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RELATIVE BIOLOGICAL EFFECTIVENESS OF AUGER EMITTERS FOR CELL INACTIVATION: *IN VITRO* VERSUS *IN VIVO*

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ABSTRACT

Clonogenic survival of Chinese hamster V79 cells and spermhead survival in mouse testes have over the years provided a wealth of data concerning quantification of the lethality of Auger electron emitters *in vitro* and *in vivo*. The radiotoxicity of Auger emitting radiopharmaceuticals has been expressed in terms of the relative biological effectiveness (RBE) compared to reference acute external photon beams, with D_{37} serving as the point of comparison. Under these conditions, RBE values as high as 7 have been observed for DNA-bound Auger emitters. Since the ultimate utility of the RBE is for radiation protection, and perhaps radiation therapy, knowledge of the magnitude of the RBE value is essential. In this work, the techniques and assumptions used to obtain these RBE values both *in vitro* and *in vivo* are reviewed. In addition, the magnitude of the RBE values obtained in each model will be compared and critically examined in terms of: 1) reference radiation (*e.g.* type and dose rate), 2) comparison with α emitters, 3) importance of the site of decay, and 4) possible artifacts. The results indicate that reference radiation plays a key role in the V79 cell model, however, no

such observance was found in the mouse testis model. Experimental results in both models show that DNA-bound ^{125}I is as effective as incorporated α emitters in causing cell death, and that ^{125}I decays on thymine or cytosine bases are equally effective. Finally, the concept of quality factor (or radiation weighting factor) for Auger emitters is explored and a means of calculating the dose equivalent is advanced.

INTRODUCTION

The extreme lethality of Auger electron emitting radionuclides is well established both *in vivo* and *in vitro*. Their effectiveness is greatest when the Auger emitter is covalently bound to the DNA as in the case of the thymidine analogs ^{77}BrUdR (bromodeoxyuridine), ^{123}IUdR (iododeoxyuridine), and ^{125}IUdR (1-3). For clonogenic survival, values of relative biological effectiveness (RBE) of 6.4 (1), 7.3 (2), and 7.2 (3), respectively, were reported for cultured Chinese hamster V79 cells. *In vivo* studies of Rao *et al.* (4,5) with ^{125}IUdR gave a similar RBE value (7.9) for spermhead survival in mouse testes. When radionuclides decaying by EC and IC are non-covalently bound to the DNA of mammalian cells, high-LET (linear energy transfer) type effects are observed, albeit with a lower value of RBE (RBE ~ 4) (6-9). It has also been shown that, for covalent binding of ^{125}IUdR to the DNA, not all sites are equally radiosensitive to the ^{125}I Auger cascades (10).

The high RBE values reported for Auger emitters have prompted discussion regarding the notion of establishing a quality factor Q for this class of radionuclides. Pomplun *et al.* (11) have proposed $Q = 1$ for ^{125}I disintegrations "outside" the DNA and the high-LET Q for disintegrations "inside" the DNA, the high-LET Q being that for multiply charged particles (*e.g.* $Q = 20$ for α particles) (12). Before definite Q factors are assigned to Auger emitters such as ^{125}I, several questions need to be addressed regarding experimental RBE values for Auger emitters: 1) In view of the chronic nature of the irradiation from incorporated Auger emitters, how appropriate is use of acute X or γ rays as the reference radiation? This question has been raised recently by Rao *et al.* (13). 2) Can the high RBE values observed for Auger emitters be substantiated by parallel experiments with α emitters (4,14,15)? 3) The RBE of Auger emitters is highly dependent on cellular localization and subcellular distribution of the radionuclide (7,9,13,16), which are governed, in turn, by the chemical form (5,9). Is there a clear relationship between the RBE of the radiochemical and its subcellular distribution? 4) Does the DNA base

site where the Auger emitter binds play any role in the expression of the biological effect? These questions concerning RBE are of importance not only in recommending Q (or w_R) for ^{125}I and other Auger emitters, but also in calculating the dose equivalent H (17). In this work, experimental studies carried out *in vivo* with the mouse testis model (18,19) and *in vitro* with cultured Chinese hamster V79 cells are reviewed and new data are provided in an attempt to answer some of the above questions concerning the RBE of Auger emitters incorporated into proliferating mammalian cells. In addition, the quality factor for Auger emitters is discussed, and suggestions for incorporating subcellular distribution into the dose equivalent are advanced.

BIOLOGICAL MODELS AND EXPERIMENTAL METHODS

I. *In Vivo* Model: Spermatogenesis in Mouse Testis

The mouse testis model is a highly sensitive and quantitative model used to study the biological effects of radiation *in vivo* (20,21). The process of spermatogenesis in the mouse and man are very similar except for their time scales: about 5 wk for mouse and 10 wk for man. As indicated in Fig. 1 (20), the process is complex, yet tractable. The stem cell (A_s) differentiates to form a pair of cells which further divide to give type A_1 spermatogonial cells. The type A_1 cells in turn divide repeatedly through several spermatogonial cell

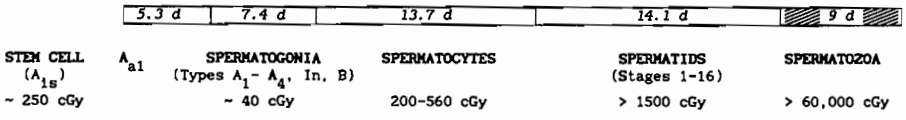

FIG. 1. The process of spermatogenesis in mouse testis is described by Meistrich *et al.* (53). There are five major populations of cells in the cycle: stem, spermatogonia, spermatocytes, spermatids, and spermatozoa. The relative radiosensitivity of the different groups to external beams of X rays is indicated by the LD_{50} values given above, the spermatogonial cells being the most radiosensitive. Timing of the spermatogenesis process as reported by Meistrich *et al.* (53) is indicated by the scale above. Approximately 29 days are required for the spermatogonia to become sonication resistant spermatids of Stages 12-16. Hence, after an initial acute radiation insult, the spermhead population in the testis reaches a minimum about 29 days later.

stages designated as A_2, A_3, A_4, In, and B. The type B spermatogonia divide to become secondary spermatocytes which mature into spermatids. Finally, the spermatids pass through 16 stages before they become functional sperm. The radiation sensitivity of these different subpopulations is markedly different (Fig. 1). When mouse testes are irradiated with external X rays, the differentiated spermatogonia (A_1-A_4, In, and B types) are found to be most sensitive $LD_{50} \sim 40$ cGy, whereas their precursors (A_{is}, A_{a1}) as well as the postgonial cells are relatively radioresistant with LD_{50} values of 100-300 cGy and 200-60,000 cGy, respectively (20). Because of this differential radiosensitivity, irradiation of the testis with low doses from external beams of X rays or from incorporated radionuclides results in a reduced testicular spermatid count 29-36 days later (see METHODS - Optimal Day Post-Administration to Assay Spermhead Survival), the time required for the spermatogonia to become sonication resistant spermatids of stages 12-16 (see below: Optimal Day) (9,18,19). Thus spermatogenesis may be used to study the effects of radiation *in vivo*.

