ^{137}Cs radiation. Means of 5 mice \pm 1 sem are presented.

The data were more variable than when iloprost was given sc. The optimal time for oral iloprost protection was 15 minutes before irradiation. There appeared to be a second increase when iloprost was given 4 hours before irradiation.

To investigate prostaglandin-induced radiation protection from neutron radiation, the most protective PG analogues were given before high energy Fermi neutrons (30) or low energy, high LET JANUS neutrons (32). The data showing 16,16 dm PGE₂-induced protection from Fermi neutrons is shown in Figure 8. These results were similar to those when misoprostol was given before irradiation.

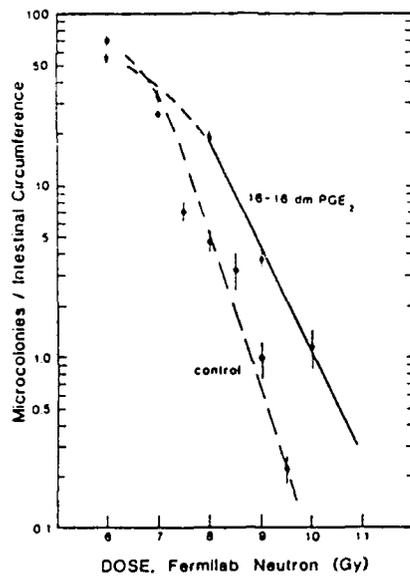


Figure 8. Microcolonies per jejunal circumference 4 days after graded doses of fermilab neutrons in controls or in animals given 10 ug 16,16 dm PGE₂ 1 hour before irradiation. Each symbol represents the mean value from 5 mice \pm 1 SEM.

Figure 9 shows the degree of protection that misoprostol afforded intestinal clonogenic cells when given before JANUS neutrons. The degree of protection was not as great as with WR-2721; however, the relationship for neutron protection was the same as seen for photon radioprotection. The combination of misoprostol and WR-2721 protected intestinal clonogenic cells to a greater extent than either compound alone.

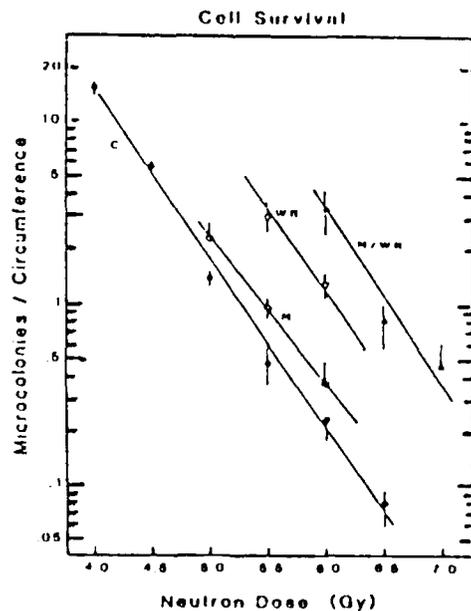


Figure 9. Microcolonies per jejunal circumference 4 days after graded doses of JANUS neutron doses in controls or in animals given misoprostol (25 ug/animal), WR-2721 (400 mg/Kg), or the combination of the two before irradiation. The values represent the mean of 5 mice \pm 1 SEM.

Additivity of radiation protection was also seen in an experiment to investigate the effect of the combination of 16,16 dm PGE₂ and WR-2721 on the LD50/6 of mice (Figure 10).

