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Biochemical studies of the rat seminiferous epithelial wave : DNA and RNA syntheses and effects of adriamycin

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Summary. When a freshly isolated unstained rat seminiferous tubule is transilluminated under a dissection stereomicroscope, its light absorption pattern varies, depending on the characteristic positions of the acrosome and maturation phase spermatids in relation to the germinal epithelium. This variation permits individual segments of the seminiferous epithelial wave to be recognized and separated, thus opening new possibilities for morphological and biochemical studies of spermatogenesis. In this study, isolated tubular segments were incubated in the presence of radioactive nucleic acid precursors to measure the rate of DNA synthesis in different segments of the seminiferous epithelial wave and to analyze the effects of adriamycin on both DNA and RNA syntheses. Two distinct peaks of DNA synthesis were seen. The first one in segments IV to VI was found in intermediate and type B spermatogonia and represented mitotic DNA synthesis. The second peak in segments VIII to IX, principally observed in preleptotene stage primary spermatocytes, represented premeiotic DNA synthesis. Adriamycin inhibited the synthesis of both nucleic acids. Premitotic DNA synthesis seemed to be slightly more sensitive than premeiotic synthesis. The synthesis of meiotic RNA was inhibited to a degree comparable with premitotic DNA synthesis. Preleptotene and midpachytene primary spermatocytes and cells performing the 1st and 2nd meiotic divisions were those most rapidly killed after administration of adriamycin.

Introduction.

Quantitative studies of spermatogenesis are based on definitions of the seminiferous epithelial cycle stages. Two different definitions are used in studies of the rat seminiferous epithelium. Roosen-Runge and Giesel (1950) distinguished 8 stages, recognized by the characteristic location of acrosome and maturation stage spermatids in relation to the seminiferous epithelium and to the Sertoli cells. A more commonly used definition was proposed by Leblond and Clermont (1952), based on the changing morphology of the acrosomic system in young spermatids and on their nuclear morphology studied after PAS-hematoxylin staining. This technique is more suitable for cytological analysis of spermatogenesis, since it is not always easy to determine the location of late spermatids in the germinal epithelium, e.g. in electron micrographs. The semineferous epithelium of several other mammalian species, including man, has later been classified in similar fashion (Courot *et al.*, 1970). The definition of the seminiferous epithelial cycle stages, combined with the use of radioactive nucleic

acid precursors, have served as a basis of our knowledge of chromosome activity and its variation during spermatogenesis.

Monesi (1962) used tritiated thymidine and found 7 distinct periods of DNA synthesis during the seminiferous epithelial cycle in mouse, which were localized at stages VIII, X, XII, II, III, V and VII-VIII and involved type A_1, A_2, A_3, A_4, intermediate and type B spermatogonia and preleptotene primary spermatocytes, respectively. Corresponding analyses in the rat (Hilscher and Hilscher, 1969) indicated the same approximate location of DNA synthesis in relation to the epithelial cycle. RNA synthesis has also been analysed by autoradiographic means (Monesi, 1964, 1965 ; Utakoji, 1966 ; Loir, 1972). The main RNA synthesis takes place in spermatogonia, mid-pachytene spermatocytes and, to a small extent, in round spermatids.

In order to measure the stage-specific DNA and RNA synthesis rates during spermatogenesis and to quantitate the effects of noxious treatments, we have used a method for the identification and isolation of different segments of the seminiferous epithelial wave in the living unstained condition. In the rat, different cell associations of the seminiferous epithelium are arranged along the seminiferous tubules in a strictly defined numerical order (Perey *et al.*, 1961). The interval from a given stage to the next same stage of the seminiferous epithelium, including all the other stages in between, is called the seminiferous epithelial wave. When a freshly isolated rat seminiferous tubule is transilluminated in a preparation stereomicroscope, the variation of light absorption caused by the different arrangements of the acrosome and maturation stage spermatids in the seminiferous epithelium allows accurate recognition of segments VI, VII, VIII and IX. If the distances between these « marker segments » are divided by the number of segments between them, taking into account their average lengths, the others (X to XIV and I to V) can be separated with a mean accuracy of $\pm$ 1 stage (Parvinen and Vanha-Perttula, 1972). If a more accurate separation is needed, the morphology of the acrosomic system can be controlled with phase contrast microscopy (Söderström and Parvinen, 1976*a*).

