The synthetic activity of primordial germ cells in normal and irradiated neonatal male rats

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INTRODUCTION

Resting primordial germ cells or gonocytes, present in the testis of the rat at birth (Beaumont & Mandl, 1963), are highly radiosensitive. A dose of 50–100 r X-rays induces complete, or almost complete, sterility, as judged by the histological appearance of the testis at 25 days post partum (Mandl et al. 1964). Studies of short-term post-irradiation changes have revealed that gonocytes, exposed to a sterilizing dose of X-rays at birth, do not degenerate immediately after exposure but differentiate normally into transitional cells (the immediate precursors of definitive germ cells; Beaumont & Mandl, 1963; Huckins, 1963; Franchi & Mandl, 1964) so that no histological abnormalities are detectable for 5 or 6 days. Subsequently, however, the irradiated transitional cells fail to divide; they increase markedly in size and form irregularly shaped giant cells which eventually become pyknotic (Franchi & Mandl, 1966; see also Sapsford, 1965a). Thus by 8 days post partum (p.p.) a number of changes are apparent in the testes of irradiated animals. At this time normal animals contain transitional cells (some of which are dividing) and spermatogonia type-A, whereas the gonads of treated specimens are populated by gonocytes, giant transitional cells and pyknotic germ cells.

These observations suggest that irradiation blocks the mitotic division of transitional cells, thus preventing the formation of spermatogonia type-A, without inhibiting the synthesis of cellular constituents (see Franchi & Mandl, 1966).

The present study was undertaken in order to examine and compare, by means of autoradiography, some of the metabolic processes of the nuclei of gonocytes and transitional cells in the testes of normal and irradiated rats aged 2–8 days p.p. The experiment was designed to determine whether the formation of giant transitional cells was correlated with changes in the pattern of synthesis of DNA, RNA and nucleoprotein.

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MATERIALS AND METHODS

Animals

One hundred and twenty-eight new-born male rats derived from fifty-seven litters were used; all belonged to the Birmingham strain.

Experimental design and procedure

On the day of birth males in each litter were grouped in pairs and the testes of one animal were exposed to 100 r X-rays, the other animal serving as a control. The techniques used for irradiation were the same as those described by Franchi & Mandl (1966). Subsequently both animals of each pair were injected at 2, 4, 5, 6 or 8 days after birth with equal doses of either tritiated [\(^{3}\)H]thymidine, uridine or phenylalanine, precursors of DNA, RNA and protein respectively. The specific activity of each precursor was 1 c/ml. (i.e. 20-7 mC/mg for thymidine, 20-5 mC/mg for uridine, and 8-5 mC/mg for phenylalanine). Compounds were administered by intraperitoneal injection. Based on results from preliminary trials, the amounts of each compound injected at each time are shown in Table 1.

Table 1. Doses of isotope used in the definitive experiment
(Figures in parentheses show number of pairs of animals used in the quantitative analysis)

<table>
<thead>
<tr>
<th>Age at injection (days p.p.)...</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{3})H-thymidine ((\mu)c)</td>
<td>20 (2)</td>
<td>30 (3)</td>
<td>40 (2)</td>
<td>40 (3)</td>
<td>50 (1)*</td>
</tr>
<tr>
<td>(^{3})H-uridine ((\mu)c)</td>
<td>50 (1)</td>
<td>60 (1)</td>
<td>70 (1)</td>
<td>80 (1)</td>
<td>80 (1)</td>
</tr>
<tr>
<td>(^{3})H-phenylalanine ((\mu)c)</td>
<td>20 (3)</td>
<td>40 (2)</td>
<td>50 (3)</td>
<td>60 (2)</td>
<td>70 (2)</td>
</tr>
</tbody>
</table>

* Three animals (one control, two irradiated) derived from one litter.

Techniques for histology and autoradiography

All animals were decapitated exactly 1 h after injection, and their testes were rapidly removed. To minimize variation, due to histological technique, between control and irradiated specimens, the gonads of each pair were always processed together. Specimens were fixed in Bouin’s aqueous fluid for 6–18 h, depending on size. On the day following autopsy they were embedded in paraffin wax and sections 5 \(\mu\) thick were subsequently cut from the middle part of each testis.