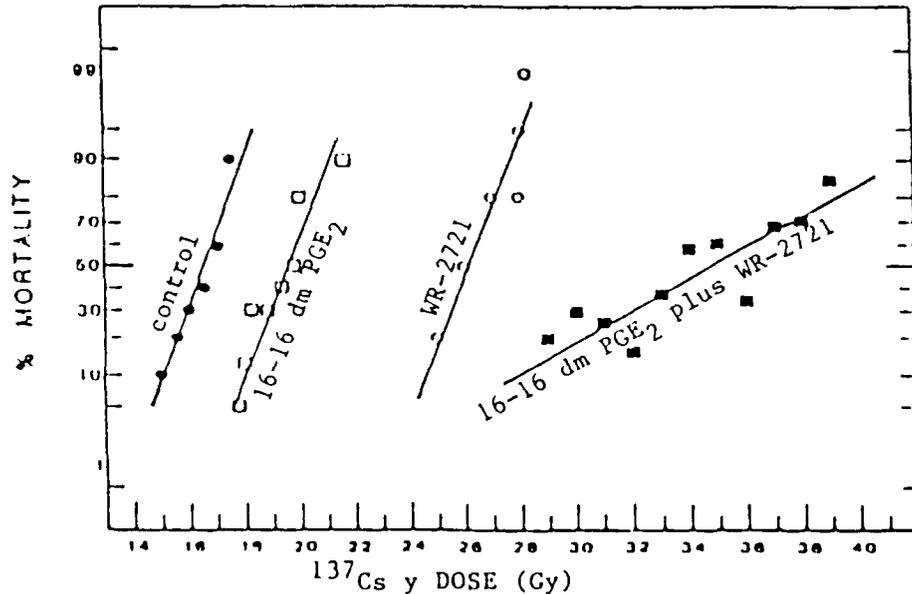


Figure 10. The 6-day mortality of controls and of mice given 10 ug 16,16 dm PGE₂, 10 mg WR-2721, or the combination of the two, before graded doses of ¹³⁷Cs gamma radiation. The doses that killed 50% in 6 days (LD50/6) and the 95% confidence limits were 16.3 ± 0.4, 20.3 ± 0.6 (16,16 dm PGE₂), 26.1 ± 1.4 (WR-2721), and 36.3 ± 1.8 Gy (combination of the two agents).

The estimated intestinal protection factor for this combination compared to controls was 2.27. The slopes of the probability curves for mortality at increasing radiation doses in PG and WR-2721 treated mice were similar to controls, however, the slope of the curve from animals given the combination was markedly decreased. Although these data reflect 6 day mortality, some animals given the combination of protective agents before 26-39 Gy lived to day 9 or 10 post-irradiation. The animals of all other treatment groups were dead by day 7 post-irradiation. Intestinal clonogenic cell survival did not reflect animal survival at 6 days (-combination paper). Therefore, animal longevity was studied at selected doses of radiation for misoprostol (Figure 11 shows the data for ¹³⁷Cs gamma radiation and Figure 12; the data for JANUS neutrons) and for iloprost (Figure 13).

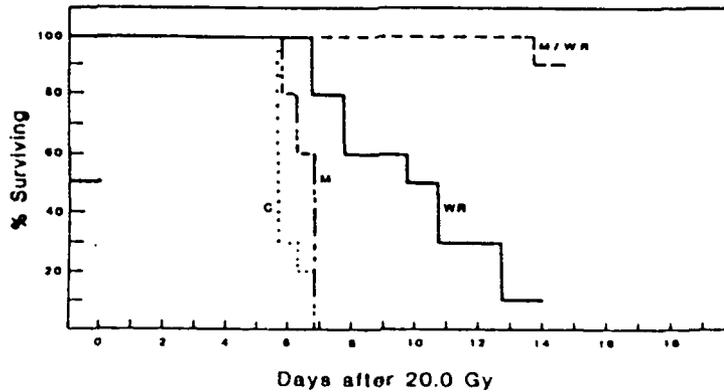


Figure 11. Percent animal survival versus days after 20.0 Gy ¹³⁷Cs gamma radiation in control (C) mice or in animals given 25 ug misoprostol (M), 10 mg WR-2721 (WR), or the combination of misoprostol followed by WR-2721 (M/WR). Each line represents a beginning population of 15 mice.

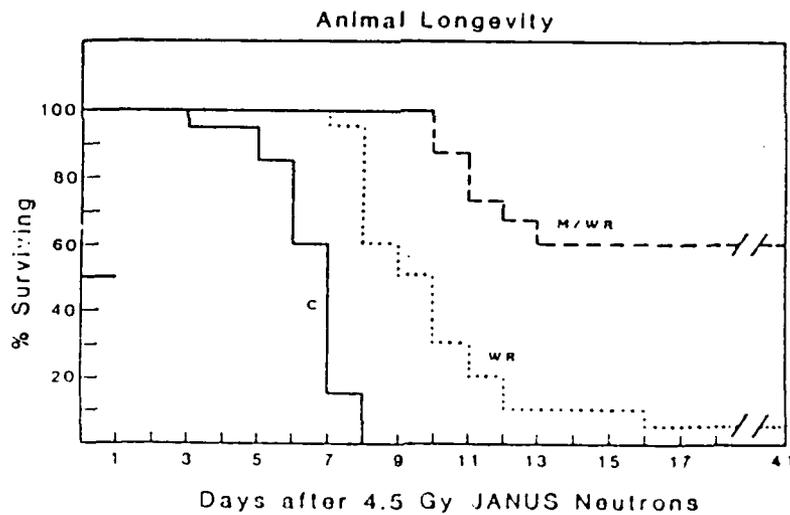


Figure 12. Percent animal survival versus days after 4.5 Gy JANUS neutron radiation in control mice or in animals given 25 ug misoprostol, 10 mg WR-2721 or the combination of misoprostol followed by WR-2721. Each line represents a starting population of 25 mice.

