PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 370–373 (1980)

Mouse Endogenous Spleen Counts as a Means of Screening for Anti-Radiation Drugs (40880)

KENNETH E. KINNAMON,[1] LYLE L. KETTERLING,[2] HERMAN F. STAMPFLI, AND MARIE M. GRENAN

Department of Biology, Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012

Abstract. Using eight radioprotective compounds, a comparison was made between the "standard" survival method and a method employing the counting of macroscopic spleen colonies in assessing the degree of radioprotection. The two methods yielded similar assessments of the degree of radioprotection afforded and required about the same amount of man-hours to conduct the procedure. The spleen colony method offers the advantage of completing the assessment after 10 days rather than 30 and yielding fresh tissues from all scored test animals. The use of a system in which macroscopic spleen colonies are counted offers an alternative to employing the method of animal survival in the quantitative assessment of compounds for radioprotective activity.

Over 20,000 compounds have been screened for radioprotective properties since the U.S. Army Anti-radiation Drug Development Program was initiated in 1959. The "standard" screen consists of exposing treated and untreated mice to the ionizing radiation, then recording the survival times for 30 days. It has long been known that estimation of the radiation dose reduction for chemical protectors was possible utilizing endogenous spleen counts (1). This suggested to us that high-volume screening of compounds may be possible using the endogenous spleen colony counting method. If feasible this method would reduce the test completion time from 30 to 10 days. To make such an assessment the present study was undertaken.

Methods. Design. A total of eight compounds with known radioprotective properties as determined in previous mouse survival studies were selected for this study (2). For each of the eight compounds separate estimates of the dose reduction factor (DRF) (2) were made by two different methods. One utilized the conventional survival time of 30 days (2); the other employed determination of the reduction in the number of macroscopic spleen colonies observed in mice that had received whole body radiation 10 days previously.[3]

In the latter method of determining the DRF, for each estimate animals were randomly assigned to 12 groups of 14 mice each. At each of six different radiation levels, two groups of animals were exposed. One of the two groups at each radiation level had received 15 min prior to exposure an intraperitoneal injection of the radioprotective compound; the other group consisted of control mice which had received 0.9% NaCl solution at the same time as their radioprotected counterparts. The radiation dose levels were selected based

[1] All correspondence and reprint requests should be directed to Kenneth E. Kinnamon, D.V.M., Ph.D., Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda Md. 20014.

[2] Present address: Officer in Charge of Laboratories, DeWitt Army Hospital, Fort Belvoir, Va. 22060.

[3] Chemical radioprotective agents act to reduce the effective radiation dose received. For example, animals protected by a drug with a DRF of 2 and exposed to 1000 rad respond as unprotected animals exposed to 500 rad. This is sometimes expressed as DRF = $LD_{50}(30)$ for protected animals/$LD_{50}(30)$ for unprotected animals, where $LD_{50}(30)$ is that radiation dose which causes 50% deaths within 30 days. The DRF determined by the spleen colony method may be expressed as DRF = LD_{50} for protected animals/LD_{50} for unprotected animals, where LD_{50} is the radiation dose necessary to reduce the confluent colony count (approximately 20) to 10 macroscopic colonies per spleen.

0037-9727/80/070370-04$01.00/0

upon preliminary studies and were 1000, 850, 650, 550, 350, and 250 rad. The animals were killed 10 days after irradiation by cervical dislocation and the spleens removed. These were fixed in Bouin's fluid for 2–4 hr before counting the spleen nodules macroscopically.

In mice in which death was used to determine the dose reduction factor (DRF), daily mortality was recorded to Day 30 following radiation exposure. The procedure was as described above except that groups of 15 animals each were employed at five or six radiation levels in increments of 50 or 100 rad. The radiation doses ranged from 650 to 1620 depending upon the expected protective properties of the test drug determined by preliminary testing.