Spermatogenesis is sensitive to various harmful treatments, such as heat (Vandemark and Free, 1970), ionizing radiation (Ellis, 1970) and a number of chemical agents, including the drugs used in anticancer chemotherapy (Gomes, 1970). Owing to the increasing use of these drugs, we need more knowledge of their action mechanisms as well as about their adverse effects on the testis.

In this study, we analyzed the early cell-killing effects of adriamycin, a new antitumor antibiotic. The effects of adriamycin on DNA synthesis in the various segments of the rat seminiferous tubules were also studied to find out if sensitivity varied at different stages in the synthesis of premitotic DNA and premeiotic DNA, and if adriamycin affected meiotic RNA synthesis.

Material and methods.

Experiments in vivo.

Three to 5-month old young adult rats of the Sprague-Dawley strain were used in the experiments. To determine which cells were the most adriamycin-sensitive, we experimented by lowering the dose and shortening the survival time. 1, 0.75, 0.50,

0.25 or 0.125 mg of adriamycin (Adriblastina R, Farmitalia, Milan, Italy) were dissolved in 0.1 ml of physiological saline solution and injected into the approximate center of the testis under a light ether anesthesia. The animals were killed at 24 or 12 hrs after injection by a blow on the head, and the proximal parts of the testes of 3 or 4 animals in each group were immediately fixed in Bouin fluid, embedded in paraffin, sectioned at 5 μm and stained with PAS-hematoxylin technique.

Experiments in vitro.

For measurement of DNA synthesis, seminiferous tubular segments containing the whole epithelial wave were isolated using a transillumination technique. They were preincubated for 1 hr at 32 °C in 100 μl of continuously shaken Krebs-Ringer solution containing glucose and 0, 0.2 or 2 mg/ml of adriamycin in an atmosphere containing 95 p. 100 O_2 and 5 p. 100 CO_2. After preincubation, 0.25 μCi of ^{125}I-deoxyuridine with 10^{-6} M of 5-fluoro-2'-deoxyuridine (Asantila and Toivanen, 1974) was added to the medium and incubation was continued for 2 hrs. The tubules were then washed carefully 3 times in Krebs-Ringer solution during 30 min. After recognition of the seminiferous epithelial segments by transillumination, 2 mm-long segments were then isolated using a special Petri dish with a 2 × 2 square bottom ; segment radioactivity was determined using a Wallac GTL 300-1000 gamma counter.

For measurement of RNA synthesis, the segments containing the cellular association VII and synthesizing RNA most actively were isolated by trans-illumination method and cut into pieces 2 mm long. They were preincubated as above in the presence of 0, 0.2, 2 and 20 mg/ml of adriamycin ; 10 μCi of tritiated uridine (5-^{3}H-uridine, spec. act. 29 Ci/mmole ; The Radiochemical Centre, Amersham, England) was then added and incubation was continued for 2 hrs. The radioactivity incorporated into RNA was assayed by a method described by Johansson (1975). Some of the samples from each incubation were fixed in Bouin fluid, embedded in paraffin and cut at 5 μm. For autoradiography, the slides were dipped in Kodak NTB 3 Nuclear Track emulsion and exposed for 24 hrs. They were developed in Kodak D-19 developer for 4 min at 20 °C and stained with Harris hematoxylin and eosin.

Results.

The *in vivo* experiments showed that preleptotene and mid-pachytene primary spermatocytes in stages VII to VIII (fig. 1) and dividing cells in stage XIV performing the Ist and 2nd meiotic divisions (fig. 2) were the most sensitive cell types ; they degenerated 24 hrs after administration of 0.125 mg of adriamycin or 12 hrs after administration of 0.25 mg of adriamycin. The preleptone spermatocytes often showed fusion to giant cells, pyknotic nuclei and PAS-positive cytoplasm. The pachytene spermatocytes, which did not degenerate as rapidly as preleptotene spermatocytes, showed chromatin condensation but no positive cytoplasm. Cells performing meiotic division displayed chromosomal clumping and often also presented PAS-positive cytoplasm as signs of degeneration.