General Procedures

Swiss Webster mice (Taconic Farms, Germantown, NY) aged 9-10 weeks were used in these studies. External irradiation of the testis with X rays was carried out by selective exposure of the organ with 60 kVp or 120 kVp X rays produced by an overhead fluoroscopy unit (9). Internal irradiation of the testis with incorporated radionuclides was achieved by intratesticular (i.t.) injection of radiochemicals in standard 3 µl volumes. This route of administration was selected over intravenous and intraperitoneal routes because essentially the entire testicular dose is attributed to energy deposited from decays occurring in the organ itself (self-dose). In the other modes of administration, the testicular dose is largely from the non-target to target dose from penetrating X and γ rays, thereby masking the effects of the low energy electrons (4,9,18,19). Intratesticular injection of the radiochemicals was performed following a minor surgical procedure to partially exteriorize the right testis (18). Using a microsyringe fitted with a 27-gauge needle, 3 µl of a solution containing the radiochemical was slowly injected along the long axis of the ellipsoidal testis as the needle was retracted. In this manner, a fairly uniform line injection was achieved. Controls were similarly injected with either saline or solutions containing the nonradioactive compounds at the same chemical concentration (18). Animals were maintained under anaesthesia throughout the procedure. The macroscopic uniformity of the resulting radionuclide distribution in the testis was confirmed by the

following method. At various times post-administration, the testes were removed, frozen with CRYOkwik™, sliced into sections, weighed, and the radioactivity per gram of tissue determined (19). The average subcellular distribution (cytoplasm, nucleus, DNA) of the radioactivity in the entire population of testicular cells was obtained 24 h postinjection using the cell fractionation methods outlined in Ref. 19.

Testicular Clearance of the Radiochemicals

The pattern of biological clearance of the radiopharmaceuticals following i.t. administration was monitored. Animals were injected with a fixed amount (~ nCi amounts) of radioactivity and sacrificed, in groups of four, at various times post-administration (18). The testes were removed immediately and the percent activity retained was determined using γ ray spectroscopy or liquid scintillation techniques. The amount of radiochemical injected did not influence the pattern of biological elimination over the range investigated.

Spermhead Count

The testicular spermhead count was determined immediately after sacrificing the animals. The testes were surgically removed (epididymis discarded), homogenized in 1 ml deionized water, and sonicated for 30 s. The sonication resistant spermheads of spermatids in Stages 12-16 were counted under a microscope to a minimum of 200.

Optimal Day Post-Administration to Assay Spermhead Survival

The optimal day post-administration to assay the spermhead survival is the day the spermhead count reaches a minimum (Fig. 2). Selective irradiation with acute external X rays yields a minimum spermhead count on the 29th day. This time scale is consistent with the spermatogenesis process (Fig. 1) in that it takes about 29 days for the radiosensitive spermatogonia to mature to spermatids of Stages 12-16. For intratesticularly injected radiochemicals, the optimal day varies between 29 and 36 days post-injection (4,9,18,19). Since this optimal day depends on the nature of the exposure (*e.g.* chronic versus acute), and perhaps on the cellular incorporation of radionuclides, it must be determined experimentally in each case (4,9,18,19).

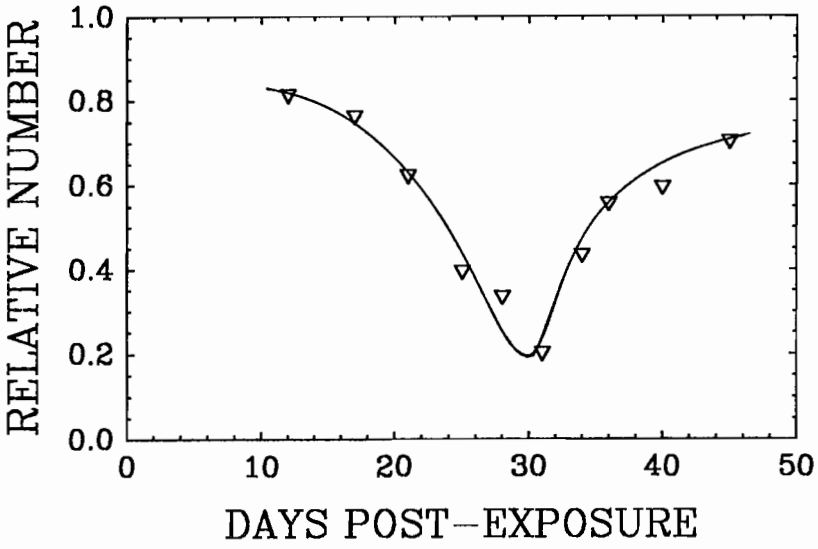

FIG. 2. Time dependence of sperm-head count in mouse testes exposed to external 120 kVp X rays (9). The spermhead count relative to unirradiated controls is plotted as a function of time post-exposure. The minimum spermhead count was observed on the 29th day post-irradiation.

Differential Radiosensitivity of Types A_1-A_4, In, and B Spermatogonia

Additional experiments were performed in order to prove that the two-component nature of the spermhead survival curves (see RESULTS) is not an artifact of the procedure, rather due to the differential radiosensitivity of the spermatogonial cells. In the spermhead survival assay described above, the killing of spermatogonial cells is measured indirectly by determining the testicular spermatid (spermhead) population 29-36 d post-injection of the radiochemical. To explore the differential radiosensitivity of the various types of spermatogonial cells (A_1-A_4, In, B), the spermatogonial cell survival was measured directly using standard histological techniques (22). Briefly, ten twelve-week old mice were sedated with sodium pentobarbital and the testes irradiated externally with 120 kVp X rays. The dose rate (9 cGy/min) was measured with a Victoreen rate meter. External X rays were employed to insure uniform irradiation of the testis and that intratesticular injections play no role in the shape of the survival curves. Mice, in groups of two, were

irradiated with 5, 25, 75, or 100 cGy. Two unirradiated mice, otherwise treated identically, served as controls. Forty-eight hours post-irradiation, the mice were sacrificed by cervical dislocation, and the testes removed. After removal of the tunica, the tubules were teased free *in toto* as they were bathed in Bouin's fixative. The tubules were kept in the fixative for 5 h and then allowed to decolorize overnight in a solution of 70% ethanol - 30% water. They were then stained in a dilute solution of Harris' hematoxylin for 5-6 min and immediately washed in tap water ten times at 30 min per wash. The sightly basic pH of the tap water facilitated removal of the excess stain. This was followed by five 30 min washes in distilled water and then a step-wise dehydration beginning at 20% ethanol up to 100%. A second wash with 100% ethanol was performed and the tubules allowed to soak overnight. Two successive washes with a 50% xylene - 50% ethanol solution and an overnight wash in 100% xylene were then performed. The tubules thus treated were mounted on glass slides with Permount and allowed to dry 3 d. With the aid of an ocular grid, the various types of spermatogonial cells were counted individually and normalized to the number per 100 Sertoli cells. At least 4 continuous grid-lengths per tubule were counted and 4 tubules were counted per animal. The entire procedure was repeated a second time with the addition of three dose points: 10, 50, and 100 cGy.

II. *In Vitro* Model: Clonogenicity of Cultured V79 Cells

Cultured mammalian cells have been widely used as a biological model to study the radiotoxicity of incorporated Auger emitters. Clonogenic survival of Chinese hamster V79 lung fibroblasts provides a simple, yet highly effective, model to examine the lethality of radionuclides in mammalian cells. Accordingly, this model has been used extensively by Kassis *et al.* (1,3,23), and is used in the present work.

