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EFFECT OF CHEMICAL PROTECTORS ON THE RESPONSE OF THE INTESTINE TO ROENTGEN OR FISSION NEUTRON IRRADIATION

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Improvement in the therapeutic index in radiation therapy requires a differential effect between normal and malignant cells. Methods to obtain this differential response are presently under active investigation. Recently, newly synthesized phosphorothioate radiation protectors have shown promise in the improvement of the therapeutic index in animals (YUHAS & STORER 1969, LOWY & BAKER 1973, HARRIS & PHILLIPS 1971, PHILLIPS et coll. 1973, and others). It has been proposed that the differential effect obtained with compounds of this type was due to poor tumor vascularity; hence little protector was delivered to the tumor (YUHAS & STORER). In addition, the tumor may have had a high proportion of hypoxic cells which have been shown to be protected less than well oxygenated cells (HARRIS & PHILLIPS).

In a series of experiments various phosphorothioates and conventional protectors were tested for their ability to modify the effects of roentgen of fission neutron irradiation. S-2-(3-amino-propylamino)ethylphorothioic acid (WR-2721) was found to be an exceptionally good protector against either roentgen radiation (SIGDESTAD et coll. 1975a) or fission neutrons (SIGDESTAD et coll. 1976). Dose modification factors (DMF) of 1.6 were found for either modality in crypt survival investigations.

Mercaptoethylamine (MEA) and its phosphorothioate derivative (WR-638) were

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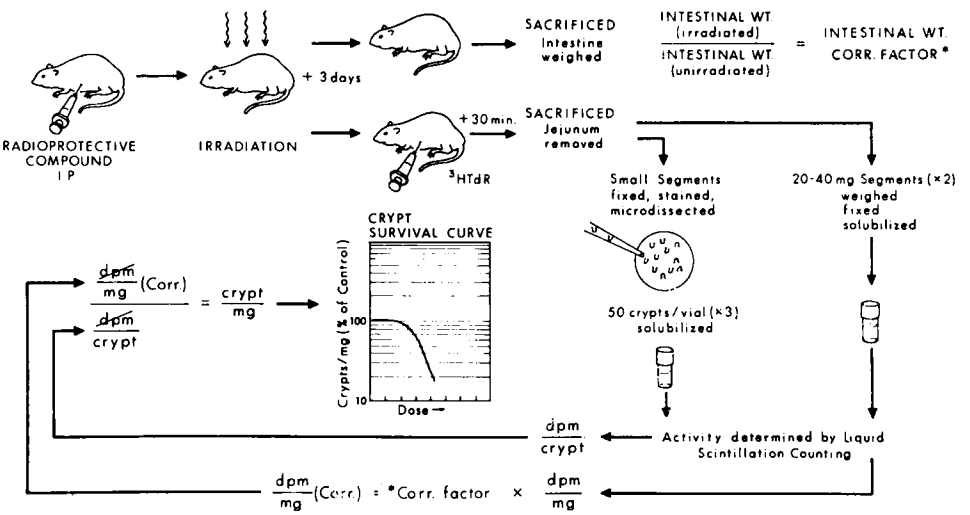

Fig. 1. Schematic drawing of the intestinal crypt survival method.

also tested (SIGDESTAD et coll. 1975b) and found to be good protectors, but less effective than WR-2721.

The present report compares another phosphorothioate, S-1-(2-hydroxy-3-amino) propylphosphorothioic acid (WR-77913) and AET for their ability to protect the intestinal cell renewal system from roentgen or fission neutron irradiation.

Material and Methods

The animals used were male, C57/Bl 6-J mice. They were approximately 100 days of age and were kept in environmentally controlled rooms and fed standard mouse pellets and water ad libitum.

Protectors. WR-77913 is the monosodium salt of S-1-(2-hydroxy-3-amino) propylphosphorothioic acid and was obtained from the Medicinal Chemistry Division of the Walter Reed Institute of Research. The toxic LD_{50} was tested and found to be 1 224 (1 119–1 320) mg/kg. Approximately two thirds of the toxic LD_{50} were used (820 mg/kg) throughout this investigation. No drug-related deaths occurred with this dosage.