DNA synthesis rate along the seminiferous epithelial wave varied considerably and had 2 distinct peaks (fig. 5). In segments I and III, the DNA synthesis rate was rela-

tively low. In the following segments IV, V and VI, a first peak was found in which synthesis rate was about twice that found in the previous stages. A low synthesis rate was again seen in the beginning of segment VII (in subsegments a and b), while the rate began to rise at the end of VII, segments VIII and IX showing the highest DNA synthesis rates. This synthesis was again at a low level during stages X, XI, XII, XIII and XIV.

When 0.2 mg/ml of adriamycin was present in the incubation medium, there was a slight decrease of DNA synthesis rate in almost all stages (fig. 5). The DNA

TABLE 1

Uptake of ^{125}IuDR *in different stages of the seminiferous epithelial wave and after incubation with different concentrations of adriamycin* (ADM)

	Control		ADM 0.2 mg/ml			ADM 2.0 mg/ml		
I	116[a] ± 13[b]	10[c]	98 ± 21	12	NS	9 ± 4	5	< 0.01[d]
II	128 ± 12	13	77 ± 11	15	NS	14 ± 4	10	< 0.15
III	121 ± 19	14	119 ± 12	15	NS	29 ± 6	5	< 0.10
IV	193 ± 23	16	174 ± 23	11	NS	17 ± 6	7	< 0.05
V	223 ± 34	16	181 ± 14	23	NS	30 ± 6	10	< 0.10
VI	208 ± 18	21	186 ± 20	20	NS	23 ± 6	12	< 0.01
VII *a-b*	70 ± 6	31	65 ± 8	25	NS	16 ± 5	16	< 0.10
VII *c-d*	128 ± 19	28	109 ± 17	24	NS	26 ± 6	17	< 0.20
VIII	353 ± 42	9	229 ± 15	12	NS	65 ± 22	8	< 0.05
IX	253 ± 32	8	246 ± 18	10	NS	91 ± 14	9	< 0.05
X	169 ± 21	7	126 ± 29	6	NS	40 ± 12	5	< 0.05
XI	98 ± 12	8	128 ± 32	6	NS	65 ± 36	3	< 0.35
XII	99 ± 20	10	58 ± 12	6	NS	38 ± 7	5	< 0.45
XIII	77 ± 7	12	42 ± 11	4	NS	38 ± 15	6	< 0.15
XIV	81 ± 11	14	55 ± 9	12	NS	17 ± 7	10	< 0.10

[a] : mean cpm value ; [b] : SE ; [c] : number of samples ; [d] : p values.

FIG. 1. — *Effects of adriamycin* (0.5 mg, 14 h) *on stage VIII.* The preleptotene spermatocytes (black arrow) and pachytene spermatocytes (white arrow) are degenerating. Magnification : 500 ×.

FIG. 2. — *Effects of adriamycin* (0.25 mg, 24 h) *in stage XIV.* Unequal spermatid nucleus size after 2nd meiotic division (white arrow) and numerous degenerating cells in meiotic division (black arrow) are seen. Magnification : 500 ×.

FIG. 3. — *Uptake of* ^{3}H-*uridine in segment VII incubated for 2 hrs without adriamycin.* The mid-pachytene spermatocytes are heavily labelled. Magnification : 500 ×.

FIG. 4. — *Uptake of* ^{3}H-*uridine by segment VII after incubation with adriamycin* (2mg/ml). Note decreased uptake of radioactivity by the pachytene spermatocytes. Magnification : 500 ×.