Cell Culture

Chinese hamster V79-513 cells (kindly provided by Dr. Athwal, UMDNJ) are maintained as monolayers under standard conditions (37°C, 5%CO_2 - 95% air). Cells are cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2mM L-glutamine. Twice weekly, the cells are subcultured.

Clonogenic Survival

Cells in exponential growth are removed from the culture flask with trypsin, suspended in calcium-free MEM (for suspension culture), and diluted to 400,000 cells/ml. One ml aliquots of cell suspension are transferred to 15 ml culture tubes and placed on a rocker-roller for 3-4 h under standard conditions. Subsequently, an additional 1 ml of MEM containing the radiochemical is added and the tubes further rolled for a predetermined period to allow the radioactivity to incorporate into the cells. Aliquots of the cells are removed and the radioactivity per cell determined according to the method of Kassis *et al.* (24). The remaining cells are washed 3 times with fresh MEM, serially diluted, and seeded in triplicate into 25 cm^2 flasks for colony formation. Aliquots of these washed cells are also assayed for intracellular radioactivity content to determine the amount of unbound radioactivity lost during the washing process. After 1 wk the colonies are washed with saline, fixed with methanol, stained with crystal violet, scored, and the survival compared to controls. Untreated cells and cells treated with the non-radioactive chemicals serve as controls.

Cellular Uptake and Clearance of Radioactivity

The cellular uptake of radioactivity is monitored as a function of time. Aliquots of cell suspension are removed at various times and the radioactivity per cell determined (25). The uptake kinetics are essential for determining the cellular self absorbed dose during the period the cells are incubated in MEM containing radioactivity.

After the cells are washed free of extracellular radioactivity and seeded for colony formation, the radioactivity per cell typically decreases exponentially in time. The effective half-life of the cellular activity is determined by seeding a large number of labeled cells into several flasks, removing the cells from the flasks with trypsin at various times later, and determining the radioactivity per cell. The clearance kinetics thus obtained are used to determine the cellular self absorbed dose during the colony forming period (25).

Subcellular Distribution of the Radiochemicals

Subcellular distribution of the radionuclide plays a critical role in dosimetry. Accordingly, using the techniques outlined in Ref. 23, the fraction

of intracellular radioactivity in the cytoplasm versus nucleus is determined as well as the fraction bound to nuclear DNA.

III. Radiochemicals and External Radiation Sources

It is well known that in some radiobiological models the effects of acute radiation are substantially greater than those of a chronic nature. According to the International Commission on Radiation Protection (ICRP), the relative biological effectiveness of radiation A with respect to reference radiation R is defined as the ratio of absorbed dose D_R in a tissue to the dose D_A that causes a qualitatively and quantitatively equal effect. Each RBE derived from a set of observations for tissue cultures or for responses of tissues, in animals or in man, refers to a defined end point produced under a specified set of exposure conditions (26). Hence, because the definition requires that the test and reference radiations be delivered under the essentially the same conditions, it is important to carefully select the reference radiation. Conventionally, photons of low-LET in the form of acute 250 kVp X rays have served as the reference radiation. However, in view of the chronic nature of the exposure from incorporated radionuclides, the conventional reference may not be adequate.

In order to establish the appropriate reference radiation for the spermhead survival assay, several sources of radiation were examined. First, two different sources of photon radiation were examined: acute external X rays (60 kVp and 120 kVp), and the radiochemical ^{7}Be chloride. The X rays were delivered over a period of only a few minutes. ^{7}Be is essentially a pure γ emitter (also emits a single 40 eV electron) which chronically irradiates the testis following i.t. injection. Secondly, the effects of the low-LET β emitter ^{131}I, localized in the cytoplasm of the testicular cells with the radiochemical H^{131}IPDM (5), was studied as a further source of low-LET chronic irradiation. Finally, the effects of H^{125}IPDM and ^{123}IMP (Spectamine™) were also examined.

In the *in vitro* model, acute and chronic irradiation of the V79 cells with photons was accomplished with external ^{137}Cs and ^{99m}Tc γ rays, respectively. The acute irradiation with ^{137}Cs γ rays was accomplished over a period of a few minutes (2.6 Gy/min). ^{99m}Tc (140.5 keV γ ray) decays with a physical half-life of 6 h and was therefore used to irradiate the cells externally over a period of hours with a dose rate which decreases exponentially in time

(initially ~ 1.2 Gy/h for a final cumulated dose of 10 Gy). This pattern is consistent with the manner in which the dose is delivered to the V79 cells from incorporated radionuclides. The details of the irradiation with a ^{99m}Tc "disk" source are given elsewhere (25).

The extreme toxicity of the Auger emitter ^{125}I, when incorporated into the DNA in the form of iododeoxyuridine (IUdR), is well known. Values of RBE as high as about 7 have been reported for cell killing both *in vivo* and *in vitro* (3,4). To confirm these high values we have compared the lethality of ^{125}IUdR with the α emitting radiochemical ^{210}Po-citrate (4,14,25). ^{210}Po emits a single 5.3 MeV α particle and is therefore an excellent source of high-LET radiation for comparison with Auger emitters in both biological models.

Auger emitters are most lethal when incorporated into DNA in the cell nucleus. Are all sites on the DNA equally radiosensitive? The highly localized action of Auger electron emitters provides a unique opportunity to examine the differential radiosensitivity of various locations on the genome through selective targeting with specific radiochemicals. Such precise delivery of the radiation insult is unattainable with external beams of radiation. We have compared the radiotoxicity of ^{125}I labeled iododeoxyuridine and iododeoxycytidine (IdC) to explore possible differential radiosensitivity of the thymine and cytosine base sites.

Finally, the dependence of the RBE of intracellularly incorporated ^{125}I on the fraction of radioactivity bound to DNA is investigated. This is accomplished by administering mixtures of two radiochemicals: 1) ^{125}IUdR which binds almost exclusively to the DNA, and 2) H^{125}IPDM which localizes in the cytoplasm of the cells. By varying the ratio of these two radiochemicals in the injectate, the fraction of radioactivity bound to nuclear DNA may be controlled.

RESULTS

I. *In Vivo* Model: Spermatogenesis in Mouse Testis

Optimal Day to Perform Spermhead Survival Assay

The testicular spermhead count is shown in Fig. 2 as a function of time post-irradiation. Acute 120 kVp X rays were employed. The minimum

spermhead count occurs on the 29th day post-irradiation (9). Hence, this is the optimal day to perform the spermhead survival assay. This same optimal day was found for all of the radiochemicals with the exception of ^{210}Po where the 36th day post-administration was the optimal day (4,5). It should be noted that this value is similar to those found in our earlier work (9) where optimal assay days for ^{111}In-oxine and ^{111}In-citrate were the 36th and 34th day, respectively.

Kinetics of the Radiochemicals in the Testis and Calculation of the Testicular Absorbed Dose

The testicular clearance patterns of the radiochemicals following intratesticular injection are shown in Fig. 3. The data were least squares fitted to a two-component exponential expression (19). Conventional dosimetry, as prescribed by the Medical Internal Radiation Dose (MIRD) Committee (27), was used to determine the absorbed dose to the testis following intratesticular injection of the radiochemical. The absorbed dose is given by $D = \tilde{A}\phi_i\Delta_i/m$, where $\tilde{A}$ is the cumulated activity, ϕ_i the absorbed fraction for the i_{th} radiation component, and Δ_i the equilibrium dose constant. Absorbed fractions were obtained using the computer code of Howell *et al.* (28) while Δ_i were obtained from Ref. 29. The cumulated activity was obtained by integrating the two-component time-activity relationships according to the provisions described in Refs. 18, 19, and 30, and the testicular dose determined (18,19,30).

