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CHEMICAL PROTECTION AGAINST RADIONUCLIDES *IN VIVO*: IMPLICATIONS TO THE MECHANISM OF THE AUGER EFFECT

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ABSTRACT

It is well known that chemical radioprotectors such as thiols are capable of mitigating acute radiation effects *in vitro*. If the effects of acute irradiation are reduced by the presence of radioprotectors, can we expect a similar response for chronic irradiation from incorporated radionuclides? Our present *in vivo* studies with the protector cysteamine (MEA), in the mouse testis model, suggest that this is indeed the case for a variety of radionuclides including α emitters. A number of mechanisms by which MEA provides protection against radiation damage have been suggested including radical scavenging, hydrogen atom donation, and induction of hypoxia. Therefore, cysteamine and similar compounds may provide an opportunity to elucidate the basic

mechanism (*i.e.* direct versus indirect interactions) of radiation damage from Auger emitters. Our results for the α emitter ^{210}Po, and the Auger emitter ^{125}I localized in the cell nucleus and bound to DNA, show that cysteamine provides considerably more protection against Auger emitters than 5.3 MeV α particles. This suggests that Auger effects may be largely due to the indirect action of radical species. Additional data for a variety of other radiopharmaceuticals and external X rays, administered in the presence and absence of cysteamine, are presented.

INTRODUCTION

For several years we have been investigating the radiotoxicity of different Auger-electron emitting radiopharmaceuticals using spermatogenesis in mouse testis as the biological model, spermhead survival and induction of sperm-shape abnormalities being the experimental end points (1-8). The extreme radiosensitivity of spermatogonial cells makes the mouse testis an effective *in vivo* experimental model to study the effects of low doses. Recently, using the same biological models, our emphasis has been to study the capacity of chemical radioprotectors to mitigate the biological effects of various incorporated radionuclides including Auger emitters (5). Such data may be helpful in elucidating the basic mechanism of radiation action by Auger electron cascades.

In order to understand how radioprotection against Auger cascades may provide basic mechanistic information, some background information concerning the biological effects of Auger emitters is necessary. Over the years it has been well documented that the effectiveness of Auger emitters depends on the subcellular localization of the radionuclide (*e.g.* 6,8-10). Specifically, our earlier *in vivo* studies showed that when the Auger emitter ^{125}I was localized in the DNA of the cell nucleus, the observed effects were much more severe when compared to ^{125}I localized in the cytoplasm (5). In fact, the biological effectiveness of ^{125}IUdR, which localizes in DNA, was observed to be as effective as 5.3 MeV α particles emitted by incorporated ^{210}Po (4). These *in vivo* results, which are consistent with several *in vitro* experiments (10-14), clearly demonstrate the high-LET type radiotoxicity of DNA incorporated Auger emitters and support the generally accepted notion that the severe biological damage caused by these radionuclides is mainly due to the direct action of Auger electrons.

Thiol compounds were first shown to protect against the effects of acute low-LET radiations by Patt *et al.* (15). Since then, many chemicals have been tested *in vitro* for their radioprotective properties against external beams of ionizing radiations. Bird (16) has shown that cysteamine (MEA) provides better protection against acute exposure to low-LET radiation compared to high-LET radiations. This suggests that MEA provides better protection against indirect effects than direct effects. Therefore, one would expect enhanced protection against cytoplasmically localized ^{125}I compared to that against DNA-bound ^{125}I. However, using the mouse testis model and MEA, Rao *et al.* (5) recently reported dose modification factors (DRF) of 3.6 for DNA incorporated $^{125}IUdR$ and 3.8 for cytoplasmically localized $H^{125}IPDM$. The fact that the both cytoplasmically and DNA incorporated ^{125}I were similarly protected against suggests that the indirect mechanism of radiation action is involved, at least in part.

This paper is an attempt to further understand the basic mechanism of radiation action of Auger electron emitters through a systematic examination of the radioprotective abilities of MEA against radionuclides which have very different radiation properties, as well as against external X rays. As before, the mouse testis model is employed, and results for intratesticular administration of MEA will be presented. In addition, the capacity of radioprotectors to mitigate the effects of chronic, versus acute, exposure will be explored. Finally, in an attempt to understand the mechanism of radioprotection, the pharmacokinetics of MEA were studied with ^{35}S labeled compound.