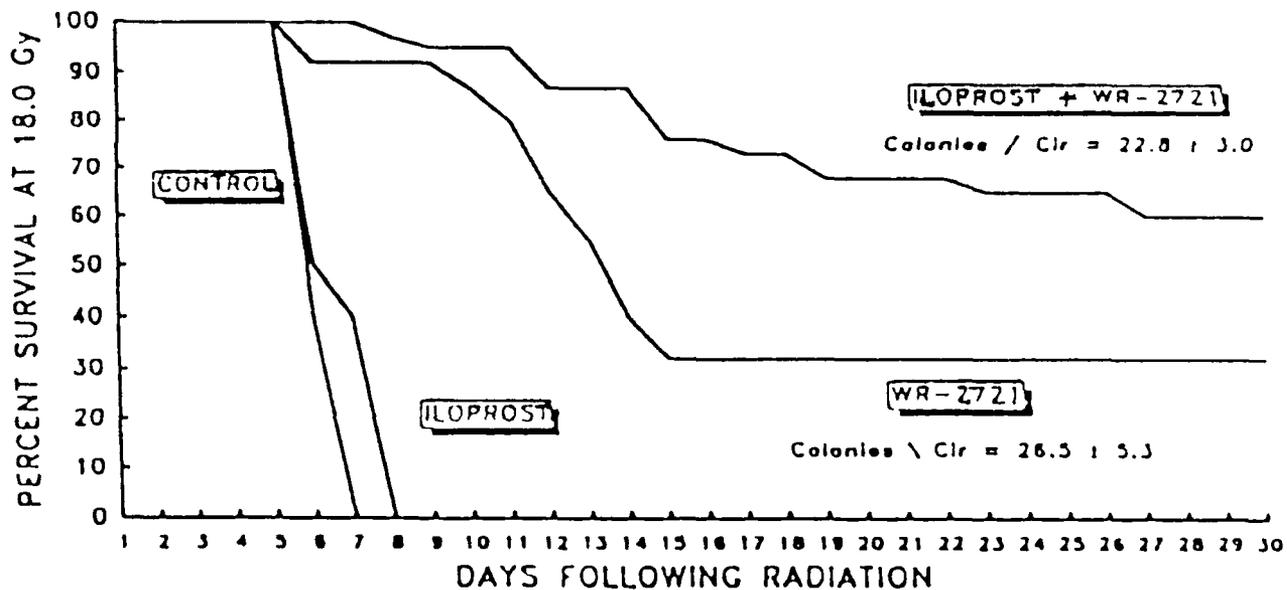


Figure 13. Longevity of control mice (vehicle injected) or mice given iloprost, WR-2721 of the combination of the two before 18.0 Gy. Forty-mice were used in each group.

These data show that PGs combined with WR-2721 are more protective than either agent alone and help confirm the contention that the mechanism for prostaglandin-induced radiation protection is different for WR-2721. Further evidence for this contention came from studies of initial DNA strand breaks in intestinal cells in mice treated with radioprotectors (33). Figure 14 shows that the single strand breaks for controls, and for animals given 5.0 or 15.0 Gy ¹³⁷Cs alone or one-half hour after WR-2721.