Spermhead Survival Curves

Figure 4 shows the fraction of surviving spermheads as a function of the testicular absorbed dose for ^{125}IUdR (4), ^{125}IdC, ^{210}Po-citrate (4), ^{7}Be chloride, H^{131}IPDM, H^{125}IPDM (5), ^{123}IMP, and X rays (9). A least squares fit to the data using a two-component exponential expression yields the following relationships where S is the survival fraction and D is the absorbed dose in cGy:

$$S(\text{X rays}) = 0.27 \exp(-D/6.5) + 0.73 \exp(-D/98)$$

$$S(^{210}\text{Po}) = 0.30 \exp(-D/0.20) + 0.70 \exp(-D/15.6)$$

$$S(^{125}\text{IUdR}) = 0.46 \exp(-D/0.41) + 0.54 \exp(-D/22.1)$$

$$S(^{7}\text{Be}) = 0.13 \exp(-D/0.70) + 0.87 \exp(-D/76.2)$$

$$S(\text{H}^{125}\text{IPDM}) = 0.21 \exp(-D/1.8) + 0.79 \exp(-D/89.2)$$

$S(^{125}\text{IdC}) = 0.45 \exp(-D/0.56) + 0.55 \exp(-D/19.3)$

$S(\text{H}^{131}\text{IPDM}) = 0.22 \exp(-D/0.67) + 0.78 \exp(-D/81.6)$

$S(^{123}\text{IMP}) = 0.12 \exp(-D/0.68) + 0.88 \exp(-D/71.3)$

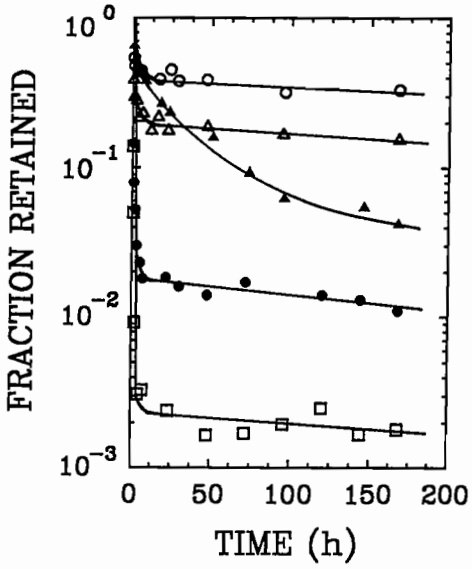

FIG. 3. Testicular clearance patterns of the radiochemicals following intratesticular injection. The fraction of injected activity retained by the testis is shown as a function of time post-injection for ^{210}Po-citrate (open circles) (4), ^{125}IUdR (solid circles) (4), ^{125}IdC (open squares), ^{7}Be chloride (open triangles) (13), H^{125}I(^{131}I)PDM (solid triangles) (5).

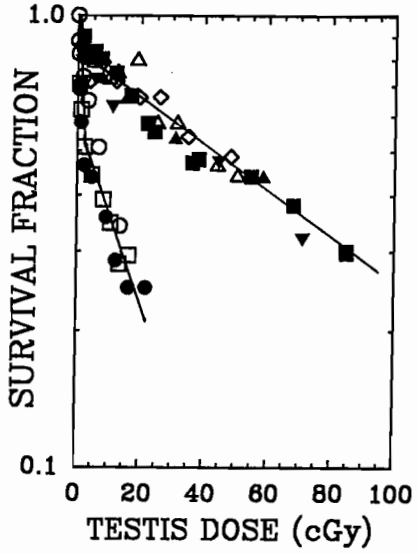

FIG. 4. Spermhead survival as a function of testicular absorbed dose following external and internal irradiation. Survival curves are shown for ^{210}Po-citrate (open circles) (4), ^{125}IUdR (solid circles) (4), ^{125}IdC (open squares), ^{7}Be chloride (open triangles), H^{125}IPDM (solid triangles) (5), H^{131}IPDM (solid inverse triangles), ^{123}IMP (open diamonds), and external X rays (solid squares) (49).

The low-LET sources of radiation (^{7}Be chloride, H^{131}IPDM, H^{125}IPDM, ^{123}IMP, X rays) all lead to comparable cell killing with similar D_{37} values (65 ± 10, 61 ± 6, 68 ± 6, 62 ± 6 and 67 ± 3 cGy, respectively). Iodine-125 labeled IUdR and IdC both bind to the DNA in the cell nucleus and yield the same 37% survival values of 8.5 ± 2.1 and 7.7 ± 1.2 cGy, respectively. This may be compared to the 37% survival value of 10 ± 1 cGy for the high-LET α particle emitter ^{210}Po (4).

Spermatogonial Cell Survival Curves

The individual survival curves for X irradiated spermatogonial cell types (A_1-A_4,In,B) are shown in Fig. 5. Each of these curves follows essentially a single-component exponential response. The large discrepancy in the slopes of the curves, particular between spermatogonial cell types A_1-A_2 and the remaining types, suggests that the first component of the spermhead survival curves (Fig. 4) is due to killing of the sensitive spermatogonial cell stages at low doses, while the second component is due to killing of the less sensitive types.

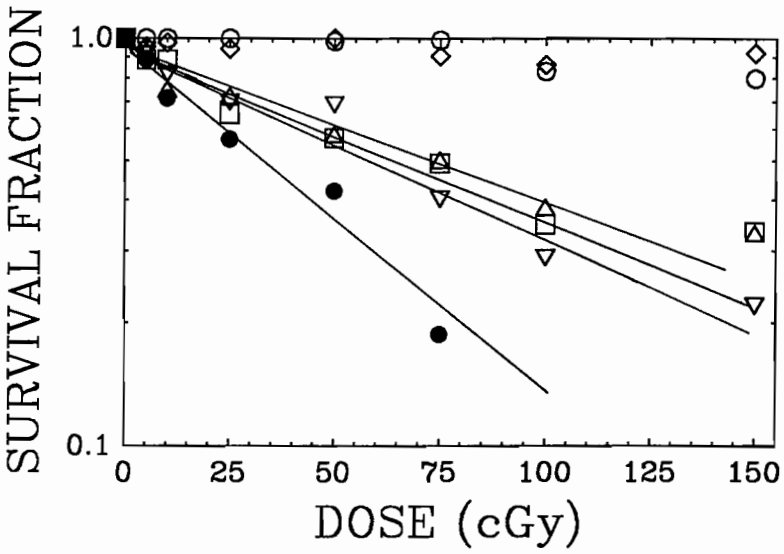

FIG. 5. Survival of spermatogonial cells as a function of testicular dose delivered externally by 120 kVp X rays. The survival curves for spermatogonial cell types A_1, A_2, A_3, A_4, In, and B, are represented by the open diamonds, open circles, open triangles, open squares, open inverse triangles, and filled circles, respectively. A least squares fit to the data yields D_o values of 103 ± 8, 96 ± 7, 86 ± 5, and 46 ± 3 cGy for types A_3, A_4, In, and B, respectively. Insufficient data was collected at high doses for types A_1 and A_2, hence, it was not possible to fit the data.