MATERIALS AND METHODS

Experimental Model

Of all the cell stages in the spermatogenic cycle of mouse testis, spermatogonial cells are the most sensitive to radiation. In contrast, the pre- and post-gonial cells are relatively radioresistent (17,18). This differential radiosensitivity provides the rationale for the mouse testis model as a means to investigate the effects of low doses of ionizing radiations. When testes are exposed to radiations externally, or with incorporated radionuclides, the initial damage to the spermatogonial cells results in a reduced spermhead population when counted after the time necessary (4 to 5 weeks) for spermatogonia to become spermatids of stages 12 to 16. It takes about 9 days for mature spermtids (spermatozoa) to migrate from the testis to the

epididymis. The epididymal sperm morphology is another sensitive indicator of radiation damage (19). The normal sperm have a hook-like head and a long tail. It is important that sperm shape abnormalities in epididymal sperm are assayed 9 days after the minimum spermhead count is reached following the radiation exposure. Since this optimal day depends on the nature of the exposure, and perhaps on cellular incorporation of the radionuclides, it must be determined experimentally in each case.

Radiochemicals

The radionuclides ^{125}I and ^{131}I were obtained from New England Nuclear (Billerica, MA) and labeled to stable HIPDM using procedures described by Lui *et al.* (20). Polonium-210 citrate was prepared according to the directions in Ref. 4. Iodine-125 iododeoxyuridine was obtained commercially from ICN Radiochemicals (Irvine, CA). Nonradioactive cysteamine was obtained from Sigma Chemical Company (St. Louis, MO). The radiolabeling of cysteamine with ^{35}S was accomplished using the methods described by Harapanhalli *et al.* (21).

Experimental Methods

Male Swiss Webster mice (8-9 weeks old) were used in the present studies. The animals (Taconic Farms, Germantown, NY) were maintained in the University animal care facility with standard food and water. The mice were anesthetized under ether and injected intratesticularly (i.t.) with known amounts of radionuclide in a suitable chemical form using a microsyringe. In the case of radioprotectors, the chemicals were also administered intratesticularly. The injected mice were sacrificed under ether after a predetermined number of days depending on the study undertaken.

Retention of the Radiochemicals

To determine the biological clearance of the injected activity from the testis, the radiochemical was administered into the right testis and the mice were sacrificed in groups of five at various times post-injection. The testis were removed and assayed for radioactivity using either a NaI well counter (^{125}I and ^{131}I) or liquid scintillation counter (^{35}S and ^{210}Po). The radionuclide clearance was checked in the presence of radioprotector.

Determination of Optimal Day for Assay

Mice, in groups of 5, were injected with the same amount of radiochemical. The animals were sacrificed at different times post-injection, and the injected testis removed and processed (see below for details) to determine the spermhead population. The day on which the spermhead count reaches a minimum is the optimal day to determine the spermhead survival. The optimal day to assay the abnormal sperm is 9 days after the minimum testicular count is reached. The optimal days for the spermtid survival assay and abnormal spermhead assay remained the same in the presence of MEA (see Table I in Ref. 6).

Determination of Spermhead Survival

The mice were intratesticularly injected with different amounts of the radionuclide of interest. In each case, a group of control mice were injected with the cold drug in amounts equal to the highest dose group. Animals injected with normal saline served as further controls. The animals were sacrificed at the predetermined optimal day. The injected testis were removed, placed in 1 ml of deionized water, homogenized for 15 s, and sonicated for 30 s. The spermheads, which are resistant to sonication, were counted in a hemocytometer using a microscope at 400X magnification.

In the case of radioprotectors, a predetermined non-toxic level of cysteamine (0.75 μg) was injected i.t. and mice were kept aside for 4 h to allow the agent to spread uniformly throughout the testis. After this time the radionuclide was administered i.t.

Abnormal Spermhead Assay

The optimal day to study the induction of sperm abnormalities was established as described above. Several groups of mice were injected i.t. with different amounts of the radiochemicals. On the predetermined optimal day, the animals were sacrificed and the epididymides removed. The epididymides of the mice in same group were pooled in 1 ml of 0.9% saline, minced with fine scissors, and pipetted vigorously (19). The solution was then filtered through four layers of gauze to separate the sperm from tissue fragments. About 20 μl of 2% eosin-Y was added to the suspension, smeared on the slide, air dried, and a cover slip mounted with Permount. At least 2000 sperm (normal + abnormal) were scored under a light microscope. The

abnormal fraction of the epididymal sperm was determined and corrected for spontaneously occurring abnormalities (~ 2%).