AET (S-2-amino ethylisothiuronium·Br·HBr) was purchased commercially. The toxic LD_{50} was found to be 295 (280–310) mg/kg.

Both WR-77913 and AET were dissolved in water and injected intraperitoneally 15 to 30 min before irradiation. Logistics of reactor operation precluded more precise timing of the injection to irradiation time.

Table

Salient data on intestinal crypt survival and lethality in mice irradiated with roentgen rays or fission neutrons with or without chemical protection

Treatment	D_0	n	D_q	GI*	$LD_{50(6)}$	95 % CL	DMF	GI**
Roentgen rays only	395	6.1	716	—	1 277	1 194–1 376	1.0	—
Roentgen rays + AET	435	14.3	1 158	488	1 813	1 715–1 908	1.42	536
Roentgen rays + WR-77913	818	3.1	938	515	1 812	1 701–1 984	1.42	535
Neutrons only	95	5.2	156	—	252	222–298	—	—
Neutrons + AET	157	3.2	181	69	302	89–341	1.20	50
Neutrons + WR-77913	136	5.4	229	102	372	340–412	1.48	120

* Gy increase at 50 % crypt survival

** Gy increase at $LD_{50(6)}$ (protected $LD_{50(6)}$—unprotected $LD_{50(6)}$)

Roentgen irradiation. The procedures were carried out with a 4 MeV linear accelerator (Varian Clinac-4). The mice were irradiated unrestrained in a lucite container 30 cm in diameter and 4 cm high. The container was rotated 3 rpm in a 32 cm × 32 cm field which was known to be extremely flat with regard to dose variation. The source–skin distance was 80 cm with a dose rate of 2.5 Gy/min (250 rad/min).

Dosimetry was accomplished with a Victoreen ionization chamber at equilibrium depth in a polystyrene calibration block with appropriate corrections.

Neutron irradiation. The Health Physics Research Reactor (Dosar), Oak Ridge National Laboratory, was used for neutron irradiation. The reactor facility has been described in some detail (AUXIER 1965). The fission spectrum had a peak energy of 0.9 MeV and a mean energy of 1.2 MeV. The mice were irradiated in nylon tubes 2 m from the unshielded core. The power level was 6 kW with a dose rate of about 0.55 Gy/min. Gamma contamination amounted to about 14 per cent of the dose. A good discussion of dose and LET distribution in small animals using this reactor was presented by WILLHOIT & JONES (1970). SIGDESTAD et coll. (1972) have previously described the RBE for this irradiation procedure.

Lethality. Groups of mice were injected with either WR-77913, AET or water 15 to 30 min before irradiation. Whole body doses ranged from 1.8 to 5 Gy for neutron irradiation and from 9 to 22 Gy for roentgen irradiation. The $LD_{50(6)}$ was calculated by probit analysis method of FINNEY (1963). The dose modification factor (DMF) was calculated as a ratio of protected to unprotected $LD_{50(6)}$ values.

Crypt assay procedure. Intestinal crypt survival, i.e. crypts per milligram wet weight jejunum, was measured 3 days after irradiation. A schematic drawing of the assay procedure appears in Fig. 1. Details of the procedure have been presented previously (HAGEMANN et coll. 1971). Briefly, crypts per milligram intestine are