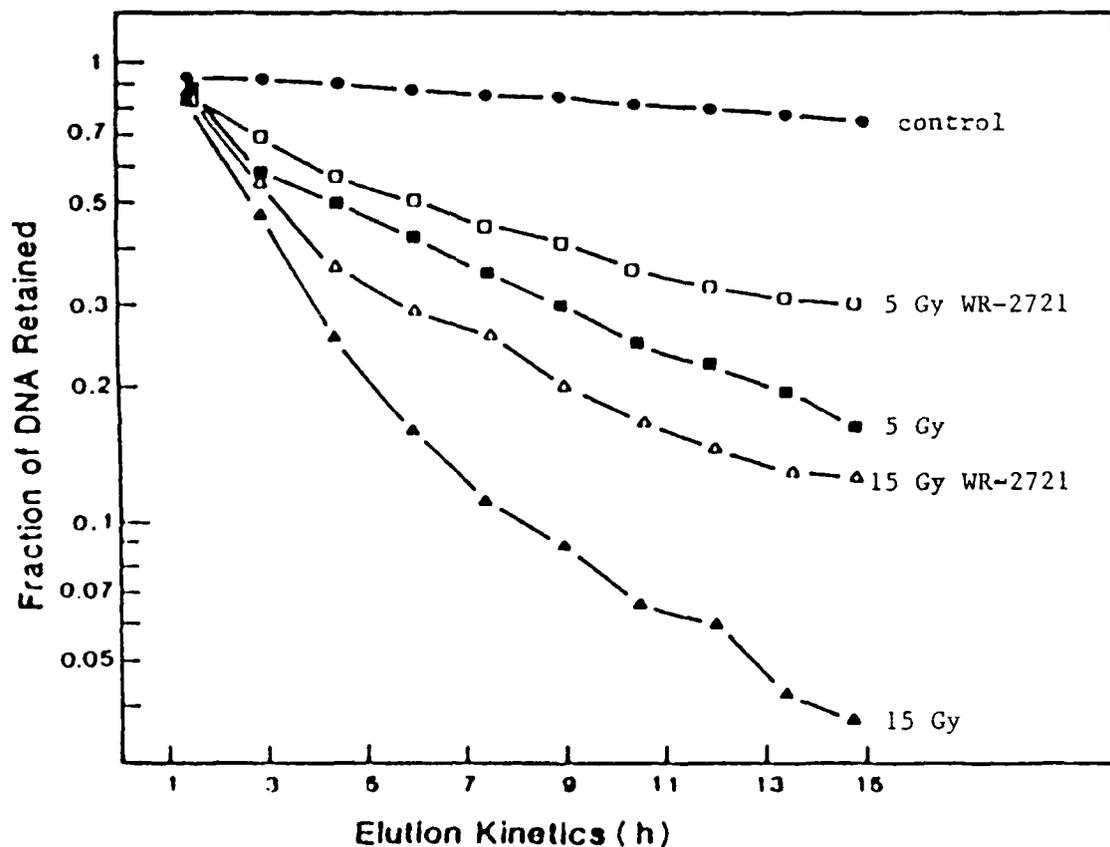


Figure 14. The fraction of DNA retained on filters versus the elution kinetics in hours in controls with or without WR-2721 that received no irradiation; and in animals that received 5 or 15 Gy with or without WR-2721. Less DNA retained on the filter indicates more single strand breaks.

WR-2721 decreased the number of initial strand breaks in DNA of jejunal mucosal cells. In contrast, 16-16 dm PGE₂ appeared to increase the number of initial strand breaks compared to triacetin treated controls (Figure 15). The number of DNA strand breaks at corresponding doses of radiation in the two control groups were the same, therefore, the data from these groups were pooled.