Dependence of RBE on Fraction of Cellular Activity Bound to DNA

Survival curves were obtained using various binary mixtures of $H^{125}IPDM$ (cytoplasmic localization) and $^{125}IUdR$ (binds to DNA in nucleus) (25). The 37% survival values and subcellular distribution for each mixture were determined. The RBE compared to external 120 kVp X rays is plotted as a function of the fraction f of ^{125}I which is bound to DNA in the testicular cells (Fig. 6). A linear relationship is observed with RBE = 1 + 7.4 f. (25)

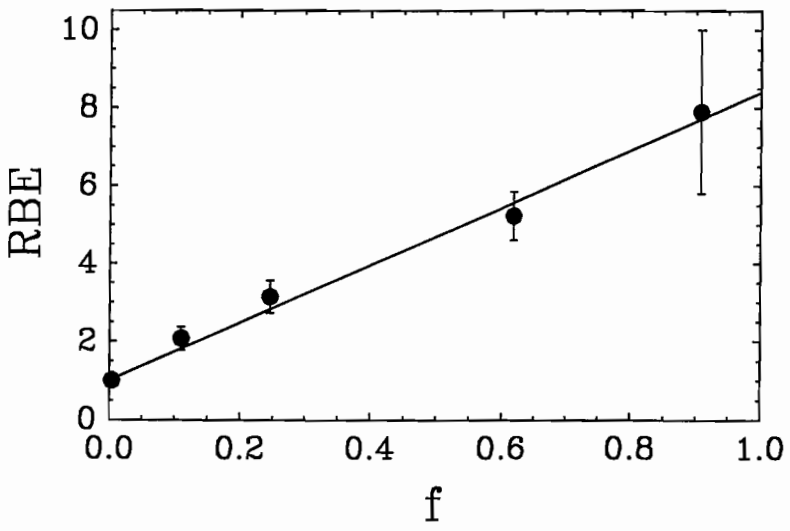

FIG. 6. Spermhead survival in the mouse testis model (5,9,18): RBE as a function of the fraction f of intracellular ^{125}I bound to DNA. The fraction of ^{125}I bound to DNA was adjusted by administering mixtures of the brain perfusion imaging agent $H^{125}IPDM$ (54) and $^{125}IUdR$ which localize in the cytoplasm (5) and nuclear DNA, respectively. The RBE is related to the DNA-bound fraction according to the relationship RBE = 1 + 7.4 f.

II. In Vitro Model: Clonogenicity of Cultured V79 Cells

Kinetics of Cellular Uptake of Radioactivity

Cellular incorporation of $^{125}IUdR$ (IdC) was directly proportional to both incubation time (up to 18 h) and radiochemical concentration κ (kBq/ml) in the extracellular medium. Based on these data, the average

cellular uptake of radioactivity in mBq/cell is given by $A(t) = \omega \kappa t$, where the constant $\omega = 0.037$ mBq/cell per h-kBq/ml and 0.0053 mBq/cell per h-kBq/ml for ^{125}IUdR and ^{125}IdC, respectively. The incorporation of these radioiodine compounds is cell cycle dependent. Therefore, since the doubling time of V79 cells is 9-12 h (23), an 18 h incubation time (T_I) was used in the survival studies with the ^{125}I radiochemicals to insure uniform labeling of the cells. The average uptake of radioactivity per cell A_I (mBq/cell) for $T_I = 18$ h is therefore given by $A_I = 0.67 \kappa$ and 0.1κ for ^{125}IUdR and ^{125}IdC, respectively. The cellular uptake of ^{210}Po-citrate was also linearly dependent on the extracellular radiochemical concentration κ, however, it was rapidly incorporated into the V79 cells, achieving saturation in only 0.5 h (14). Accordingly, cell survival studies with ^{210}Po-citrate called for only a 0.5 h exposure to the radiochemical before washing the cells and seeding them for colony formation. In this instance, the average uptake of radioactivity per cell A_I (mBq/cell) at $T_I = 0.5$ h is given by $A_I = 0.04 \kappa$.

Clonogenic Survival

The survival fraction S is related to the cellular uptake A_I (at T_I) according to the expression $S = \exp(-A_I / A_o)$, where A_o is the mean lethal uptake. A least squares fit to the data (data not shown, see Fig. 8 for dose-response curve) yielded A_o values of 0.13 ± 0.01 mBq/cell and 0.72 ± 0.05 mBq/cell for ^{210}Po-citrate, and ^{125}IUdR and ^{125}IdC, respectively. Characteristic of high-LET radiation effects, these curves had no initial shoulder.

Cellular Retention of Radioactivity During the Colony Forming Period

The radioactivity per cell during the colony forming period decreased exponentially with time for all three radiochemicals (Fig. 7). The effective half-lives (τ_e) were 13.8 h and 12 h, for ^{210}Po-citrate and the radioiodines, respectively. These values were independent of the cellular uptake of radioactivity for the range of uptakes studied.

Subcellular Distribution of Radioactivity

The subcellular distribution of the radiochemicals following incorporation into V79 cells is given in Table II. In all cases, the relative subcellular distributions remained the same during the uptake and colony forming period.

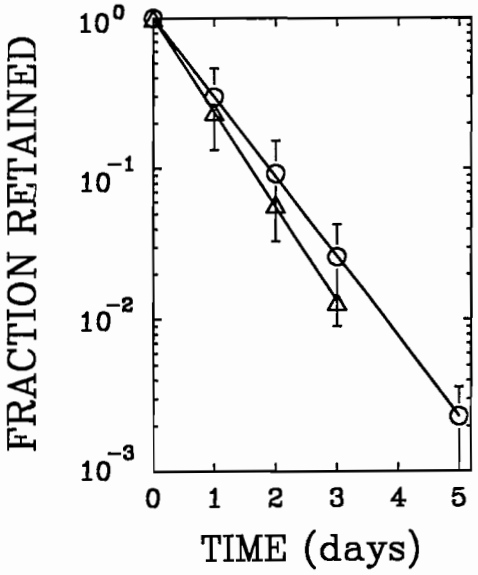

FIG. 7. Elimination of the radiochemicals from exponentially growing V79 cells during the one week colony forming period. The average amount of radioactivity per cell decreases with an effective half-life of 12 h for ^{125}IUdR and ^{125}IdC (solid circles), and 13.8 h for ^{210}Po-citrate (open circles) (Data taken from Ref. 25). Three experiments were averaged, the error bars representing the SDM.

Dosimetry for Internal Irradiation with Incorporated Radionuclides

Because the cell nucleus is widely believed to contain the radiosensitive targets, the absorbed dose to the nucleus is determined (3,16,23,25). There are four contributions to the average dose to the cell nucleus: 1) non-target to target dose from decays occurring in the extracellular medium during the incubation period T_I, 2) self-dose from decays within the target cell during the incubation period T_I, 3) self-dose from decays within the target cell during the colony forming period, and 4) cross irradiation from decays in neighboring cells of the colony. Since κ is small in these studies, contribution (1) is negligible (< 1% of the total dose). Calculation of contributions (2) - (4) requires determination of the cumulated cellular activity during the uptake ($\tilde{A}_I$) and colony forming periods ($\tilde{A}_{CF}$). Details of this calculation are

given in Ref. 25. For ^{125}IUdR (IdC) $\tilde{A}_I = 9\ A_I$ mBq-h, while for ^{210}Po-citrate $\tilde{A}_I$ is negligible compared to the cumulated cellular activity during the colony forming period $\tilde{A}_{CF}$. The values of $\tilde{A}_{CF}$ are 19.9 A_I mBq-h and 17.3 A_I mBq-h for ^{210}Po-citrate and the radioiodines, respectively. The cumulated intracellular disintegrations N are correspondingly 71.6 A_I and 94.6 A_I.