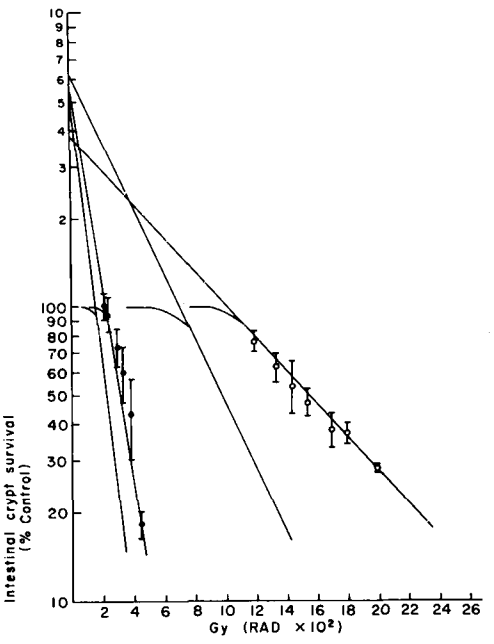

Fig. 2

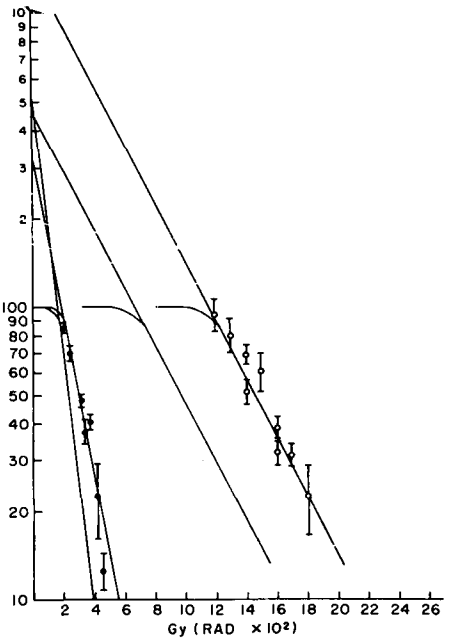

Fig. 3

Fig. 2. Crypt survival curves for WR-77913 treated mice irradiated with either 4 MeV roentgen rays or fission neutrons. Lines without data points represent unprotected but irradiated controls.

Fig. 3. Crypt survival curves for AET treated mice irradiated with either 4 MeV roentgen rays or fission neutrons. Lines without data points represent unprotected but irradiated controls.

determined by the ratio of dpm/mg intestine and dpm/crypt 30 min after injection of ^{3}HTdR. The crypts/mg intestine determined in groups of mice given various doses of roentgen or neutron radiation are compared to unirradiated controls. A semi-log plot of crypt/mg in per cent of control versus dose described the crypt survival curve.

Total and per crypt cellularity. Previously reported data (HAGEMANN et coll., HAGEMANN & LESHER 1971) indicate that while dpm/mg and dpm/crypt differ from unirradiated controls at 3 days after irradiation, the dpm/labeled nucleus does not. These results demonstrate that the dpm/mg and dpm/crypt values are good indicators for the total and per crypt DNA-synthesizing cell population. This, in turn, is a reflection of the size of the proliferative compartment.

Results

Lethality. Unprotected mice exposed to 4 MeV roentgen radiation were found to have an $LD_{50(6)}$ value (Table) of 12.77 (11.94–13.76) Gy. Mice protected with WR-77913 or AET had $LD_{50(6)}$ values of 18.12 Gy and 18.13 Gy, respectively. This resulted in a DMF value of 1.42 for each protector. The so-called Gy increase (GI) values (YUHAS 1971) were 5.35 and 5.36 Gy, respectively.

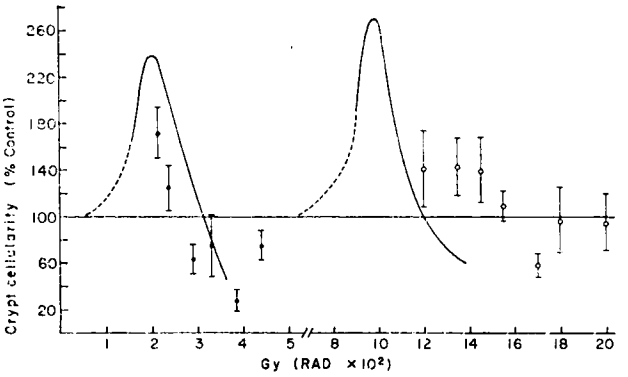

Fig. 4. Crypt cellularity as a function of dose in roentgen or neutron irradiated mice protected with WR-77913. The curves without data points represent unprotected but irradiated control.

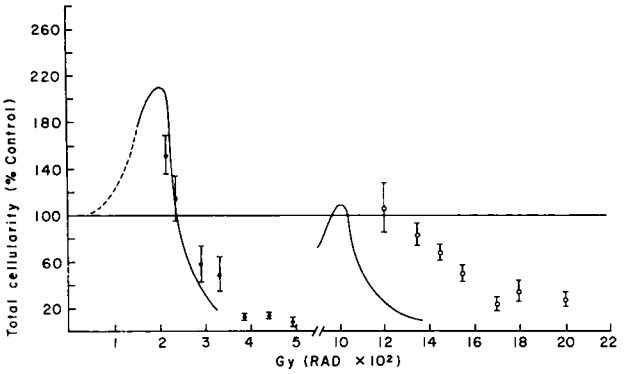

Fig. 5. Total cellularity as a function of dose in roentgen or neutron irradiated mice protected with WR-77913. The curves without data points represent unprotected but irradiated control.