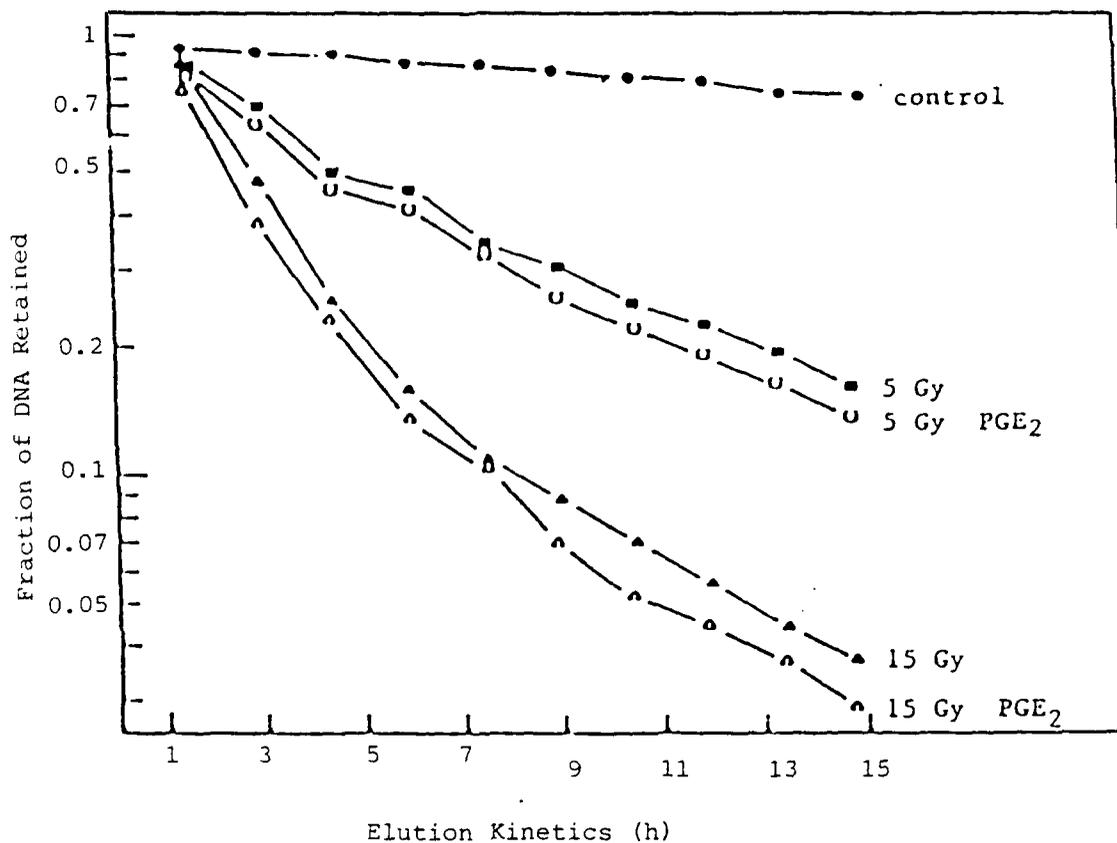


Figure 15. The fraction of DNA retained on filters versus the elution kinetics in hours in controls with or without 16-16 dm PGE₂ and in animals that received 5 or 15 Gy with or without 16-16 dm PGE₂.

In contrast to the observed difference in effect of these two agents on the formation of DNA single strand breaks, the two agents appeared to act similarly with one another in inhibiting the rate of strand break rejoining (Figure 16).

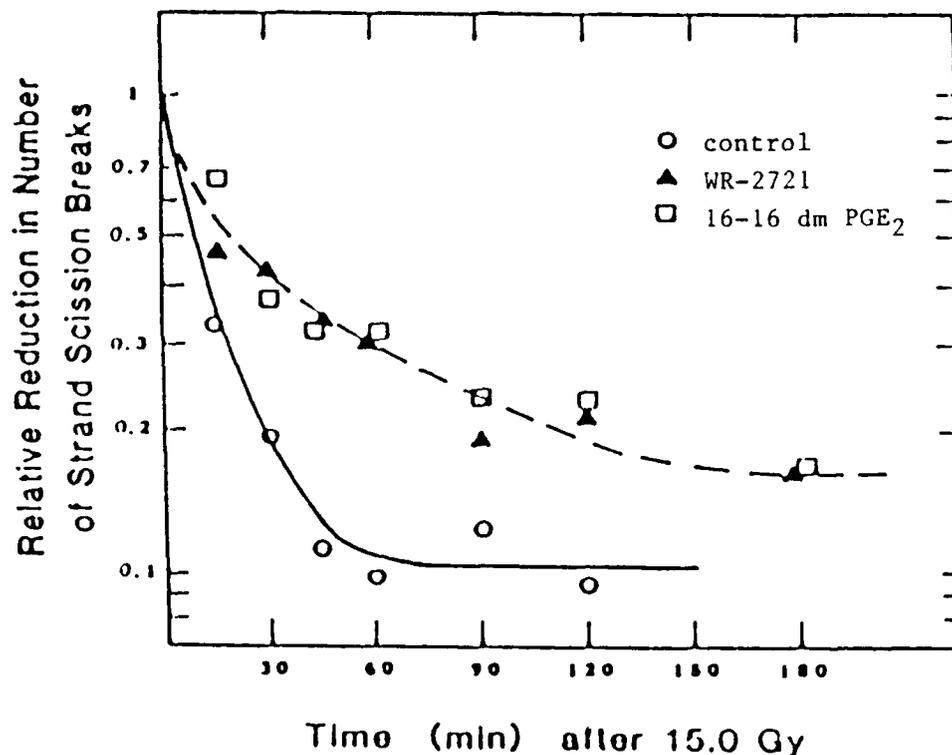


Figure 16. Relative reduction in number of strand scission breaks with time after 15 Gy ¹³⁷Cs irradiation. A reduction to about 0.1 within 45 minutes in control animals represents nearly complete repair. DNA in animals treated with WR-2721 or 16-16 dm PGE₂ did not show complete repair within 3 hours post-exposure.

The strand scission factors were similar for the two agents as a function of time after ¹³⁷Cs exposure and were consistently elevated compared to the control values. The rejoining of DNA breaks was at its maximum by 45 min. following irradiation in controls, however, in animals treated with either WR-2721 or 16-16 dm PGE₂, rejoining of the strand breaks was less than controls, even at 3 hours after exposure.

SECTION 4

DISCUSSION

Radiation protection of military and civilian populations in a hostile radiation environment is one of the primary objectives of research on radioprotective compounds and their mechanisms of action. In addition to the potential advantage that radioprotectants may offer to field operations within a radiation environment, radioprotectants may become more important to personnel in the relatively unknown radiation environment of space as the length of space activities of both civilians and military personnel increase. Investigations into the alteration of radiation sensitivity of cells and organisms by protectors also may lead to a better understanding of the nature of radiation injury that may eventually lead to better protection from radiation-induced mutations or oncogenic transformation and better cancer treatment.