Macroscopic Distribution

Mice were injected i.t. with the radiochemical and the testes removed 24 h later, frozen with CRYOkwik, and sliced into 10-15 sections. Each section was weighed and assayed for radioactivity. The activity per gram of tissue was relatively constant in the sections, indicating that the radionuclide was distributed fairly uniformly in the testis.

Subcellular Distribution

Following intratesticular injection of the radionuclide, the subcellular distribution was performed 1 day post-injection according to procedures described in Ref. 7. Briefly, the injected testes were removed and the testicular cells isolated promptly to separate cytoplasmic and nuclear fractions. The nuclear fraction was further separated into protein and DNA components. The activity in each fraction was obtained by counting aliquots of these fractions using an appropriate radiation detector. Subcellular distribution of the ^{35}S-cysteamine was also performed using the same procedures.

Irradiation with External X rays

For external irradiation of the testis, an overhead fluoroscopy X ray unit was employed (8). The selective irradiation of mouse testes was accomplished after the animals were anesthetized with sodium pentobarbital (60 mg/kg of body weight) and placed in custom-made lead shields to expose only the testes to the X ray beam. Exposure levels were determined using a Victoreen R meter.

RESULTS

Biological Clearance and Dosimetry

The biological clearance of ^{125}IUdR, H^{125}IPDM, and ^{210}Po-citrate were reported elsewhere (4,5). The biological clearance of intratesticularly injected ^{35}S-MEA yields a similar two-component exponential expression:

$$f = 0.54\, e^{-0.693t/0.43} + 0.46\, e^{-0.693t/40},$$

where f is the fraction of injected activity remaining in the testis, and t is the time (h) post-injection. Using the clearance data for the various radiochemicals, the average absorbed dose to the testis was calculated following MIRD formalism (22). Details of the calculations are given elsewhere (1,5).

Optimal Day for the Assays

The minimum spermhead count for the two iodine compounds and for external X rays was on 29th day (5,8), whereas for ^{210}Po it was on 36th day (4). The optimal day in the presence of radioprotectors was checked in each case and found to be the same. Hence, the spermhead survival assay with and without MEA was performed on these days. The abnormal spermhead assay was performed 9 days later as described in Ref. 6.

Subcellular Distribution of the Radiochemicals

The subcellular distributions of all the radiochemicals, taken from our earlier reports (4-6), are given in Table I. All of the ^{125}IUdR activity was bound to the DNA of the testicular cells. In the case of H^{125}IPDM and H^{131}IPDM, about 30% of the injected activity was localized in the testicular cells, of which more than 95% was found in the cytoplasm of the cells. About 20% of the ^{210}Po-citrate activity was found in the cell nucleus and the remaining 80% in the cytoplasm. In all cases, the distribution of the radionuclides was not influenced by the presence of the radioprotector. The subcellular distribution of ^{35}S-MEA in the testis following i.t. administration is given in Table II.

Spermhead Survival

The mean lethal doses at 37% survival (D_{37}) are 8.5 ± 2.1, 10 ± 1, 68 ± 6, and 61 ± 6 cGy for ^{125}IUdR, ^{210}Po-citrate, H^{125}IPDM, and H^{131}IPDM, respectively (4,5). The D_{37} value for external X irradiation of the testes is 67 ± 3 cGy (8). These results show that the Auger emitter ^{125}I bound to the DNA is at least as effective as the high-LET α particles of ^{210}Po. On the other hand, the cytoplasmically localized H^{125}IPDM is only as lethal as external X rays. The dose response curves are shown in Figs. 1A-1E for these radiochemicals and external X rays in the presence and absence of MEA. The observed D_{37} values in presence of MEA are 26.4 ± 5.6 cGy for ^{210}Po-citrate,

237 ± 48 cGy for $H^{131}IPDM$, 260 ± 32 cGy for $H^{125}IPDM$ (5), 31 ± 6 cGy for $^{125}IUdR$ (5), and 154 ± 53 cGy for X rays.