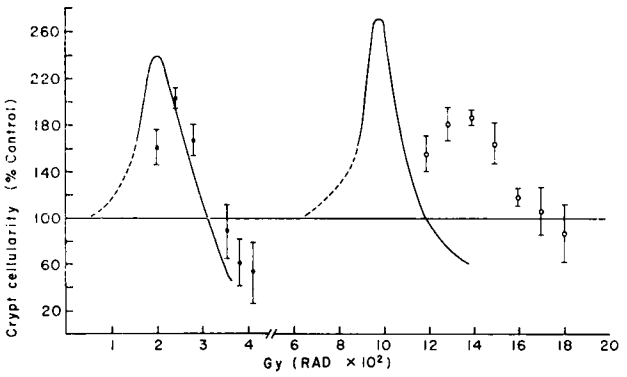

Fig. 6. Crypt cellularity as a function of dose in roentgen or neutron irradiated mice protected with AET. The curves without data points represent unprotected but irradiated control.

Mice irradiated with fission neutrons without the benefit of protection had an $LD_{50(6)}$ value of 2.52 (2.22–2.98) Gy. With WR-77913 the $LD_{50(6)}$ was increased to 3.72 Gy which resulted in a DMF of 1.42 and GI of 1.20 Gy. Mice pretreated with AET had an $LD_{50(6)}$ of 3.02 Gy which resulted in a DMF of 1.2.

Intestinal crypt survival. Salient data for intestinal crypt survival is presented in the Table and Figs 2 and 3. The curves presented without data points are results

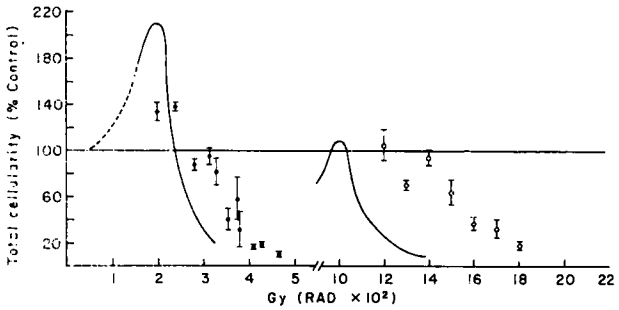

Fig. 7. Total cellularity as a function of dose in roentgen or neutron irradiated mice protected with AET. The curves without data points represent unprotected but irradiated control.

obtained from animals irradiated without benefit of chemical protection. Crypt survival curves in mice pretreated with WR-77913 is presented in Fig. 2. The slope is significantly different from the unprotected group irradiated with roentgen rays. This is also seen, but to a lesser extent, in the neutron irradiated group protected with WR-77913. These results correlate with the probit curves obtained in the lethality experiments inasmuch as linearity but not parallelism was found.

Roentgen irradiated mice pretreated with AET (Fig. 3) showed a crypt survival curve which was parallel to the unprotected curve. The GI for crypt survival (difference between protected and unprotected curves at the 50 per cent crypt survival level) was 5.15 Gy. For unexplained reasons the crypt survival curve for AET protected neutron irradiated mice was not parallel to the unprotected but showed a slight increase in slope.

Intestinal cellularity. The cellular dose response in the crypt 3 days after irradiation has been described previously (SIGDESTAD et coll. 1972, 1973, 1975).

The effects of WR-77913 and AET in the cellular response appear in Figs 4 to 7. The curves without data points are results obtained in irradiated but unprotected mice (SIGDESTAD et coll. 1975).

It is apparent in these data, as seen in crypt survival, that protection from neutron irradiation is slight with these agents. However, when low LET radiation is used protection is seen as a shifting of the cellularity curves to higher doses.