Slides for ordinary microscopic examination were stained with Weigert’s haematoxylin and ‘Chromotrop 2R’.

Sections for autoradiography were mounted on slides which had been thoroughly cleaned and covered with a layer of gelatin. The slides were coated with a layer of photographic emulsion (Ilford K2), using the ‘dipping’ method described by Berry & Rogers (1965). The emulsion was allowed to dry, and the slides were exposed for 14 days at 4 °C. Then they were developed in ‘Amidol’ solution, fixed, stained with Harris’s haematoxylin and mounted with DPX.
In order to reduce possible bias during the quantitative analysis each specimen carried a code number.

**Histology**

Sections were scanned using high-dry and oil-immersion objectives (magnification \(\times 640\) and \(\times 1600\)).

The size of the nuclei of fifteen primordial germ cells was measured in both irradiated and control testes of one pair of animals in each group, as described by Franchi & Mandl (1966).

**Quantitative analysis of autoradiographs**

*Standardization of observations*

Two main sources of error are inherent in a comparison of autoradiographs prepared from the testes of control and irradiated animals: (1) variations in the concentration of isotope available to the testes; and (2) differences in the level of ‘background’ grains (Ficq, 1959). An attempt was made to correct for these two factors.

(1) Since the morphology of supporting cells in the sex cords appears unchanged at 2–8 days after exposure, it seems reasonable to suppose that their synthetic activity also is little affected, and is comparable in control and irradiated litter-mates. The amount of labelled precursor in the supporting cells was therefore used as an index of the quantity of isotope available to each animal, and the number of grains over the nuclei of germ cells (indicative of the amount of isotope incorporated) was expressed as a ratio of the number over the nuclei of supporting cells.

Owing to the overlapping of supporting cells it was impossible to define the number of grains overlying individual nuclei. Thus the number of grains lying within 100 units of area was determined (a unit, \(U_1\), being approximately equal to the area of cross-section of a somatic cell nucleus).

(2) The level of background label was assessed for each specimen by counting the number of grains in 100 units of area (\(U_2\), equivalent to the area of cross-section of a germinal nucleus at 4 days \(p.p.\)) in emulsion not overlying histological material. The value obtained for specimens aged 2, 5, 6 and 8 days was adjusted to correct for the mean area of nuclear cross-section (cf. \(U_2\)). A factor for somatic cells was calculated by a similar procedure, relating the unit (\(U_1\)) used to define the number of grains over somatic nuclei and the unit (\(U_2\)) used to measure background. A correction was then applied in order to eliminate from the analysis nuclei—of both germinal and somatic cells—which had a number of grains equivalent only to the general background level (see Levi, 1964).
Measurement of the synthetic activity of germinal nuclei

Slides were viewed using an oil-immersion objective ($\times 900$) on a Leitz Laborlux microscope fitted with a Heine phase-contrast condenser. Tubules were scanned, and nuclei of gonocytes and transitional cells, judged to have been sectioned approximately through the centre, were selected using phase-contrast illumination. The lighting was then adjusted to the dark-ground position, which showed up the silver grains as bright spots against a dark field. The number of silver grains lying over the nuclei of 100 germ cells was recorded.

Two methods were used to compare the synthetic activity of germinal nuclei in control and irradiated specimens.

(1) The percentage of germinal nuclei which had incorporated radioactive precursor was determined for each specimen (a correction factor being applied to exclude those nuclei which were labelled only by background grains; see above). $\chi^2$ tests were carried out to see whether there were any significant differences in the proportion of labelled nuclei in control and irradiated testes.

(2) The concentration of isotope in labelled nuclei was estimated. The ratio (mean number of grains per labelled germinal nucleus)/(mean number of grains per somatic unit) was determined (each value being corrected by subtracting the appropriate value for background). Taking this ratio ($R$) as an index of the amount of precursor incorporated by the nuclei of germ cells in any one animal, the mean concentration of precursor in cells in irradiated animals ($RI$) was compared with that in the controls ($RC$) by taking the ratio of the two factors ($RI/RC$).