Radioprotective compounds. Structures for the radioprotective compounds are shown in Fig. 1. Compounds were administered as aqueous solutions in a volume of 0.01 ml/g of body weight at approximately two-thirds of the 50% lethal toxic dose, i.e., that dose which kills 50% of unirradiated animals, as determined previously. Solutions were made within 1 hr of the time they were injected.

Animals. Female mice of the ICR strain (Charles River Breeding Laboratories, Wilmington, Mass.), 9–12 weeks of age were used in this study.[4] They were housed in air-conditioned quarters, seven to a group in stainless-steel wire (front and bottom) cages (24.8 cm long, 20.3 cm wide, 17.8 cm high). A standard pelleted laboratory chow (Purina Mouse Chow, Ralston Purina, St. Louis, Mo.) and tap water were available *ad libitum*.

Irradiation procedure. Whole body radiation was delivered by a Gamma-cell 40 cesium-137 irradiation unit (Atomic Energy of Canada Limited, Ottawa, Canada) at a dose rate of approximately 140 rad/min.

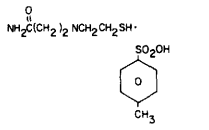
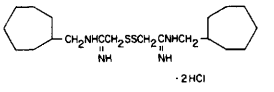
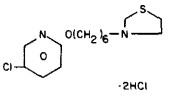
Designation	Structure
1. WR 2721	$H_2N(CH_2)_3NHCH_2CH_2SPO_3H_2$ 1.28·H_2O
2. WR 3689	$CH_3NH(CH_2)_3NHCH_2CH_2SPO_3H_2 \cdot H_2O$
3. WR 2823	$H_2N(CH_2)_5NHCH_2CH_2SPO_3H_2 \cdot 2H_2O$
4. WR 638	$NH_2CH_2CH_2SPO_3HNa$
5. WR 347	$NH_2CH_2CH_2SH \cdot HCl$
6. WR 2529	
7. WR 151 331	
8. WR 108 503	

FIG. 1. Chemical structures of compounds used in these studies.

The radiation dose to be delivered to each group of animals was calibrated before, then individually confirmed during each "run" with the aid of a thermoluminescent dosimetry (TLD) system (Model E-IV, Madison Research, Middleton, Wisc.). A pair of these dosimeters were taped to the animal restrainer at each exposure. Mice were irradiated in groups of 14–21 by placing them in a cylindrical 30.5-cm (diameter) by 3.2-cm (height), Plexiglas restrainer having walls approximately 0.5 cm in thickness.

Statistical analyses. The estimate of the $LD_{50}(30)$ in the "standard survival study" was determined using standard log-probit analysis procedures (3). The estimate for the endogenous spleen colony counts was by a modified logarithmic transformation method reported by Smith *et al.* (1). The index employed for describing the magnitude of chemical radioprotection was the dose reduction factor (4). A statistical comparison of the dose reduction factors obtained by the two methods was accomplished by a paired *t* test.

Results. Results are summarized in Table 1. A comparison of the dose reduction factors (DRFs) when determined by survival (column 4) and endogenous colonies (column 5) showed that the values obtained by

[4] The authors in conducting the research described in this report, adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Committee of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

TABLE I. DOSE REDUCTION FACTORS (DRFs)[a] AS DETERMINED BY 30-DAY SURVIVAL AND ENDOGENOUS SPLEEN COLONY REDUCTION FOR EIGHT RADIOPROTECTIVE COMPOUNDS[b]

(1) Compound	(2) WR No.	(3) Dose administered (mg/kg)	Dose reduction factor		Rank order		(8) Remarks
			(4) Survival	(5) Colonies	(6) Survival	(7) Colonies	
1	2721	400	2.17	2.22	1	2	Gammaphos
2	3689	500	1.81	1.77	2	4	
3	2823	300	1.66	2.87	3	1	
4	638	375	1.60	1.75	4	5	Cystaphos
5	2529	500	1.59	1.56	5	6	
6	347	150	1.46	2.11	6	3	MEA
7	151 331	10	1.30	1.46	7	7	
8	108 503	75	1.05	1.24	8	8	