FIG. 5. — *Uptake of* ^{125}IuDR *in different segments of the semineferous epithelial wave and the effects of adriamycin.*

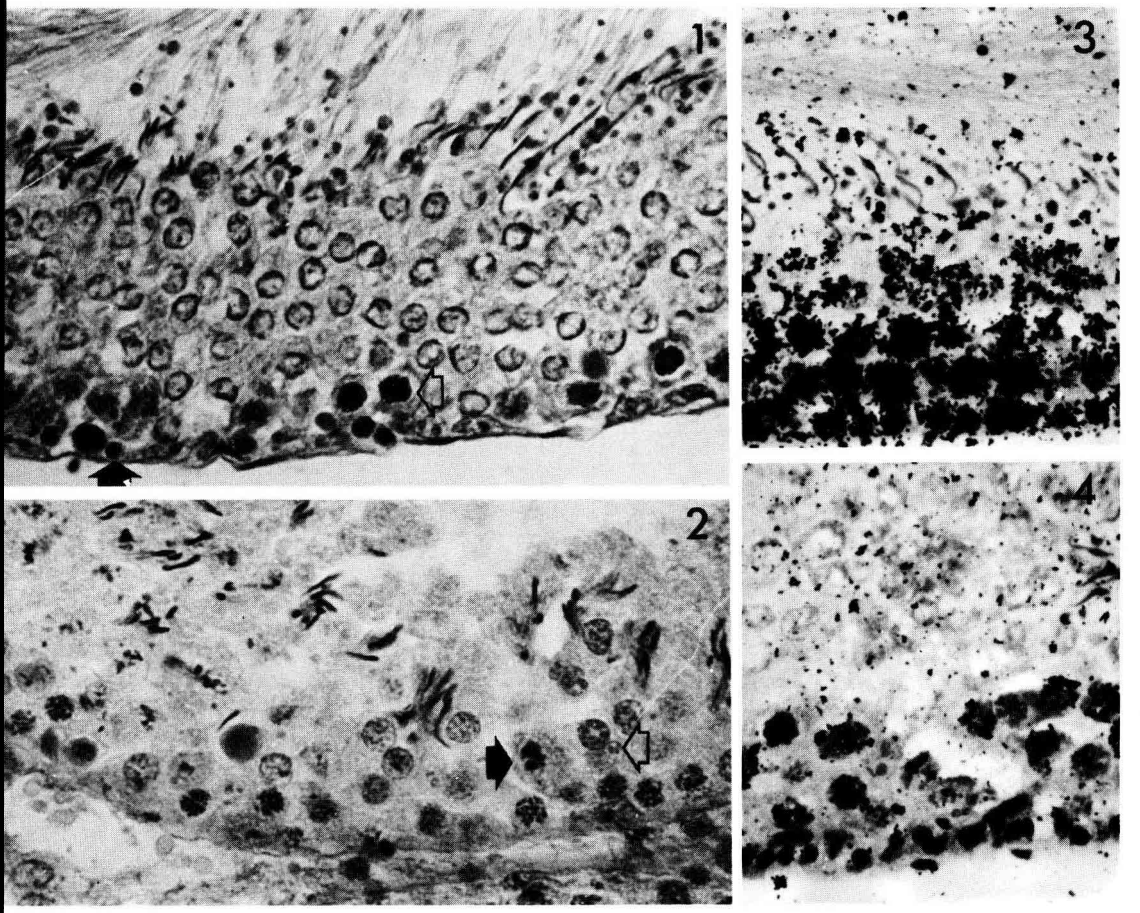

FIG. 1, 2, 3, 4.

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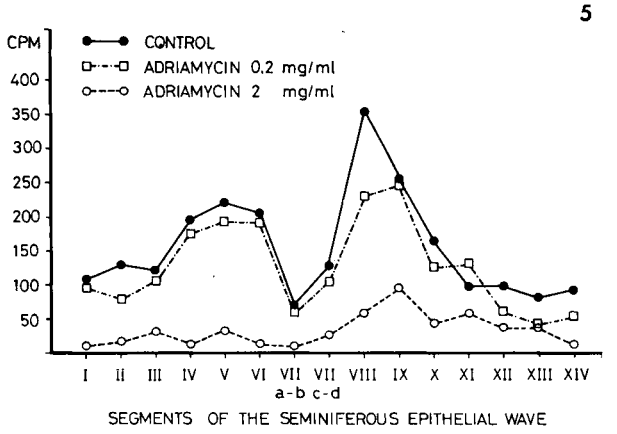

FIG. 5.