Discussion

The continued improvement of irradiation procedures depends upon a difference in effectiveness of radiation on normal and malignant cells. This can be accomplished by either radiation sensitizers which preferentially protect sensitized malignant cells or by radiation protectors which preferentially protect normal cells.

The phosphorothioate class of chemical protectors are receiving considerable attention for possible use in radiation therapy. Primarily this is due to a differential protection of normal cells first reported by YUHAS & STORER. These results were confirmed and expanded by HARRIS & PHILLIPS. The proposed mechanism of this differential effect is thought to be due to a reduced concentration of the protector in

tumor tissue (YUHAS & STORER) and to a reduction in the oxygen enhancement ratio (HARRIS & PHILLIPS). The differential distribution of labeled WR-2721 was demonstrated by whole body autoradiography by UTLEY et coll. (1975). They found that the tumor uptake of the protector was less than normal tissue uptake.

Previously the protective effects of WR-2721 against roentgen radiation were reported (SIGDESTAD et coll. 1976). Mercaptoethylamine (MEA) and its phosphorothioate derivative (WR-638) were also compared for protective effects (SIGDESTAD et coll. 1975b) on the intestinal epithelium. Of the compounds tested to date, WR-2721 is the most effective protector against effects of roentgen radiation on the gut with a DMF of 1.64. DMF obtained with other protectors in descending order was WR-638, 1.57; WR-77913, 1.42; AET, 1.42 and MEA, 1.26. When fission neutrons were used the DMF was somewhat reduced; WR-2721, 1.56; WR-77913, 1.48; WR-638, 1.42; MEA, 1.39 and AET, 1.20. It is apparent from these data that the phosphorothioate class of protector is more effective than conventional protectors (MEA and AET).

An interesting and possibly important finding in the present report is the increased slope in the roentgen crypt survival curve in animals pretreated with WR-77913. This slope change may indicate that the drug is affecting oxygen concentration in the crypt cells. This finding is substantiated by the probit lethality curves which were linear but not parallel. Non-parallelism indicates a difference in the mechanism of killing. If tissue hypoxia were induced and the distribution of the agent was similar to WR-2721, then the potential use in a clinical situation may be enhanced. An additional advantage is that WR-77913 is less toxic (LD_{50} = 1 224 mg/kg) than WR-2721 (LD_{50} = 704 mg/kg).

The data obtained from the cellularity examinations demonstrate the effectiveness of these protectors at the cell level. Three distinct phases of compensation occur in the crypt 3 days after irradiation. (1) At low doses the crypt begins the compensatory response which is seen with a greater than normal S-phase cell content. This response is directly related to the dose. (2) The peak response where the crypt is maximally stimulated (commensurate with radiation injury) to maintain cell input to the villus. (3) The final phase appears at higher doses where the crypt cells are so depopulated that an effective response is not possible. In this phase the response is inversely related to the dose; however, it rarely returns to levels less than control. These 3 phases are observed subjectively during the crypt dissection procedure.

Total intestinal cellularity (dpm/mg in per cent of unirradiated control) responds somewhat differently than crypt cellularity. The compensatory response of the entire intestine is due to (1) the effect of irradiation on surviving crypts, i.e. radiation injury and compensatory response and (2) the inability of some crypts to survive the radiation insult. Both effects are responsible for an overshoot somewhat less than observed in the crypts while the loss of entire crypts reduces total S-phase cellularity to levels much lower than in the crypt.

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SUMMARY

The monosodium salt S-1-(2-hydroxy-3-amino) propylphosphorothioic acid (WR-77913) and S-2-amino ethylisothiuronium·Br·HBr (AET) were tested for protective effects against 4 MeV roentgen irradiation and fission neutrons in the mouse intestine. The parameters tested were intestinal crypt survival, lethality and intestinal crypt cellularity. The results showed both compounds to be good protectors in animals. The crypt survival curve for roentgen-irradiated mice treated with AET was parallel to that of the untreated group and was displaced to the right by 4.88 Gy (488 rad). Protection from neutron irradiation was less effective with a displacement of only 0.69 Gy (69 rad). Pretreatment with WR-77913 increased the slope of the crypt survival curve in roentgen-irradiated mice. This was also seen to a much less extent in neutron-irradiated animals. The displacements of the curves (at 50 per cent crypt survival) were found to be 5.15 and 1.02 Gy (515 and 102 rad) for roentgen and neutron irradiation, respectively. The lethality experiments showed a dose modification factor (DMF) of 1.42 for both drug-tested groups of roentgen-irradiated mice. The dose modification factors for fission neutron irradiated mice were 1.48 and 1.2 for WR-77913 and AET-treated mice, respectively. The effect of these protectors on crypt cellularity is also discussed.