TABLE I
Summary of Subcellular Distribution Studies[a]

Radiochemical	Subcellular Distributions[b]
120 kVp X rays	-
$H^{125}IPDM$	100% Cy, 0% N, 0% D
$H^{131}IPDM$	100% Cy, 0% N, 0% D
$^{125}IUdR$	0% Cy, 100% N, 100% D
^{210}Po-citrate	80% Cy, 20% N, 45% D
$^{125}IUdR$+MEA	0% Cy, 100% N, 100% D
^{210}Po-citrate+MEA	80% Cy, 20% N, 45% D

[a] The numbers are reproduced from Ref. 6.

[b] % Cy is percentage of activity in cytoplasm, % N is percentage of activity in nucleus and % D is percentage of activity in cell nucleus bound to DNA.

TABLE II
Subcellular Distribution of ^{35}S-Cysteamine in Mouse Testis[a]

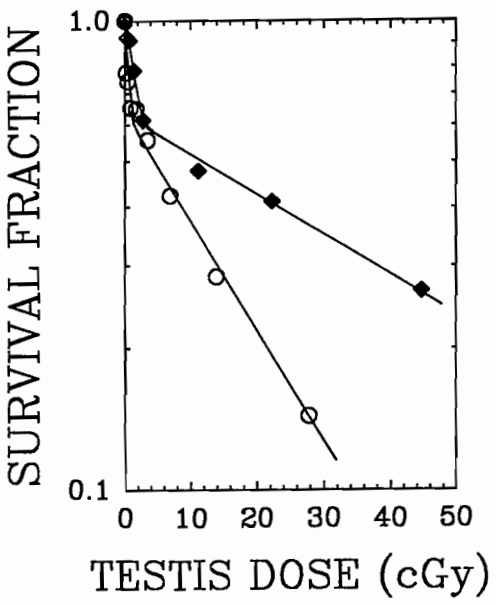
%Testis Activity in Cells	% Cellular Activity in Nucleus	% Nuclear Activity in DNA
25	10	53

[a] The data represents the average of two experiments.

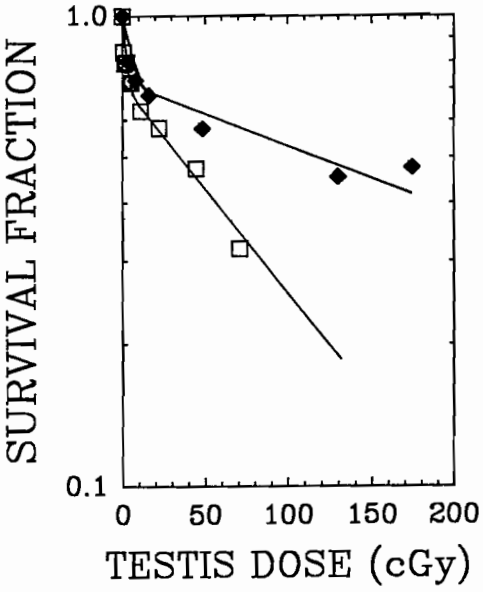
Induction of Sperm-shape Abnormalities

Figures 2A and 2B show the fraction of abnormal epididymal sperm as a function of average absorbed dose to the testis for $^{125}IUdR$ and ^{210}Po-citrate, respectively (6). These curves exhibit a linear increase in the abnormal fraction in the low-dose region and saturate at higher doses. This saturation is

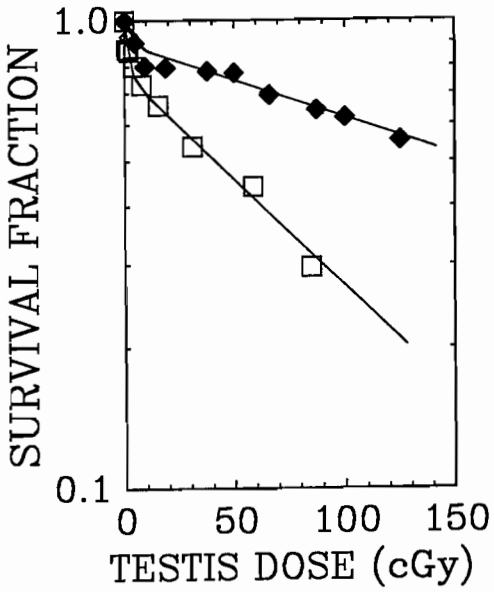
A


SURVIVAL FRACTION
1.0
0.1
0
10
20
30
40
50
TESTIS DOSE (cGy)