The average dose D to the V79 cell nucleus is given by $D = \xi/v_N$, where v_N is the volume of the nucleus, and ξ is the total energy deposited in the nucleus. If the cross irradiation component of the dose is ignored (see later), then $\xi = N\ (f_N\varepsilon_{NN} + f_{CY}\varepsilon_{NCY})$, where f_N and f_{CY} are the fraction of cellular radioactivity in the nucleus and cytoplasm, respectively. The parameters e_{NN} and e_{NCY} are the average energy deposited in the nucleus per decay in the cell nucleus and cytoplasm, respectively (31). The quantity e_{NN} is 10.9 keV for ^{125}I in V79 cells where the average diameter of the cell and cell nucleus are 10 μm and 8 μm, respectively (25,31). Since $f_N \sim 1$ for ^{125}IUdR and ^{125}IdC, $\xi = 1030\ A_I$ keV. For ^{210}Po $e_{NN} = 216$ keV and $e_{NCY} = 93$ keV. With $f_N = 0.28$ and $f_{CY} = 0.72$, $\xi = 9120\ A_I$ keV for ^{210}Po (25). With $v_N = 270\ \mu m^3$, the average dose to the V79 cell nucleus is 5.4 A_I Gy and 0.61 A_I Gy, for ^{210}Po-citrate and ^{125}IUdR (IdC), respectively. The corresponding dose response curves for cell survival are shown as a function of absorbed dose to the cell nucleus in Fig. 8. Fitting the data to $S = \exp(-\alpha D)$ yields $\alpha = 1.4 \pm 0.5$ Gy^{-1} for ^{210}Po-citrate, and 2.3 ± 0.8 Gy^{-1} for ^{125}IUdR (IdC). The corresponding D_{37} are 0.70 ± 0.25 Gy and 0.44 ± 0.15 Gy, respectively. For ^{210}Po, this corresponds to about 9 intracellular disintegrations. When the subcellular radionuclide distribution is taken into account, this is equivalent to about 2 α particle traversals across the cell nucleus. This number (9 disintegrations) is substantially fewer than the 66 disintegrations required for the ^{125}I compounds.

External Irradiation with γ rays

Dose response curves for V79 cells irradiated either acutely with a single exposure to 662 keV ^{137}Cs γ rays (2.6 Gy/min), or chronically with 140.5 keV ^{99m}Tc γ rays (initially ~ 1.2 Gy/h for final cumulated dose of 10 Gy), are shown in Fig. 8. The data were least squares fitted to the linear-quadratic model $S = \exp -(\alpha D + \beta D^2)$ (25). The resulting D_{37} values were 400 cGy and 880 cGy for ^{137}Cs and ^{99m}Tc γ rays, respectively. Based on these data, it is clear that the chronic irradiation, delivered in a manner somewhat similar to that for incorporated radionuclides, is significantly less effective than acute irradiation.

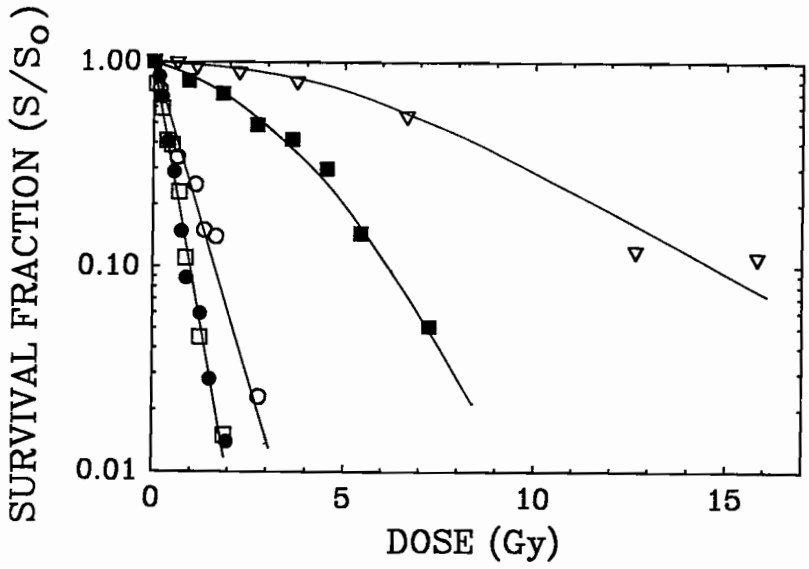

FIG. 8. Dose response curves for cultured V79 cells following incorporation of ^{210}Po-citrate (open circles), ^{125}IUdR (solid circles), and ^{125}IdC (open squares). The survival of Chinese hamster V79 cells following external irradiation with acute or chronic γ rays is also shown. The solid squares represent the cell survival fraction as a function of absorbed dose from ^{137}Cs γ rays delivered acutely (2.6 Gy/min). The open inverse triangles are for ^{99m}Tc γ rays delivered chronically with the dose rate decreasing exponentially over several days according to the 6 h physical half-life of ^{99m}Tc (initial dose rate ~ 1.2 Gy/h for final cumulative dose of 10 Gy). The SDM is indicated by the error bars. Data taken from Ref. 25.

DISCUSSION

Delineation of the biological effects of incorporated Auger electron emitters is complicated indeed. Therefore, the parallel *in vivo* and *in vitro* experiments presented in this work, namely spermhead survival in mouse testis and clonogenic survival of cultured V79 cells, provide valuable complementary data regarding the lethality of these radionuclides. These parallel data address several aspects including: 1) role of reference radiation for determining RBE values - dependence on dose rate, 2) RBE of DNA incorporated Auger emitters compared to α emitters, 3) dependence of RBE on decay site in DNA, 4) dependence of RBE on absorbed dose at which

comparison is made, and 5) calculation of the dose equivalent. In addition, experimental data are provided to support the validity of using the mouse testis model as a unique assay to study the effects of incorporated radionuclides *in vivo* at low doses.

Survival Curves

Before discussing the issue of reference radiation, it is perhaps useful to critically examine the dose-response (survival) curves obtained with the *in vivo* and *in vitro* models. As expected, the survival curves obtained following external irradiation of V79 cells with γ rays, delivered chronically or acutely, have a distinct shoulder (Fig. 8). Similarly, the exponential response of V79 cells (Fig. 8) to doses delivered by internal α and DNA-bound Auger emitters is also expected based on the observations of others (3,32). These shouldered and non-shouldered curves are classic radiobiological cell-survival curves for low- and high-LET radiations, respectively. In contrast, the spermhead survival curves obtained *in vivo* with the mouse testis model are strikingly different in their functional form (Fig. 4). The observed two-component exponential dose-response curves are not due to artifacts caused by intratesticular injection. Indeed, the same two-component nature is seen when the testis are uniformly irradiated with external X rays as shown in Fig. 4. Similar two-component curves were also observed by Oakberg (33), Gasinska (34), Hacker *et al.* (35), and Spano *et al.* (36) when spermhead survival was measured following exposure to external X rays. Furthermore, the data in Fig. 5, which show the response of spermatogonial cell types A_1-A_4, In, and B to external X irradiation, strongly suggest that the two-component nature of the spermhead survival curves is due to differential radiosensitivity of the spermatogonia. Perhaps even more important is the equivalence of survival curves for external X rays and internally administered radionuclides which impart low-LET type damage (Fig 4). Among these radiochemicals are ^{7}Be chloride (γ emitter), H^{131}IPDM (β emitter), and H^{125}IPDM & ^{123}IMP (cytoplasmically localized Auger emitters). The essentially identical nature of these curves (Fig. 4), which encompass the effects of intratesticular injection of extremely short range electron emitters (^{125}I) to very long range γ emitters (^{7}Be), clearly demonstrates that intratesticular injection of the radiochemicals does not lead to macroscopic inhomogeneities in activity distribution which in turn influence the shape of the survival curves. Even further support for this conclusion is provided by our studies with intraperitoneally administered ^{210}Po-citrate (data not shown) which result in the same mean lethal dose as