The results of a preliminary study showed that there was no significant difference between the nuclei of gonocytes and of transitional cells in any one specimen, as judged either by the percentage of each type labelled ($P > 0.8$), or by the concentration of precursor in each type of nucleus. Hence observations on the two cell types have been pooled.

RESULTS

Histological observations

The testes of normal and irradiated animals appeared similar to those described by Franchi & Mandl (1966).

The mean volume of the nuclei of germ cells was calculated for animals in each age-group (Table 2). The results show that there was very little growth of germinal nuclei in normal animals after 5 days p.p., whereas those in irradiated specimens continued to enlarge and by 8 days had reached 'giant' proportions. Estimates of the mean volume of germinal nuclei made in the present study are in close agreement with those previously reported for normal (Beaumont & Mandl, 1963) and irradiated specimens (Franchi & Mandl, 1966) of equivalent age.
Quantitative study of autoradiographs

Incorporation of $^3$H-thymidine

In the normal testis the percentage of germ cells which incorporate $^3$H-thymidine, and thus by inference synthesize DNA, rises from c. 10% at 2 and 4 days to c. 30% at 5 days and 70% at 8 days p.p. (Fig. 1). Up to 5 days there is no significant difference between control and irradiated animals in the proportion of nuclei labelled ($P > 0.3$). At 6 and 8 days, however, the percentage of nuclei incorporating $^3$H-thymidine in treated specimens is much lower than in controls ($P = 0.001–0.05$).

Table 2. Mean volume ($\mu^3$) of the nuclei of germ cells

<table>
<thead>
<tr>
<th>Age (days p.p.)</th>
<th>Control specimen ($\mu^3$)</th>
<th>Irradiated specimen ($\mu^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>4</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>5</td>
<td>670</td>
<td>650</td>
</tr>
<tr>
<td>6</td>
<td>710</td>
<td>830</td>
</tr>
<tr>
<td>8</td>
<td>720</td>
<td>1440</td>
</tr>
</tbody>
</table>

Although the relative concentration of isotope in germinal nuclei ($RI/RC$) varies widely between different pairs of animals in the 2-, 4- and 5-day groups, the results of this analysis suggest that nuclei in irradiated specimens take up more thymidine at 2 and 4 days than those in controls (Fig. 2). This trend is reversed at 5 days, and at 6 and 8 days the nuclei of irradiated germ cells con-
in consistently incorporate less precursor than those in testes which had not been exposed (Fig. 2). It seems probable that the majority of irradiated germ cells pass through only one synthetic period, and although the present observations give no information on the amount of DNA accumulated, they suggest that synthesis, although prolonged, does not continue indefinitely.

**Incorporation of $^3$H-uridine**

The pattern of incorporation of $^3$H-uridine is similar in irradiated and control specimens, although it should be noted that the quantitative assessment was limited to one pair of specimens in each age-group. In both treated and untreated testes the percentage of nuclei labelled rose from low values (5–15 %) at 2 and 4 days to a maximum of c. 75 % at 5 days, then fell to 50 % at 6 days and returned to 5–15 % at 8 days p.p. The relative concentration of the isotope in germinal nuclei appears unaffected by irradiation ($RI/RC = 1.0$ at 2 days, 0.84 at 4 days, 0.99 at 5 days and 1.06 at 6 days). At 8 days supporting cells did not incorporate uridine in either control or irradiated specimens; hence it has not been possible to derive a 'standard' for the general level of synthetic activity in these animals (see above). The ratio $RI/RC$ was calculated, however, and a value of 1.4 was obtained, suggesting a higher level of incorporation in irradiated nuclei.

**Incorporation of $^3$H-phenylalanine**

The percentage of germinal nuclei labelled with $^3$H-phenylalanine increases in the normal animal from c. 55 % at 2 days to 90 % at 8 days. The proportion of nuclei synthesizing protein in irradiated specimens shows a similar trend.
Rat primordial germ cells

(Fig. 3); at 6 and 8 days, however, a significantly higher percentage of labelled cells was recorded in these animals \( P = 0.05-0.001 \).

The concentration of isotope in the nuclei of irradiated germ cells is lower than that in controls at 2 and 4 days, but by 8 days irradiated nuclei show an increased concentration of the isotope (Fig. 4).