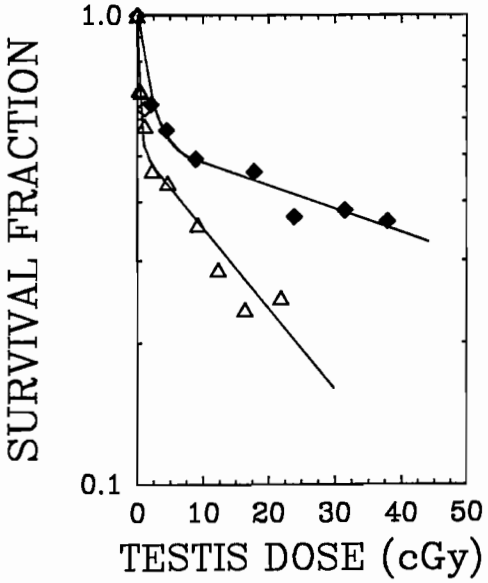
B


SURVIVAL FRACTION
1.0
0.1
0
50
100
150
200
TESTIS DOSE (cGy)

C


1.0
0.1
SURVIVAL FRACTION
0
50
100
150
TESTIS DOSE (cGy)

D


1.0
0.1
SURVIVAL FRACTION
0
10
20
30
40
50
TESTIS DOSE (cGy)

E

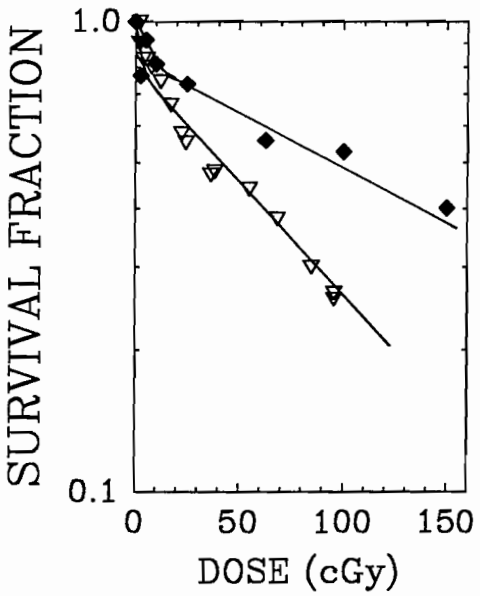


FIG. 1. Spermhead survival as a function of average testicular absorbed dose from intratesticularly administered radiochemicals. A) ^{210}Po-citrate: open circles without MEA and closed diamonds with MEA. B) H^{131}IPDM: open squares without MEA and closed diamonds with MEA. C) H^{125}IPDM: open squares without MEA and closed diamonds with MEA. Data taken from Ref. 5. D) ^{125}IUdR: open triangles without MEA and closed diamonds with MEA. Figure reproduced from Ref. 5. E) X rays: open reverse triangles without MEA and closed diamonds with MEA.

perhaps due to increased spermatogonial cell killing at high doses. However, the slope of the initial portion of the curve provides a sensitive comparison of the relative efficacy of the various types of radiations. Therefore, in each figure, the low-dose region of the curve is displayed as an inset. The initial slopes for ^{125}IUdR with and without MEA are 0.12 ± 0.01 %abnormals/cGy and 1.7 ± 0.1 %abnormals/cGy, respectively (6). For ^{210}Po-citrate, the corresponding initial slopes are 0.7 ± 0.04 %abnormals/cGy and 7.1 ± 0.5 %abnormals/cGy (6).

