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Differential Effect of i.p.- and i.v.-injected Adriamycin on the Clonogenic Spermatogonia of Murine Testis*

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Abstract—*The response of murine clonogenic spermatogonia has been measured after intraperitoneal or intravenous injections of adriamycin (ADR). When measured over the whole testis, the survival curve for regenerating tubules after i.p. ADR had a* 10-15% *lower* LD_{37} *dose (that dose which sterilises* 37% *of tubules) but a two-fold higher average* D_0 *for the clonogenic cells than that for i.v. ADR. Survival was then measured in concentric zones of these equatorial sections of testis. For i.v. injection survival parameters varied little between zones in the centre of the testis and zones adjoining the testicular capsule. In contrast, for i.p. ADR central zones had a* 40% *higher* LD_{37} *but the same* D_0 *as for i.v. ADR, while peripheral zones had* LD_{37}*s as little as* 35% *of the i.v. figure and mean* D_0*s* 2-3 *times higher. Pharmacokinetic considerations that might lead to these differences in clonogenic response are discussed.*

INTRODUCTION

FOR A number of normal tissues, *in vivo* and *in situ* assays have been developed that measure the clonogenic response of cells or assemblages of cells to cytocidal agents [1]. In these assays a range of doses may be administered and hence dose-survival curves may be constructed from which threshold dose and slope can be calculated. These assays were developed to measure the response to radiation. Increasingly, they are also being employed to measure the response to cytotoxic drugs, e.g. for primitive cells in bone marrow [2], intestinal mucosa [3] and the spermatogenic element of the testis [4]. In all of these examples the independent variable of the dose-response curves has been the *administered* dose of drug. It is assumed implicitly that these graded, injected doses bear some constant relationship to the concentration-time products that, for each tissue, actually determine cytotoxicity to the cells (presumably by supposed analogy with the well-established relationships of 'exposure' dose and 'absorbed' dose of radiation). However, to an extent far greater than for radiation, the quantitative outcome of assay by cytotoxic drug is the resultant of a complex interaction between the mode of administration of the agent, its pharmacokinetics and the anatomical organisation of the tissues. We have shown that the shapes of survival curves for clonogenic cells in drug-treated intestinal mucosa and bone marrow, both individually and relative to each other, may be strongly conditioned by experimental technique [5]. We demonstrate here that similar considerations can apply in another *in situ* clonogenic assay, of the testis. The agent employed, adriamycin, was selected because it has been shown that this drug yields reproducible curves of testis clonogenic cell survival over 2-3 logs of cell depletion, when injected by the intravenous route [4].

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MATERIALS AND METHODS

Mice

First-generation hybrids of the C57BL6 × DBA2 cross ($B6D2F_1$) were used. Mice were treated at 9-10 weeks of age (mean body wt 25 g, 1 S.D. = 2 g). They were housed under a light regimen of 12 hr dark (1800-0600 hr), 12 hr light, and were given tap water and food *ad libitum*.

Drug

Adriamycin (ADR) was kindly donated by Farmitalia U.K. (Barnet). The drug was dissolved in sterile 0.9% saline and the concentration was adjusted to give injection volumes of 0.25–0.3 ml. Dose was expressed as mg ADR/kg of individual mouse body wt. Injections were made between 1000 and 1100 hr as a single bolus given either i.p. into the right posterior quadrant of the abdomen or i.v. via the lateral vein of the tail.

Experimental

Four mice were injected per dose point for ADR doses between 5 and 9 mg/kg and six per point between 10 and 12 mg/kg. The clonogenic response of cells within spermatogenic tubules was measured by the assay of Withers *et al.* [6]. Thirty-five days after injection both testes were removed from each animal and fixed in Bouin's solution. Five-micron-thick sections were cut transversely to the long axis of the testis, at the equator. Thirty-two sections in total were taken from the four mice (eight testes) of the low-dose groups and 48 sections from the six mice of the high-dose groups. The sections were stained with haematoxylin and eosin. Tubules that were approximately circular in cross-section were examined microscopically for the presence or absence of foci of regeneration, defined as a cluster of at least three cells with densely staining, non-necrotic and non-Sertoli cell nuclei. For the present analysis each testis section was divided arbitrarily into concentric annular zones by means of an eyepiece graticule (Fig. 1). The proportion of regenerating tubules was measured separately for each zone. Also for each zone of each section, the area of one randomly selected tubule was measured using a 'MOP-Videoplan' image analyser (Reichert-Jung, Slough).