**DISCUSSION**

The present observations suggest that the majority of the gonocytes present in the testis of the normal rat between birth and 4 days p.p. are in the presynthetic (G1) phase of the mitotic cycle. The number of nuclei synthesizing DNA (as shown by the uptake of ³H-thymidine) increases between 5 and 8 days p.p., coinciding with the initiation of division and the differentiation of gonocytes into transitional cells. Up to 4 days p.p. very few gonocytes synthesize RNA; the proportion of cells labelled with ³H-uridine rises at 5 days but declines again, so that by 8 days the proportion of active cells is as low as on the day of birth. In contrast, a high percentage of germinal nuclei synthesize protein (i.e., incorporate ³H-phenylalanine) at all ages studied.

The formation of giant transitional cells appears to be due to the fact that synthesis continues in cells whose division has been inhibited by irradiation. The formation of giant nuclei cannot, however, be closely correlated with DNA synthesis. At 6 and 8 days p.p. when germinal nuclei in treated testes grow very
rapidly to twice the size found in normal specimens, the irradiated nuclei synthesize less DNA than the controls. On the other hand, the rapid increase in nuclear volume appears to be associated with an enhanced rate of synthesis and accumulation of nucleo-protein, and possibly (to a lesser extent) nuclear RNA. Thus it appears from the present study that the amount of protein synthesized by the nuclei of primordial germ cells is proportional to nuclear size. For example, at 8 days p.p. almost all nuclei incorporate phenylalanine, but the quantity of isotope in irradiated nuclei is greater than that in controls. The ratio $RI/RC$ is $c~1.5$ and compares very closely with the ratio (mean volume of germinal nucleus in section of irradiated testis)/(mean volume of germinal nucleus in section of control testis): $2.8/1.7 = 1.6$. (The volume of nuclear material in a section equals the mean area of cross-section of nucleus $\times$ section thickness). Thus the rate of synthesis of protein per nucleus can be said to increase, although volume for volume, control and irradiated nuclei take up equivalent amounts of the isotope.

The present findings have confirmed that the biosynthetic activities of male primordial germ cells continue for a period after exposure to 100 r on the day of birth, although mitotic activity is almost completely inhibited (Franchi & Mandl, 1966). The formation of giant cells was at first thought to be due to prolonged synthesis of DNA, so that cells in which division has been blocked become polyploid (see e.g. Sapsford, 1964; Yu & Sinclair, 1964; Franchi & Mandl, 1966). Observations using autoradiography have demonstrated that the time of onset and the initial rate of DNA synthesis are unaffected, and have supported the view that the period of synthesis may be considerably prolonged in some irradiated cells. Sapsford (1965b) was the first to provide unequivocal evidence that the giant cells formed in the testes of irradiated rats (300 r administered on day 4) accumulate abnormally high amounts of DNA. This author used a microspectrophotometric method to determine the DNA and protein content of individual nuclei. He found that at 5 days p.p. the majority of germ cells in both control and treated testes were diploid (i.e. had not entered the S phase) although some were tetraploid; by 8 days about 30% of the irradiated germ cells were octoploid and many nuclei contained a quantity of DNA intermediate between the tetraploid and octoploid amounts.

As in the present study, Sapsford (1965b) found that DNA content and nuclear size were only partially correlated; he reported that although many nuclei had an abnormally high content of DNA, this substance became progressively diluted after about 6 days p.p. by other nuclear constituents. In contrast, the results of the two studies have shown that nuclear enlargement can be directly correlated with the synthesis of nucleoprotein. It seems probable that RNA plays a similar though subsidiary role in the formation of giant cells.

The metabolic characteristics of giant primordial germ cells appear to be similar to those of giant somatic cells in tissue culture. DNA, RNA and protein have all been found to accumulate in cells blocked by irradiation from entering
Rat primordial germ cells

mitosis. DNA synthesis proceeds for many days at approximately the same rate as in normal, actively dividing cells (Sheek, Des Armier, Sagik & Magee, 1960; Dickson & Paul, 1961; Nias & Paul, 1961). Eventually, however, DNA synthesis fails to match the rate of increase in cell size and the DNA becomes progressively more dilute (Klein & Forsberg, 1954; Sheek et al. 1960). On the other hand, synthesis of RNA and protein parallels the increase in cell size, and the concentration of these substances is constant (e.g. Dickson, Paul & Davidson, 1958). It should be noted, however, that both nucleus and cytoplasm were considered in these studies.