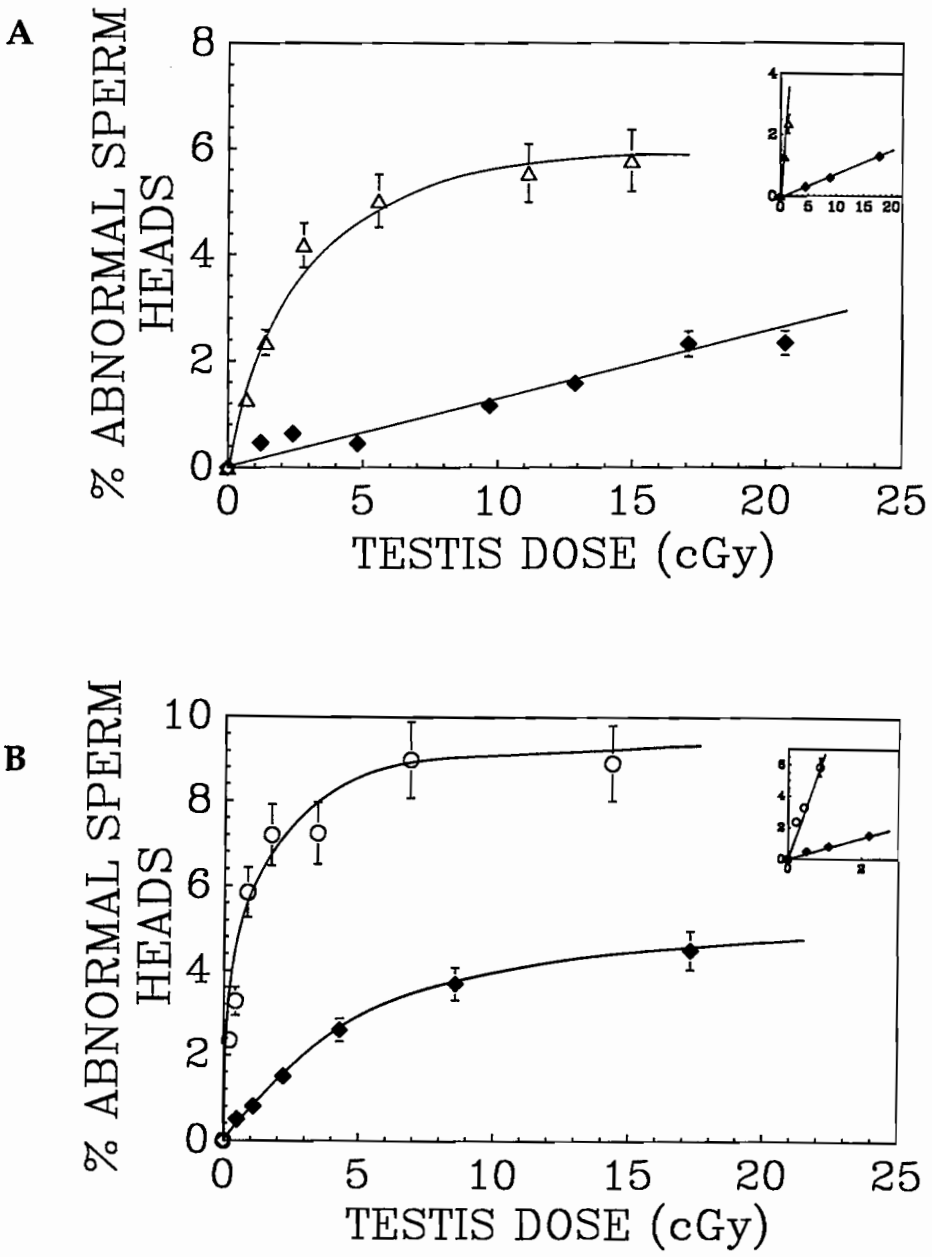


FIG. 2. Fraction of abnormal spermhead versus testicular absorbed dose from the intratesticular injection of radiochemicals. A) ^{125}IUdR: open triangles without MEA and closed diamonds with MEA. B) ^{210}Po-citrate: closed circles without MEA and closed diamonds with MEA. Data in A and B are reproduced from Ref. 6.