synthesis of stage VIII was affected most, as it dropped to 64.9 p. 100 ot the control, while a slight stimulation was seen in stage XI. The mean inhibition of DNA synthesis was 81.7 p. 100 of the control value mean. None of these differences, however, were statistically significant (table 1). A significant decrease of the DNA synthesis rate was observed in most segments when 2 mg/ml of adriamycin was added to the incubation medium (fig. 5). There was a highly significant difference in segments I, IV, VI, VIII, IX and X while the differences were hardly significant or entirely non-significant in segments X to XIV (table 1). The absolute radioactivity values, especially in segments VIII to IX but also in segments X to XIII, were considerably higher than in other segments after incubation with 2 mg/ml of adriamycin.

Stage VII RNA synthesis was slightly, but not significantly, inhibited by an adriamycin concentration of 0.2 mg/ml, while a concentration of 2 mg/ml of adriamycin caused the RNA synthesis rate to drop to 9.0 p. 100 of the control, and a concentration of 20 mg/ml almost totally stopped synthesis (table 2). Autoradiographic analysis also clearly showed the inhibition, although it varied in individual cells (fig. 3 and 4).

TABLE 2

Uptake of ^{3}H-uridine in the segment VII of the seminiferous epithelial wave after incubation different concentrations of adriamycin (ADM)

Control	14 561[a] ± 1 359[b]	10[c]	
ADM 0.2 mg/ml	8 816 ± 1 052	10	0.40[d]
ADM 2.0 mg/ml	1 317 ± 140	10	< 0.01
ADM 20.0 mg/ml	158 ± 50	10	< 0.01

[a] : mean cpm value ; [b] : SE ; [c] : number of the samples ; [d] : p values.

Discussion.

The distribution of the DNA synthesis rate along the seminiferous epithelial wave reflects the relative number of cells in S-phase in various generations of spermatogonia and preleptotene primary spermatocytes. The renewal of the spermatogonial stem cells has been a subject of intensive research in recent years, partly owing to the great radiobiological interest of this phenomenon. Spermatogonia involved in the cell cycle belong to the most radio-sensitive cells of the organism (Ellis, 1970). Radioautography with tritiated thymidine has been the most commonly used method (Monesi 1962 ; de Rooij and Kramer, 1968 ; Hilscher *et al.*, 1969 ; Oakberg, 1971 ; Huckins, 1971). The mitotic peaks are accurately localized in given cell associations in the rat, as well as in other mammals thus far studied. In the rat, the duration of the S-phase of the spermatogonia is 10.5 to 25.5 hrs, while the duration of the subsequent G_2-phase varies from 5.5 hrs (type B spermatogonia) to 11.0 hrs (type A spermatogonia) (Hilscher and Hilscher, 1969). The rise of the DNA synthesis rate in stage IV thus reflects the S-phase of the intermediate type spermatogonia, which divide to produce type B spermatogonia. The S-phase of type B spermatogonia lasts 25.5 hrs (Hilscher and Hils-

cher, 1969), and this may in part also extend to stage V which has a high DNA synthesis rate too. The type B spermatogonia divide mitotically in stage VI to produce the preleptotene primary spermatocytes. The DNA synthesis rates observed in this study also express the relative numbers of spermatogonia present in each stage. Although spermatogonia in stages XII and XIV also show mitotic peaks (A_2, A_3, respectively ; Huckins, 1971), the number of spermatogonia does not increase very much, presumably because of their extensive degeneration (Clermont, 1962) as compared to A_4, In and type B spermatogonia. While only 4 to 5 cells/frame of type A_1 to A_3 spermatogonia can be calculated, the corresponding number of In spermatogonia is 15 and that of B spermatogonia 30 (Huckins, 1971). Being the starting point of spermatogenesis (Monesi, 1962 ; Huckins, 1971), the mitotic activity of A_1 spermatogonia in segment IX is masked in our study by the active DNA synthesis of preleptotene spermatocytes occurring in the same segments. There is also some DNA synthesis during the meiotic prophase (Hotta *et al.*, 1966 ; Kofman-Alfaro and Chandley, 1970) showing a maximum in pachytene spermatocytes in segments VII, VIII, IX (Söderström and Parvinen, 1976*b*). However, in relation to the premeiotic DNA synthesis of the preleptotene spermatocytes, this rate of synthesis is negligible and apparently has only a small influence on total DNA synthesis rate in these segments.