The Walter Reed research and development program on radioprotectors developed a number of mostly sulfur-containing compounds (34), some of which were the most effective radioprotectants found to date; however, one of the limitations to the effectiveness of these agents is the toxicity. Hypotension is the most severe side effect that occurred in both experimental animals and in humans at radioprotective doses. New WR compounds may offer a similar degree of protection with less toxicity; however, another approach is to develop new agents with different mechanisms or to combine radioprotectants that have additive effects to increase the degree of radioprotection and decrease the toxicity of each individual agent. The evidence summarized in this report suggests that prostaglandins are radioprotective agents that are additive to radioprotection by WR-2721 and likely work through separate mechanisms compared to thiol compounds. PGs and WR-2721 appear to have separate properties comparing their effects on cell survival and animal survival.

There are several factors that influence the shape of the post-irradiation murine intestinal cell survival curve. The extent of the shoulder is associated, in part, with both multiplicity (the number of clonogenic cells per crypt) and the ability of clonogenic cells to repair sublethal damage (SLD). The slope of the terminal exponential portion of the curve is thought to define the inherent radiosensitivity of the clonogenic cells surviving the higher radiation doses; however, the slope also may be influenced by repair of SLD. One measure of the efficiency of radiation protective agents is the degree to which the shape of the cell survival curve is changed. WR-2721 increased both the shoulder and the slope of the radiation survival curve. Since these effects were seen one-half hour after WR-2721 administration, it was unlikely that there was time for a change in multiplicity. WR-2721 may increase intestinal clonogenic cell survival and thus alter the shoulder and the slope of the curve by increasing either the extent or the fidelity of repair. Further changes in the survival curve in WR-2721 treated mice may come from increased radiation resistance of clonogenic cells by free radical scavenging, hydrogen atom donation, induced hypoxia, or a combination of these mechanisms.

A similar scenario for 16-16 dm PGE₂-induced radiation protection can be argued since both the shoulder and the slope of the clonogenic survival curve were increased, even though to a lesser extent than that seen after WR-2721. A change in multiplicity of clonogenic cells was again unlikely since 16-16 dm PGE₂ protection of the intestine has been seen within 10 min. after a SC injection in the dorsal neck of mice.

However, there were several notable differences when comparing radiation protection by WR-2721 and 16-16 dm PGE₂. First the drug dose-response for protection was different, both in regard to the shape of the drug dose-response curve and the drug concentrations to protect cells from radiation injury. The increase in intestinal clonogenic

cell survival at a single dose of 13.5 Gy was linear with increasing dose of WR-2721. WR-2721 toxicity was encountered at doses above 12.5 mg/mouse. In contrast, there was a rapid 400% increase in intestinal clonogenic cell survival in mice given from 0 to 10.0 ug/mouse of 16-16 dm PGE₂, followed by a long plateau. Although the PG treated mice exhibited acute diarrhea and lethargy, there were no deaths even at the highest PG doses. Animals recovered from these side effects within 3-4 hours after the PG injection. This marked increase in cell survival followed by a plateau; and the low PG dose to induce radiation protection suggests that the 16-16 dm PGE₂ may have receptor sites that are saturated above a dose of 10 ug/mouse. These results suggest that the mechanism for radioprotection by 16-16 dm PGE₂ may be receptor site mediated and different from that of WR-2721. Alternatively, the mechanisms may be similar; that is, both agents may protect through free radical scavenging, atom donation, or the induction of hypoxia, but the two compounds may partition to separate areas within the cell and protect separate but critical targets for cell survival. WR-2721 may protect predominately the DNA whereas the PG may protect mainly membranes or membrane associated structures.

The contention that one of these two possibilities exists is strengthened by the results showing that there is additivity of radiation protection of intestinal clonogenic cells by the two agents given in combination for doses of WR-2721 from 2.0 to 8.0 mg/mouse. However, when 10 ug 16-16 dm PGE₂ was added to 10 mg WR-2721, the clonogenic cell survival curve was not altered from that of the WR-2721 treatment alone. These results suggest that at high WR-2721 doses, the mechanism by which 16-16 dm PGE₂ exerts its protective effect on clonogenic cells was overwhelmed. Alternatively, if the mechanisms were the same, but the sites of action were separate; subcellular partitioning of the two agents may break down at high doses of WR-2721, and WR-2721 may reach and exert a

greater protective effect that masked the more modest protection by the PG.