DISCUSSION

The radioprotective capacity of a variety of chemicals has been well established in various biological models using acute external beams of radiation. However, with the exception of the recent work by Rao *et al.* (5,6), to the best of our knowledge the protective action of chemicals against the effects of chronic irradiation by tissue incorporated radionuclides or external beams have not been studied. The effectiveness of a radioprotector, usually referred to as the dose modifying factor (DMF), is the ratio of absorbed doses at a chosen biological end point in the presence and absence of the chemical protector. The present spermhead survival studies which utilized the agent HIPDM to cytoplasmically localize ^{125}I (Auger emitter) and ^{131}I (β emitter) yielded DMF values of 3.8 and 3.9, respectively when MEA was used as the radioprotector (Figs. 1B & 1C). For DNA incorporated ^{125}I, we previously reported a DMF value of 3.6 (5). In the case of the α emitter ^{210}Po, a DMF of only 2.6 was observed for spermhead survival (Fig. 1A). This result for ^{210}Po is in good agreement with that of Bird (16) who obtained a similar DMF value for α particles. It is interesting to note that there is no significant difference in the protection offered by MEA for cytoplasmically localized and DNA incorporated ^{125}I. Furthermore, MEA offers equal protection against the low-LET β emitting radiochemical $^{131}IUdR$. This suggests that the high-LET type effects of $^{125}IUdR$ may be largely due to indirect action of radical species as opposed to direct deposition of energy in the critical targets. This suggestion is supported by the recent Monte Carlo calculations of Wright *et al.* (23) and Pomplun *et al.* (24, this volume), which simulate the direct and indirect interactions with DNA resulting from an ^{125}I decay on the DNA.

As indicated above, the data in this work also sheds light on the potential of MEA to protect against chronic versus acute irradiation. For acute exposure with external X rays where the testicular dose is delivered over a few minutes, a DMF of 2.4 was obtained with the spermhead survival assay (Fig. 1E). In contrast, DMF values of 3.9 and 3.8 were obtained for cytoplasmically localized ^{131}I and ^{125}I, respectively. Here, the dose is delivered over a period of several days. This suggests that superior protection is offered against chronic low-LET irradiation from incorporated radionuclides than against acute external beams. It should he noted, however, that the protective effect of MEA may be dependent not only on the dose rate, but also on its concentration in the tissue during the time the dose is delivered. Therefore, it may be necessary to have information regarding the cumulative

concentration (*i.e.* μg-h) of the chemical protector in the organ during the time of exposure, and perhaps its subcellular distribution. In fact, Smoluk *et al.* (25) showed that MEA binds to DNA and suggested that the protective action of chemical may indeed be related to the extent it binds to the DNA. Our subcellular distribution studies with radiolabeled MEA also indicate some binding of MEA to the DNA in the testicular cells. It is essential that further data be collected to investigate the dependence of radioprotection on these parameters.

Using sperm-shape abnormality as the biological end point, we obtained DMF values of 14 and 10 for ^{125}IUdR and ^{210}Po-citrate, respectively (6). These values are significantly higher than those observed for spermhead survival, and point to the dependence of DMF values on the biological end point. Furthermore, the increased protection observed with ^{125}IUdR compared to ^{210}Po α particles supports our earlier conclusion that the biological damage caused by DNA incorporated ^{125}I is largely due to the indirect action of radical species produced in the immediate vicinity of the decay site.

Further studies are underway with other radioprotectors such as vitamin C in order to get a clearer understanding of the mechanism of the Auger effect. The limiting factor in these studies is the high degree of chemotoxicity associated with most protectors. Some compounds, even in small quantities (~ ng), can affect spermatogonial cell survival and therefore are not useful for these studies. For instance, WR compounds may not work in this model because of their severe chemotoxicity to spermatogonial cells. Nevertheless, there are numerous compounds available which may shed light on the mechanism of the Auger effect.

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DISCUSSION

Yasui, L. S. LeMotte and Little showed a 5 fold increase in DNA damage induction from ^{125}I decay when decays were accumulated in cells without radioprotector (no glycerol). But cell survival does not change when decays are accumulated in the presence or absence of glycerol, suggesting an indirect effect is not a major factor in cell survival. How do you respond to this past data with respect to your results?

Narra, V. R. The experiments of LeMotte and Little were carried out with cultured cells in the frozen state. The efficiency of radical scavenging in the frozen state may be very different than *in vivo* at 37°C. This may explain the difference between our results and those of Lemotte & Little. It also

should be pointed out that radioprotection of cultured cells at 37°C is much more difficult than for spermatogonial cells. Experiments with cultured cells at 37°C necessarily involves prolonged exposure (1 wk) to the protector. Our experience has been that the cytotoxic nature of protectors precludes exposing the cells to concentrations of protectors that are high enough to afford appreciable protection. The testis, on the other hand, seem to be fairly resistant to the chemical toxicity of various radioprotectors.

Martin, R. F. Can you estimate the concentration of cysteamine in the cells at risk?

Narra, V. R. No, we have not directly measured the concentration of cysteamine in the spermatogonial cells, however, we are presently working on techniques which will allow us to accomplish this.