Analysis

Objective curves were fitted to the data for the proportion of regenerating tubules relative to regenerating plus ablated tubules (the 'surviving fraction', SF) after graded doses of ADR, using minimum chi-squared methods [7]. Two parameters were calculated from the fitted curves: (i) LD_{37}, the dose required to reduce the population of *tubules* to an SF of 0.63, which is also the dose that would be expected to leave on average one surviving clonogenic cell per tubule; and D_0, the inverse of the final slope of the survival curve for the clonogenic *cells*. This is the (administered) dose of drug that reduces survival of a cell population to 37% of an initial value. The calculated mean values of LD_{37} and D_0 have an associated standard error, whose relative size is a measure of the scatter of the data points around the fitted line.

RESULTS

Tubule number and area

The nine zones in cross-sections of untreated testes varied in relative area from 1 for zone 1 to 4.75 for zone 9 (Fig. 1). The average area for each tubule was constant across the nine zones (0.04044 ± 0.00007 mm²). Thus the number of tubules measured in each zone, i.e. the relative number at risk', varied as the relative area of that zone. Treatment by ADR caused gross shrinkage of the testis, which reflected a reduction in the average area of each tubule by approximately 70% (range from 0.01451 ± 0.0004 mm² at 5 mg/kg to 0.01102 ± 0.0004 mm² at 12 mg/kg). This shrinkage was centripetal and symmetric as the total numbers of tubules per testis did not alter and the relative numbers per zone remained approximately the same as in untreated testes.

Clonogenic assay

Survival curves were plotted (Fig. 2) and survival parameters calculated for individual zones. Results for i.v. ADR were very consistent between zones, with only a slight trend towards higher LD_{37} (by less than 10%) in going from zone 1 (6.0 ± 0.3 mg/kg) to zone 5 (6.6 ± 0.1 mg/kg). The D_0s were similarly invariant between zones (range 1.1 ± 0.1 to 1.3 ± 0.2 mg/kg). The results for i.p. injection were markedly different. The calculated

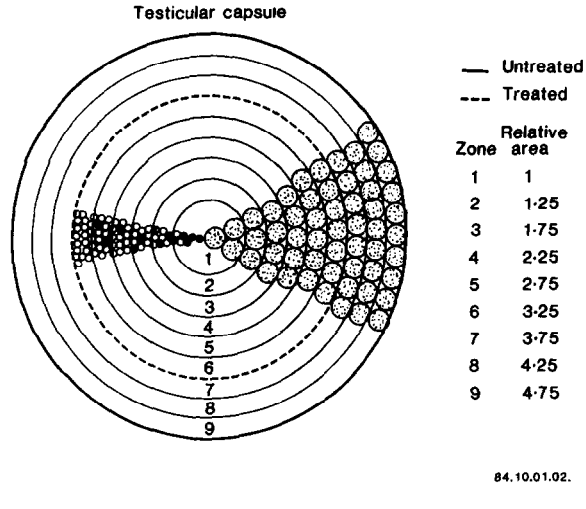

Fig. 1. A schematic illustration of equatorial cross-section of testis. The outer, heavy circle represents the perimeter of untreated testis, and the inner, dashed circle the perimeter of ADR-treated testis (shrinkage was relatively dose-independent). Light circles delimit the various concentric scoring zones. The radius of zone 1 was 0.4 mm (area 0.5 mm²), radial thickness of each of zones 2–9 0.2 mm. Large, stippled circles (right) indicate full tubules in untreated testis. Small circles (left) indicate regenerating tubules (stippled) and ablated tubules (open) in ADR-treated testis.

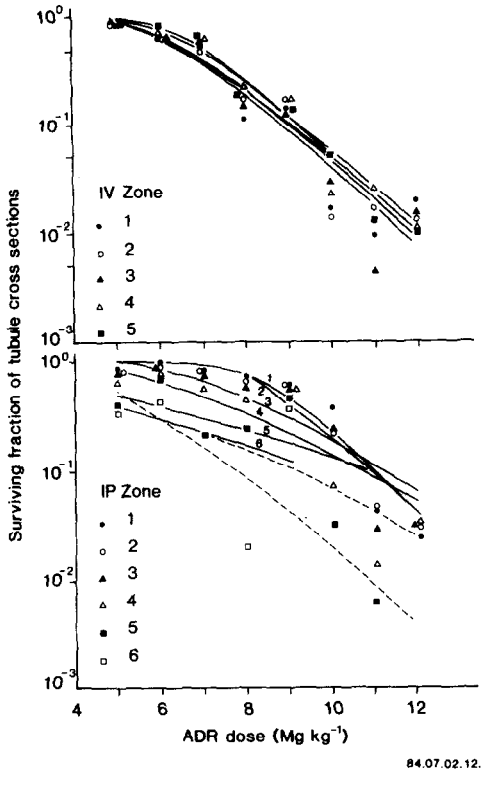

Fig. 2. Upper panel: surviving fraction of regenerating testis tubules in concentric zones of equatorial testis section after i.v. ADR. Zone 1 is central, zone 5 underlies the testicular capsule after drug treatment by this route. For the individual data points the average coefficient of variation (= 1 S.D./SF) was 37% (range 26–51%). Lower panel: surviving fraction of tubules after i.p. ADR. Solid lines are best fits to the data points shown, dashed lines are fits that include upper and lower estimates for possible surviving fractions in zone 6 after high doses of ADR (see text). The average coefficient of variation on the data points was 44% (range 27–66%).