[a] See Methods for definition of dose reduction factor (DRF).
[b] Source of compounds: from the inventory maintained at the Walter Reed Army Institute of Research, Washington, D.C. 20012.

the two methods were not statistically different. Rank ordering the eight compounds by different means of determining the DRFs yielded some differences. By survival the most effective compound was WR 2721 (Table 1, column 6); by colony formation the most effective was WR 2823. Both are phosphorothioates. WR 2721 (compound 1) ranked second as determined by endogenous spleen colony formation. By both methods, the four phosphorothioate compounds ranked as the top four (survival method) or five (colony method) in DRF. By colony determination the thiol, 2-mercaptoethylamine (MEA) (WR 347) ranked third. The disulfide (compound 7) and the thiazolidine (compound 8) ranked seventh and eighth, respectively, by both methods.

Discussion. Although others have noted the association of endogenous spleen colony formation and survival of the whole animal (5–10), the present work has shown a quantitative relationship between the assessment of the degree of radioprotection afforded by several compounds as determined by these two methods. Eight known radioprotective drugs representing four different chemical types were used.

It is recognized that the net effect in the assessment of the degree of protection afforded by a radioprotective agent depends upon a number of factors including the type, total dose and rate of radiation exposure (4, 11), the types and concentrations of other radioprotectants and other radiation-modifying agents (11–13), and the endpoint chosen for the assessment (4, 14–16). Even when studying survival curves of cell cultures it is clear that in reality these curves are the sum of a number of survival curves pertaining to various subpopulations (11). And, although theories are offered (4) regarding the means by which these different chemical types afford radiation protection, the exact mechanism is not known. However, the present data support the contention that under the condition of this experimental design these radioprotective substances afford the same protection to the event or series of events leading to endogenous spleen colony formation as to the event or series of events that lead to survival of the whole animal. In addition, the method employing spleen colony formation offers a more direct assessment of stem cell survival, although the relationship of stem cells and colony forming units is a subject of extensive investigation (17–21).

The man-hours of time spent in conducting the assessment by the two methods is approximately the same. In the two methodologies, the procedures are identical until Day 10 following radiation exposure. To "process" 100 mice employing the endogenous method requires approximately 3 hr (to kill, remove spleens, and place the spleens in Bouin's fluid = 2 hr; to count the macroscopic colonies = 1 hr); to pro-

cess 100 mice by the survival method requires observation and recording of deaths for 20 additional days at approximately 10 min per day (20 days × 10 min/day = 3½ hr). The analysis of the data after collection using either method requires about the same amount of time. In addition to being able to determine the results more rapidly (10 days rather than 30), the endogenous spleen colony method of assessing DRFs offers an advantage if fresh tissues from all the scored mice are required. Employing the endogenous colony method, animals are killed at the end of the 10-day test period, thus, yielding the fresh tissues; by using the survival method significant postmortem autolysis may have occurred before the dead animals are found for scoring.

To characterize and appropriately assess any agent as a chemical radioprotectant requires extensive evaluation. However, in the quest for new agents or classes of agents, it is necessary to have a mechanism to rapidly screen and identify compounds with potential radioprotective activity so that these may then be subjected to evaluation in other test systems. It is believed that screening by the method reported here offers a reasonable alternative to that employing survival of the whole animal.

The authors wish to thank Ms. June A. Page, Director, Computer Operations; Dr. David F. Cruess, Department of Preventive Medicine, Uniformed Services University of the Health Sciences; and Dr. Douglas B. Tang, Chief, Department of Biostatistics and Applied Mathematics, Division of Biometrics, Walter Reed Army Institute of Research, for their assistance in conducting the statistical analyses.

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Received February 4, 1980. P.S.E.B.M. 1980, Vol. 164.