intratesticular administration. Finally, it should be noted that the sharply different slopes (compared to X rays) of the individual components of the curves for the high-LET radiations (^{210}Po-citrate and ^{125}IUdR & ^{125}IdC) are expected based on similar curves given by Spano *et al.* (36) and Oakberg (33) for neutron irradiation of mouse testes.

Reference Radiation

The issue of selecting a reference radiation for the purpose of calculating RBE values takes on very different degrees of importance for the *in vitro* and *in vivo* models utilized in this work. We established above for the mouse testis model that chronic internal irradiation from radionuclides which impart low-LET type damage are just as lethal as acute external X rays. Hence, selection of the reference radiation is not critical for the *in vivo* survival assay and therefore external X rays perhaps are the best choice in view of the conventional 250 kVp X rays. Conversely, the acute versus chronic nature of the irradiation plays a major role in the response of cultured V79 cells. As shown in Fig. 8, acute ^{137}Cs γ rays are more than twice as effective in killing V79 cells than ^{99m}Tc γ rays delivered chronically in a manner consistent with incorporated radionuclides. Therefore, ^{99m}Tc γ rays are perhaps the more relevant reference radiation for the *in vitro* model.

Relative Biological Effectiveness of the Radiochemicals

As indicated earlier, the relative biological effectiveness is defined as $RBE = D_R/D_A$ for radiation A relative to the reference radiation R for equal biological effect (26). With 120 kVp X rays serving as the reference radiation for the mouse testis model, the RBE at 37% survival for the radiochemicals ^{7}Be chloride, H^{131}IPDM, H^{125}IPDM, and ^{123}IMP is ~ 1.0 (Table I). The RBE values for ^{210}Po-citrate and ^{125}IUdR & ^{125}IdC are 6.7 and 7.9, respectively (4). Similarly, if we use ^{99m}Tc γ rays as the reference radiation (chronic) for Chinese hamster V79 cells (25), the RBE at 37% survival is 13 ± 5 for ^{210}Po-citrate and 20 ± 7 for ^{125}IUdR and ^{125}IdC (Table II). Although these values may be quite high for cell killing, we note that our data give RBE = 6 ± 2 and 9 ± 3 for the respective radiochemicals when acute γ ray exposure is used as the reference (Table II). These results are in good agreement with other published work with V79 cells (3,14,32,37,38). Similarly, a value of 12 was reported for mouse embryos (39). It is important to note that these RBE values also depend strongly on the end point. This is emphasized in Figs. 9A & 9B where the RBE is plotted as a function of the absorbed dose. For both the *in vivo* and *in vitro*

models, the RBE is greatest in the low-dose region and substantially smaller (4-6 times) in the high-dose region. Therefore, caution is needed when interpreting reported RBE values for incorporated Auger emitters.

TABLE I

RBE Values Obtained with Spermhead Survival Assay

Radiochemical	*In vivo* Subcellular Distribution	RBE Spermhead Survival
120 kVp X rays	-	-
^{7}Be chloride (9)	46% Cy, 54% N	1.0
H^{131}IPDM (5)	100% Cy	1.1
H^{125}IPDM (5)	100% Cy	1.0
^{123}IMP	100% Cy	1.1
^{125}IdC	100% N; 100% D	8.7
^{125}IUdR (4)	100% N; 100% D	7.9
^{210}Po-citrate (4)	80% Cy, 20% N; 45% D	6.7

TABLE II

RBE Values Obtained with Clonogenic Survival of V79 Cells

Radiochemical	*In vitro* Subcellular Distribution	RBE Compared Acute ^{137}Cs γ rays	RBE Compared to chronic ^{99m}Tc γ rays
^{125}IUdR (25)	100% N; 100% D	9	20
^{125}IdC (25)	100% N; 100% D	9	20
^{210}Po-citrate (25)	72% Cy, 28% N	6	13