Another criterion for the assessment of the efficiency of radiation protective agents is their effect on animal survival. Ten micrograms 16-16 dm PGE₂/mouse increased the LD50/6 by 4 Gy or about 125% of the control value and the slope of the probit curves were similar. WR-2721 increased the LD50/6 value to about 26 Gy or about 160% of the control value, also with a similar slope. The combination of WR-2721 and 16-16 dm PGE₂ not only increased the LD50/6 value to about 36 GY or 225% of controls, but the slope was markedly decreased. Some of these animals lived 9 to 10 days after radiation doses above 30 Gy. These doses were well above those where any clonogenic cell survival was observed. These results show a marked increase in animal survival that does not correlate with clonogenic cell survival.

The change in the slope of the probit curve in animals treated with the combination of agents compared to the controls and each agent given alone may result from alterations in the histopathological changes within the intestine following irradiation. The increased length of villi and the increased number and more normal appearance of columnar epithelial cells may prolong the presence of a functional mucosal barrier that, in turn, may lead to decreased protein loss, better maintenance of electrolyte balance, better absorptive function, and less bacterial invasion; processes that are all believed to contribute to death from the gastrointestinal syndrome. The presence of a large number of grossly normal appearing epithelial cells 4 days after radiation doses far about those where clonogenic cells survive in animals given both agents suggests that normal migration and epithelial extrusion from the tips of villi were inhibited. The PG-induced changes in villus cell morphology may explain the reduction in the slope of the radiation mortality curve following treatment by the combination of agents. A possible explanation for the large slope change is that one

protective agent may influence the action of the other in animals treated with the combination of compounds. This would explain the change in the slope of the mortality curve with the combination treatment that was not seen with either agent alone. For example, PGs are known to influence plasma membrane fluidity that may alter the distribution or availability of WR-2721 to different parts of the cell or to different cell populations. Further studies of drug localization and the migration and fate of the epithelial cells given the various regimens of radiation protectors are indicated.

An increase in the integrity of the mucosal barrier may be the factor that contributes most to the heterogeneity of the response and, therefore, the most likely explanation for the decreased slope of the post-irradiation mortality curve and the extended life of mice treated with both agents; however, the increased structural integrity of the mucosa may not result from protection of the epithelium directly. The results also may be explained by the possible protection of other structures or cell systems such as the hematopoietic and immune systems, although this seems unlikely at these high radiation doses. Protection of another more likely system that may account for these results is the radiation protection of the supporting vascular structure.

Given the data showing increased animal survival with no increase in intestinal clonogenic cell survival, the use of LD50/6 values to assess radiation protection can be questioned. Likewise, survival of a specific number of intestinal clonogenic cells does not necessarily predict animal survival. Certainly, the survival of clonogenic cells is important for tissue recovery and for long term survival of the mice; however, these data show that animal survival over the short post-irradiation time associated with the gastrointestinal syndrome can be manipulated independently from intestinal clonogenic cell survival. Aspects of intestinal regeneration, not reflected totally by the clonogenic assay, may be

important.

There are several implications of these results. First, the observed additivity of the two agents may have application in situations where toxicity of WR-2721 given alone is limiting. WR-2721 drug doses could be reduced to less toxic levels but radiation protection could be increased back to useful levels by the addition of less toxic PGs. The realization of a beneficial use of this combination treatment to increase protection will depend upon the combined toxicity of the agents. A second implication is that the mechanisms of radioprotection by the two agents may be different or that the drugs may affect different cells within tissues, or separate subcellular targets that are critical to cell survival. Evidence showing that WR-2721 reduced the number of initial DNA single strand breaks but 16-16 dm PGE₂ had no effect on initial strand breaks in murine intestinal mucosa favors the hypothesis that separate targets may be affected by the two agents. However, single strand breaks are only one measure of DNA effects and caution must be exercised in assuming that 16-16 dm PGE₂ does not influence DNA in another way.

With the development of stable and long lasting PG analogues with similar PG activities, a mixture of the most effective WR agent with the most effective PG may offer the best field-ready radioprotection. In addition to photon radioprotection, protection from intermediate and high LET irradiation is also an important consideration. The mixture of PGs and WR compounds may provide the best protection from high LET radiation as well as for photons.