LD_{37} fell by 65% between zone 1 (8.4 ± 1.3 mg/kg) and zone 6 (3.0 ± 3.2 mg/kg). Conversely, the average D_0 increased by a factor of 2.6 (1.1 ± 0.8 vs 3.0 mg/kg). The D_0 for zone 6 may be artificially high because at doses of 10–12 mg/kg i.p. no tubules with regenerating foci were found, and these dose-points were not included in the initial calculation of D_0. Upper estimates can be made of surviving fractions in this zone after high doses, using two sets of arbitrary assumptions: (i) that there was one surviving tubule relative to numbers actually scored; or (ii) that there was one surviving tubule relative to the maximum possible number had all the testes that were scored contained a zone 6. This calculation tends to increase the LD_{37} [(i) 4.4, (ii) 3.6 mg/kg] and decrease the D_0 [(i) 1.4, (ii) 2.4 mg/kg]. Nevertheless, the trend to lower the LD_{37} from inner to outer zones remains apparent (Fig. 2).

The data for the different zones were analysed together by obtaining a common computer fit to all the curves, to yield a survival curve for 'whole testis'. For i.v. injection the LD_{37} was 6.3 ± 0.1 mg/kg and the D_0 was 1.2 ± 0.1 mg/kg. For i.p. injection the LD_{37} was 5.4 ± 0.5 mg/kg and the D_0 was 2.5 ± 0.4 mg/kg. Greater scatter of data, and therefore larger errors on fitted values, was always seen after i.p. than i.v. injection, despite otherwise identical procedures.

DISCUSSION

The steepness of dose-survival curves in irradiated systems is almost universally described in terms of the D_0, which is measured in units of *absorbed* dose, i.e. the amount of energy imparted to unit mass of a given tissue. It has become customary to describe the steepness of dose-response curves for drugs also by D_0 (e.g. 2, 4, 5), but the units here are of *administered* dose. We have shown for ADR that this drug 'D_0' may vary markedly as a function of route of injection and anatomy of the target tissue.

Lu and Meistrich [4] reported that for i.v.-injected ADR clonogenic cells of the testis of C3H mice had a D_0 of 1.3 mg/kg, and we have inferred from their data an LD_{37} of 5.6 mg/kg. These values are similar to those in our experiments. The i.v. route appears to result in an even distribution of the cytotoxic moiety throughout the testis, as reflected by the killing of clonogenic cells (Fig. 2). For i.p. injection we propose a working hypothesis in which the drug may reach the organ by two routes: (i) 'i.v.' via the portal vessels and the liver (the major site of ADR metabolism), thence to the testis parenchyma via its blood vessels; and (ii) 'directly' from the peritoneal cavity through the inguinal canals into the scrotal sacs, and across the testicular capsule into the testis.

Levels of peak concentration of ADR may be important for its toxicity [8]. If the peak concentration in the testis of the 'i.v.' component of i.p. injection is less than that for true i.v. injection (e.g. as a result of 'first pass' clearance by the liver), but the kinetics of accumulation are first-order and the same, then in zone 1 the curve of effect vs administered dose for i.p. will be shifted to the right of that for i.v., i.e. a higher LD_{37} but not necessarily a different D_0. This component of injury will also occur in the outer zones of the testis, but here there may also be a high peak concentration from direct absorption across the testicular capsule. This would tend to reduce the LD_{37}. However, Ozols *et al.* [9] have shown that the time at which peak concentration is achieved and the rate of subsequent clearance of ADR and/or its metabolites is slower after i.p. than after i.v. injection, for liver and kidney of mice. This may result in a different *slope* of the survival curve where the independent variable is administered dose. van Putten *et al.* [2] observed just such a change in the slope of dose-survival curves for

murine marrow CFUs treated by BCNU or nitrogen mustard, injected i.p. or s.c. The i.p. route yielded D_0s for both drugs that were three times higher than for s.c. In the present experiments, where the assay was *in situ* and relatively long-term, the final SFs would be determined by the integrated dose received by the testis, i.e. the product of concentration and the natural time of availability of ADR. In the outer zones of the testis in i.p. treated mice the final SF may be the outcome of particularly complex dose-time, dose-effect relationships.

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