ZUSAMMENFASSUNG

Es wurden das Mononatrium Salz S-1-(2-Hydroxy-3-Amino) Propylphosphorothioic Säure (WR-77913) und S-2-Amino Aethylisothiuronium·Br·HBr (AET) hinsichtlich ihres Schutzeffektes gegenüber 4 MeV Röntgenstrahlung und Fusionsneutronen am Mäusedarm untersucht. Die untersuchten Parameter waren das Überleben der Darmkrypten, die Lethalität und die Zellularität der Darmkrypten. Die Ergebnisse zeigen für beide Substanzen einen guten Schutz bei Tieren. Die Überlebenskurve der Krypten der Röntgenbestrahlten und mit AET behandelten Tiere lag parallel zu derjenigen der unbehandelten Tiere und war um 4,88 Gy (488 rad) verschoben. Der Schutz gegenüber Neutronenbestrahlung war weniger wirksam mit einer Verschiebung um nur 0,69 Gy (69 rad). Die Vorbehandlung mit WR-77913 hob die Neigung der Überlebenskurve der Krypten bei den Röntgenbestrahlten Mäusen. Das war auch in geringerem Umfang bei den Neutronenbestrahlten Tieren zu sehen. Die Verschiebungen der Kurven (bei 50% Überleben der Krypten) betrug 5,15 und 1,02 Gy (515 und 102 rad) für Röntgen bzw. Neutronebestrahlung. Die Lethalitätsuntersuchungen zeigten einen Dosis-Modifikations-Faktor (DMF) von 1,42 für beide Testsubstanzen bei den Röntgenbestrahlten Gruppen von Mäusen. Die Dosis-Modifikations-Faktoren für die mit Fusionsneutronen bestrahlten Mäusen betrugen 1,48 und 1,2 für die mit WR-77913 bzw. AET-behandelten Mäuse. Es wird ebenfalls der Effekt der Schutzsubstanzen auf die Zellularität der Krypten diskutiert.

RÉSUMÉ

Les auteurs ont testé sur l'intestin de la souris l'effet protecteur contre l'irradiation par des rayons roentgen de 4 MeV et contre les neutrons de fission le sel monosodé de l'acide S-1-(2-hydroxy-3-amino) propylphosphorothioïque (WR-77913) et du S-2-amino éthylisothiuronium·Br·HBr (AET). Les paramètres testés ont été la survie des cryptes intestinales, la léthalité et la cellularité des cryptes intestinales. Les résultats ont montré que ces deux

corps sont de bons protecteurs chez les animaux. La courbe de survie des cryptes pour les souris irradiées par les rayons roentgen et traitées avec AET a été parallèle à celle du groupe non traité et est déplacée vers la droite de 4,88 Gy (488 rad). La protection contre l'irradiation par les neutrons a été moins efficace avec un déplacement de 0,69 Gy (69 rad). Le traitement préalable par le WR-77913 augmente la pente de la courbe de survie des cryptes chez les souris irradiées par les rayons roentgen. Cet effet est constaté aussi, dans une beaucoup moins grande mesure, chez les animaux irradiés par les neutrons. Les déplacements des courbes (pour une survie des cryptes de 50 %) sont de 5,15 et 1,02 Gy (515 et 102 rad) respectivement pour les rayons de roentgen et pour les neutrons. Les expériences sur la léthalité ont montré un facteur de modification de doses (DMF) de 1,42 pour les deux groupes de souris irradiées par les rayons roentgen traitées par ces 2 agents. Les facteurs de modification de doses pour les souris irradiées par les neutrons de fission sont 1,48 et 1,2 respectivement pour les souris traitées par le WR-77913 et par AET. Les auteurs examinent aussi l'effet de ces protecteurs sur la cellularité des cryptes.

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