It seems evident that the failure of gonocytes to divide, and their eventual death, is not due to a complete breakdown of synthetic processes. Franchi & Mandl (1966) reported that irradiation did not inhibit the formation of centrioles (which normally precedes the onset of mitotic prophase: Mazia, 1961), but rather prevented the cells from proceeding further into the division cycle. It is possible that irradiation during interphase damages the DNA template and thus impairs the ability of the cell to produce enzymes essential for cell division. Thus, although biosynthesis continues, the structure of at least some products may be altered (see e.g. Smith & Shore, 1966).

As Franchi & Mandl (1966) have pointed out, however, the incidence of giant cells in a population can be increased by a number of environmental changes and hence the response may be a non-specific one. This inference is supported by the finding that the response of the testis to irradiation is clearly dose-dependent. Following exposure to 19 r on the day of birth, Mandl (1966) reported that some germ cells survive to multiply and repopulate the testis, some giant cells are formed, and other germ cells degenerate in the course of a mitotic division, a type of cell death seen very infrequently after exposure to 100 r (see also Franchi & Mandl, 1966). Differences in the response of cells have been evident in the present study, and include variations in nuclear size and synthetic activity (see also Sapsford, 1965a, b), and in the time at which death occurs.

SUMMARY

1. Exposure of the testes of rats to 100 r of X-rays at birth inhibits the division of gonocytes but does not prevent them from differentiating into transitional cells. The latter, however, fail to divide to form spermatogonia type-A, but increase markedly in size so that by 8 days post partum (p.p.) the testes of irradiated animals contain numerous giant transitional cells which are destined to degenerate.

2. The metabolic activity of germ cell nuclei in normal and irradiated rats has been studied by the technique of autoradiography, using radioactive precursors of DNA, RNA and protein.

3. The results have demonstrated that synthetic processes continue in cells whose division has been blocked. The synthesis of RNA is largely unaffected by
irradiation. DNA synthesis may be prolonged after exposure, but the amount of
$^3$H-thymidine incorporated by the nucleus declines from 5 days p.p. and does
not keep pace with nuclear growth. The synthesis of nuclear protein, on the
other hand, increases from 5 days after irradiation and the quantity of phenyl-
alanine taken up by giant cells seems to be proportional to nuclear size. Thus the
accumulation of nuclear protein appears to be the main factor in nuclear en-
largement.

RESUME

Les activités de synthèse dans les gonocytes primordiaux de rats mâles
nouveau-nés normaux et irradiés.

1. L'exposition des testicules de rats à des doses de rayons X de 100 r, à la
naissance, inhibe la division des gonocytes mais ne les empêche pas de se
différencier en cellules de transition. Ces dernières, cependant, ne se divisent plus
pour former des spermatogonies de type A mais subissent un net accroissement
de taille, de telle sorte que 8 jours après la naissance (post-partum, p.p.) les
testicules des animaux irradiés contiennent de nombreuses cellules géantes de
transition qui sont destinées à dégénérer.

2. L'activité métabolique des noyaux des gonocytes chez des rats normaux et
irradiés a été étudiée par autoradiographie, à l'aide de précurseurs de l'ADN, de
l'ARN et des protéines.

3. Les résultats ont démontré que les processus de synthèse se poursuivent
dans les cellules dont la division a été bloquée. La synthèse d'ARN est peu
affectée par l'irradiation. La synthèse d'ADN peut se prolonger après l'irradia-
tion mais la quantité de thymidine-$^3$H incorporée par le noyau diminue à
partir du 5ème jour p.p. et ne va pas de pair avec la croissance nucléaire. La
synthèse de nucléoprotéines, d'autre part, augmente à partir du 5ème jour
après l'irradiation et la quantité de phénylalanine absorbée par les cellules
géantes semble être proportionnelle à la taille du noyau. Ainsi, l'accumulation de
nucléoprotéines apparaît comme le facteur principal de l'accroissement de
taille du noyau.

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Rat primordial germ cells


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