Adriamycin, a new antitumor antibiotic used increasingly in the treatment of different types of malignant tumors, is known to have a primary action on DNA replication and transcription. Its interaction with DNA may be described as an insertion of the planar tetracyclic chromophore between adjacent nucleotide pairs (Di Marco *et al.*, 1971 ; Zunino *et al.*, 1972, 1975, 1977). The incorporation of nucleic acid precursors into DNA and RNA are inhibited by this drug, and it has been shown to inhibit mitosis and induce chromosome aberrations and tumors *in vivo* (Vig, 1971, 1977 ; Newsome and Littlefield, 1975 ; Marquardt *et al.*, 1976). In small doses, adriamycin has been demonstrated to have an effect on mitosis and when given in larger amounts, on DNA and RNA (Tobey, 1972). Cycling cells have been shown to be far more sensitive to the effects of adriamycin than non-cycling cells (Tobey *et al.*, 1976). When DNA and RNA syntheses are compared in different experimental conditions, adriamycin was shown to have varying inhibitory effects. In L 1210 cells, adriamycin inhibited DNA and RNA syntheses to the same extent (Meriwether and Bachur, 1972), while DNA synthesis of the HeLa cells was inhibited more (Kim and Kim, 1972). Although the effects of adriamycin on human and other mammalian chromosomes have been studied (Vig, 1977), there are no earlier reports on the effects of adriamycin on mammalian spermatogenesis.

Preleptotene spermatocytes and the cells performing meiotic division seemed to be the most sensitive cell types. Mid-pachytene spermatocytes synthesizing RNA were not killed as rapidly, although adriamycin clearly affected their RNA synthesis. Premeiotic DNA synthesis seemed to be less sensitive to the action of adriamycin than premitotic synthesis, thus suggesting the selective action of adriamycin in the DNA replication of different cell types. This is also supported by the observation that DNA synthesis in segments XIV, I, III (A_4 spermatogonia) was inhibited more than in segments XI to XII (A_2 spermatogonia). More detailed studies are needed to determine the deleterious effects of mutagenic drugs on germ cells. The quantitative determina-

tion of the effects of these drugs on DNA and RNA syntheses in various stages of germ cell development may be used in toxicological analysis as well as in morphological analysis of mitotic and meiotic chromosomes.

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Résumé. En éclairant sous un stéréomicroscope à dissection un fragment de tube séminifère fraîchement isolé, on observe une variation d'absorption de la lumière le long du tube. Elle est due aux changements de l'acrosome et à la maturation des spermatides. Cette technique permet de reconnaître les segments occupés par différents stades du cycle de l'épithélium séminifère. On peut donc les séparer. Cela ouvre de nouvelles possibilités pour des études morphologiques et biochimiques de la spermatogenèse. Dans ce travail on a incubé des segments ainsi isolés de tube séminifère en présence de précurseurs radioactifs des acides nucléiques pour mesurer la synthèse d'ADN dans les différents stades du cycle de l'épithélium séminifère et pour analyser les effets de l'adriamycine sur la synthèse d'ADN et d'ARN. On a observé deux pics de synthèse d'ADN : le premier pendant les stades IV à VI, représente la synthèse prémitotique d'ADN des spermatogonies intermédiaires et B ; le deuxième, durant les stades VIII à IX est lié principalement à la synthèse préméiotique d'ADN des spermatocytes au stade préleptotène. L'adriamycine inhibe les synthèses d'ADN et d'ARN. La synthèse prémitotique d'ADN semble plus sensible à cet inhibiteur que la préméiotique. La synthèse d'ARN préméiotique est inhibée à un degré comparable à celle de l'ADN prémitotique. Les spermatocytes primaires aux stades préleptotène, mi-pachytène, ainsi que les spermatocytes en première ou seconde division sont tués très rapidement après l'injection d'adriamycine.

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