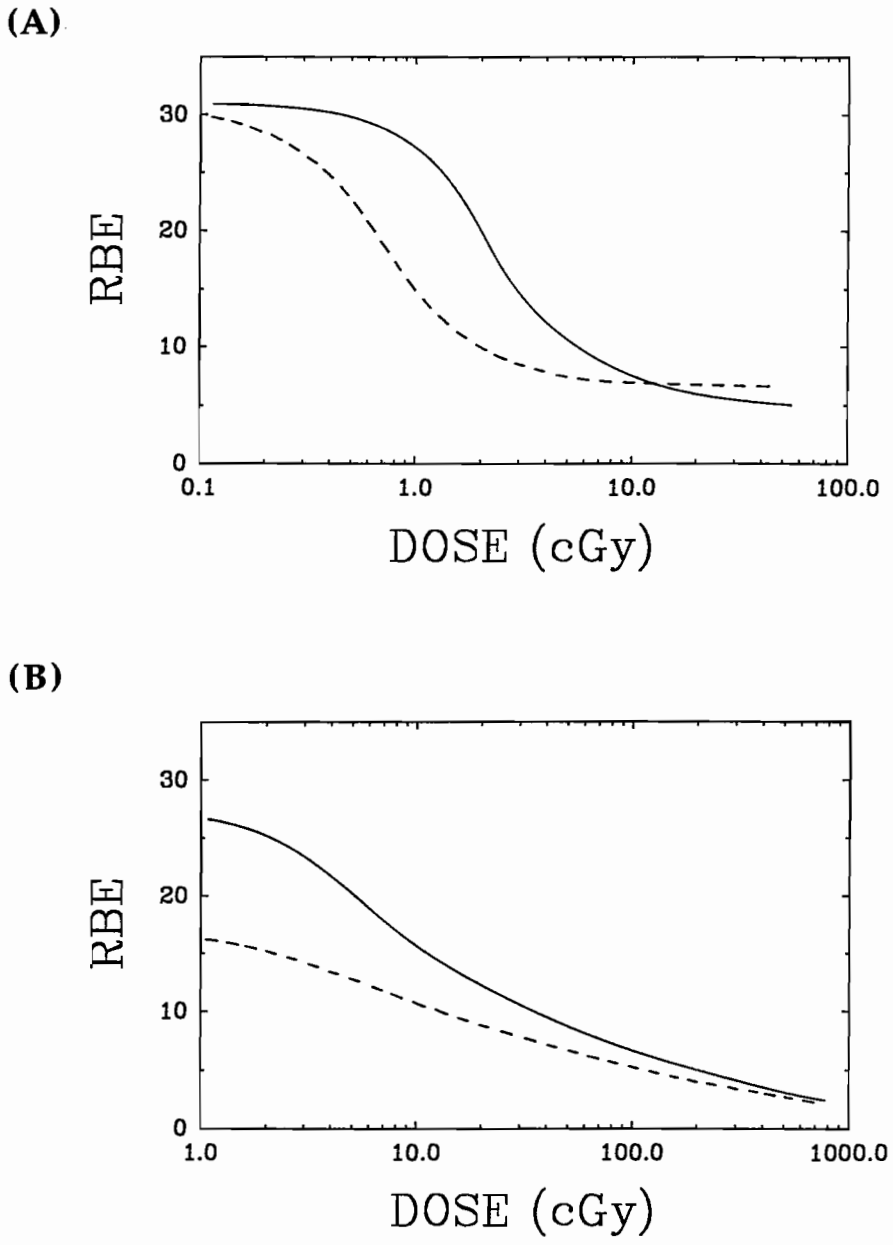

FIG. 9. Dependence of RBE on absorbed dose. The RBE for ^{210}Po-citrate (dashed line) and ^{125}IUdR (solid line) is plotted as a function of the absorbed dose for A) spermhead survival in mouse testis, and B) clonogenic survival of cultured V79 cells. For each assay and each radiochemical, the RBE is maximum (~ 20-30) at low doses and minimum (~ 5) in the high dose region.

Lethality of Auger Cascades Compared to High-LET α Particles

The data shown in Figs. 4 & 8 and Tables I & II allow a direct comparison between the lethality of incorporated high-LET α emitters and the Auger emitter ^{125}I covalently bound to the DNA in the form of ^{125}IUdR and ^{125}IdC. Both the *in vivo* and *in vitro* data indicate that ^{125}I Auger cascades are at least as lethal as 5.3 MeV α particles from ^{210}Po when compared on the basis of absorbed dose to the testis or V79 cell nucleus. This is consistent with theoretical expectations based on the very large energy densities (40-44) and number of chemical species ($OH\cdot$, H_3O^+, *etc.*) produced in the immediate vicinity of the decay site of ^{125}I in comparison with those along the track of a 5.3 MeV α particle (45).

Radiosensitivity of Different DNA Base Sites

The biological effects of Auger emitters are most severe when the radionuclide is covalently bound to the nuclear DNA (*e.g.* ^{125}IUdR) (1-5,13,46,47). However, Yasui *et al.* (10) have demonstrated that not all sites to which ^{125}IUdR binds are equally radiosensitive with some sites being over 3 times more sensitive than others. This suggests that thymine sites are not equally sensitive to radiation. One may ask, then, whether the different DNA base sites (*e.g.* adenine, thymine, guanine, cytosine) are differentially radiosensitive to Auger cascades. Inasmuch as ^{125}IUdR and ^{125}IdC covalently bind to DNA at the thymine and cytosine base sites, respectively, our experimental results for these radiochemicals (Figs. 4, 8; Tables I, II) suggest that thymine and cytosine sites are equivalent in terms of their radiosensitivity. This finding highlights the potential of Auger electron emitters to probe the radiosensitive targets in the DNA by directing them to particular sites with radiochemicals which damage only a highly localized region. This remarkable capacity for differential and site-specific damage, unattainable by conventional beams of external radiation, may have a significant impact on ultimately elucidating the radiosensitive targets in the mammalian cell.

The Quality Factor *Q* for Auger Electron Emitting Radiochemicals

The question of assigning quality factors Q (or radiation weighting factors w_R) to Auger-electron emitters is a complex issue. Unlike external beams of radiation where the variables which govern the biological response

of the system are easily controlled (*i.e.* type of radiation, dose rate, *etc.*), the effects of radiations from incorporated radionuclides are complicated by additional factors. These include the macroscopic and subcellular distribution of the radionuclide (40,41,48-52), as well as the patterns of biological clearance. These additional factors may affect the radiotoxicity of Auger emitters, and therefore must be considered when addressing the issue of quality factor and calculation of dose equivalent H. Such factors were considered by Pomplun *et al.* (11) who proposed a quality factor equal to that of high-LET radiations when "inside" the DNA. They have further suggested $Q = 1$ when the ^{125}I decay occurs "outside" the DNA. These suggestions are supported by our *in vivo* and *in vitro* data comparing the biological effects of ^{125}IUdR and ^{210}Po-citrate (Tables I and II). However, since the quality factor is intended to reflect the nature of the radiation (17), the value of Q for Auger emitters should not depend on where the decay occurs relative to the radiosensitive targets. Hence, the Q for Auger emitters should simply be equal to Q for high-LET radiations regardless of the decay site. With Q a constant, subcellular distribution of Auger emitters may in turn be built into the dose equivalent. The dose equivalent H has been defined by the ICRU (17) as $H = D\,Q\,N$, where D is the absorbed dose, and N is the product of any other factors influencing the dose equivalent. Since subcellular distribution of Auger emitters is clearly the primary determinant of their effects (5,7,9,16), the quantity H must also depend on their subcellular distribution. The data shown in Fig. 6 shows that the RBE is directly proportional to the fraction f of intracellular ^{125}I bound to DNA ($RBE = 1 + 7.4\,f$). Therefore, it is not unreasonable to suggest that H for ^{125}I, or other Auger emitters for that matter, might also be directly proportional to f. Insofar as H is linear in f, the relation $H = D\,(1 + f\,(Q - 1))$ may be a reasonable first step toward establishing meaningful radiation protection guidelines for Auger-electron emitters (25). This of course would be subject to confirmation of these conclusions with respect to experimental end points more pertinent to carcinogenesis.

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DISCUSSION

Schneiderman, M. From the kinetics of cell production can you tell if the stem cells are being affected by your ^{125}IUdR treatment?

Howell, R. W. It is unlikely that the stem cells are being affected significantly because they are 5-10 times less radiosensitive than the rapidly dividing spermatogonial cells which actively incorporate the radiochemical. Furthermore, we assay the effects on the 29th day which is well before the time necessary for stem cells to become sonication resistant spermatids.

Humm, J. L. The use of RBE with an *in vivo* study can be dangerous in particular when one injects an isotope releasing short range particles directly into the testes. Did you perform autoradiography to investigate the degree of heterogeneity in the testes, and what influence do you think any heterogeneity will have on your determination of RBE's?

Howell, R. W. This question has been addressed extensively in our manuscript. Yes, we do perform autoradiographic studies and macroscopic distribution studies, but our survival curves perhaps yield more information in this regard. Testicular injection of various "low-LET radiochemicals", including those labeled with short-range electron emitters such as ^{125}I, invariably results in a survival curve that closely matches that obtained following external irradiation with 120 kVp X rays. Therefore, any heterogeneity caused by intratesticular injection of the radiochemicals does not lead to any appreciable effect on the shape of the survival curve and correspondingly no effect on the RBE.

Adelstein, S. J. For radiation protection purposes, it is the RBE for carcinogenesis that is most important, hence, one should look at such biological end points as transformations, mutagenesis, in defining the value of Q or, more lately, w_R.

Howell, R. W. I agree. Because of this, we took care not to specifically assign a Q value for Auger emitters, although we did suggest that a value of the order of that for α emitters may be appropriate based on the survival data. It also remains to be seen if a linear relationship between RBE and fraction of DNA-bound activity (Auger emitter) holds true when transformations and mutations are taken as the biological end points.