Although it is now well established that many PGs and their analogues protect tissue from a variety of injurious agents including radiation, the mechanism to account for the experimental observations of PG-induced radioprotection presented above is unclear. The dose response data (Figure 5) is not consistent with a direct radioprotective effect of the

PG molecule itself. There appears to be a limited number of binding sites on the cell surface membrane that become saturated at low PG levels. Even if there were sufficient numbers of PG molecules to protect directly, there does not appear to be a PG structural explanation for why this molecule should "scavenge" free radicals produced by ionizing radiation. Other possibilities can be eliminated, such as a shift in the cell cycle distribution toward a less sensitive stage since PGs have been found to be radioprotective in the gut within 5-10 minutes of sc administration. A possibility and perhaps the most likely one to account for the data comes from evidence showing that many PGs increase adenyl cyclase and cAMP that has been shown to protect the intestine from radiation injury (35). There are several other PG-induced physiological changes that may account for the results such as the known alterations in intra and extra-cellular calcium or cGMP. There is also the possibility that PG-induced alterations of intracellular levels of enzymes or compounds such as superoxide dismutase or glutathione may influence tissue radiosensitivity. Irrespective of the mechanism, studies of PG-induced radioprotection alone and particularly in combination with the WR compounds may have rapid and practical applications.

4.1 Directions for Future Research.

Future goals include research on: 1) the structure-activity relationships for PGs and LTs, 2) the basic mechanism of protection by these compounds and, 3) their interaction with exogenous or endogenous sulfhydryl compounds as radiation protectors.

4.1.1 Structure-Activity Relationships.

A comparison of radiation protection by the E-series PGs suggests that the structures of the alpha and beta side chains are important in determining the degree to which these compounds protected (36). PGE₁, for example, produced no protection at any time or

concentration tested, whereas PGE₂ increased intestinal clonogenic cell survival to 200% of control values. These data suggested that a C5-C6 double bond is critical for protective activity. However, the same chemical difference between the E₁ analogue, misoprostol, and its E₂ form, SC-30071, did not increase activity. Furthermore, the closely related C4-C5 unsaturated analogue, SC-34301, showed equivalent activity to misoprostol. Therefore, the presence of a double bond in the alpha chain does not significantly enhance the radiation protection by the misoprostol series of prostaglandins. The addition of a second double bond (SC-36729) or the presence of a methyl group adjacent to the carboxyl functionality (SC-39932) severely reduced protective activity (37). These latter findings indicate that somewhat minor structural variations can have significant impact on the protective activity of prostaglandins. The alterations in PG structure that diminished or eliminated the protective activity may reduce either the receptor binding or the ability of the PG to activate second messengers. The differences in PG structure-activity also suggest that other analogues may show greater protective activity than those studied to date. Thus, there is an enormous number of permutations on the single theme of PG analogue-induced radiation protection.

The studies summarized in this report show that currently the three most protective PGs are 16,16 dm PGE₂, misoprostol, and iloprost. By careful analysis of structure-activity relationships, it may be possible to design and synthesize more potent PG radioprotectors.

4.1.2 Mechanism of Action.

An important goal for future work is the elucidation of the mechanism of PG or LT radiation protection. Since PGs and LTs appear to produce their effects through receptor proteins, studies of the nature of these receptors is one of the critical steps. The receptor

sites appear to be saturated above a certain concentration when PGs are given in a single dose, however, it is possible that additional receptor proteins could be induced. In some cells, for example lymphocytes, receptor sites for interleukin-2 can be induced and increased over time (38). It is possible that if a way were found to increase the number of PG receptors, the degree of PG-induced radiation protection could be increased. In addition to the nature of the receptors, research on the secondary signals involved in PG-or LT-induced protection is a future goal.

The location of the actual site where protection occurs also must be investigated. As discussed above, alkaline elution studies have shown that WR-2721 reduced the number of initial single strand breaks in DNA following gamma irradiation. In contrast, 16-16 dm PGE₂ did not reduce the number of DNA breaks. The PG-induced radioprotection, therefore, may not be associated directly with DNA strand breaks. Since strand breaks are only one measure of DNA damage by radiation, caution must be exercised in assuming that PGs do not play a role in protection by interacting either directly or indirectly with DNA to protect cells. However, these data suggest that PGs may protect targets other than DNA.

Perhaps the most informative data so far regarding the nature of PG-induced radiation protection is the observation that some PG analogues given before but not after WR-2721 protect more than each agent alone. The evidence presented in this report and that of Walden (39) showing that combined treatment of animals with WR-2721 and PGs were more effective than either agent alone suggests that PGs may protect through a separate mechanism than the sulfhydryl compounds. Future studies may find ways of exploiting this possibility to extend the degree of radiation protection beyond the present maximum levels while maintaining toxicity within acceptable limits.

As a result of the array of biological effects and the observed radiation protection by the family of PGs and LTs, the metabolism, pharmacology, mode of action, stimulation, and inhibition of the AA cascade have important implications in cell biology and clinical medicine.

SECTION 5

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