Immunohistochemical detection of the proliferative activity of spermatogonia in *Xenopus laevis* by application of bromodeoxyuridine

YOHKO ASAKAWA¹, TOHRU KOBAYASHI^{1,2} and HISAAKI IWASAWA^{1,3}

¹Biological Institute, Faculty of Science, and ²Graduate School of Science and Technology, Niigata University, Niigata 950-21, Japan

ABSTRACT

The activity of spermatogonial proliferation was examined immunohistochemically by means of the incorporation of bromodeoxyuridine (BrdU) into S-phase spermatogonia in *Xenopus laevis*. All the labelled germ cells were spermatogonia 1 h after the injection of BrdU, and the percentage of labelled cells was 20-25% in the secondary spermatogonia, irrespective of the time of the injection. Spermatogonia-supporting Sertoli cells also incorporated BrdU.

Spermatogenesis is one of the most attractive systems for the study of differentiation and morphogenesis. Concerning the proliferation of spermatogonia, the cell cycle in each class of spermatogonia has been examined in mammals (4). In anuran amphibians, on the other hand, the normal timing of these processes is hard to deduce from morphological observations, and there is little information about spermatogonial proliferation. In anurans, however, it is known that clonally derived clusters of germ cells are surrounded by Sertoli cells to form spermatogenic cysts, which are the basic morphological unit within the seminiferous tubules (11), and the progression of spermatogenesis synchronously occurs within these cysts.

In general, studies on cell proliferation have been performed by means of the autoradiographic technique with tritiated thymidine (³H]Tdr). However, this method is rather time-consuming, and it is not always easy to interpret the results, because of the lack of clear discrimination between labelled and unlabelled cells in not a few cases. Recently, as an alternative method for the labelling of dividing cells, the use of bromodeoxyuridine (BrdU), taken up and incorporated into newly synthesized DNA via the same pyrimidine salvage pathway as [³H]Tdr

(thymidine kinase), has been developed (2, 3, 5, 6, 10, 15).

In the present study, as a first step in clarifying details of spermatogonial proliferation in anurans, we used BrdU to label S-phase spermatogenic cells and investigated the activity of spermatogonial proliferation in *Xenopus laevis*.

Adult male *Xenopus laevis*, 15-17 g in body weight, were used. Prior to and during the experiment, the frogs were kept in dechlorinated tap water at 22°C on a 12L:12D cycle with the light phase from 06 00 to 18 00. The frogs were given 5-bromo-2'-deoxyuridine (Sigma), 0.05 mg/g body weight, in a single intraperitoneal injection at 00 00, 04 00, 08 00, 12 00, 16 00 or 20 00, and were killed by decapitation 1 h after the injection. Each group consisted of four frogs. The testes were excised, fixed in Bouin's fluid for 12 h at room temperature, embedded in Paraplast (Sherwood Medical, St. Louis, MO, U.S.A.), and sectioned at 4 µm. The sections were deparaffinized, stained immunohistochemically with the reagents in a Cell Proliferation Kit (Amersham), and in some cases were counterstained with Carazzi's hematoxylin. The whole staining procedure was performed at 20 ± 1°C.

After two 5-min washes in 0.02 M phosphate-buffered saline (PBS), pH 7.4, tissue sections on slides were incubated for 60 min with a solution

³To whom reprint requests should be addressed

Fig. 1 Testicular histology 1 h after a single injection of BrdU. a: Control. Carazzi's hematoxylin staining. b: Immunohistochemical detection of BrdU. Small arrows, large arrows and arrowheads indicate primary spermatogonia, cysts of secondary spermatogonia, and Sertoli cells, respectively. $\times 440$

of primary antibody, purified mouse anti-BrdU monoclonal antibody, containing a nuclease for DNA denaturation. After three 5-min washes in PBS, the secondary antibody, sheep anti-mouse immunoglobulin conjugated to peroxidase, was applied for 30 min. After being washed in PBS again (5 min, 3 times), the sections were immersed for 5 min in a staining solution of diaminobenzidine and hydrogen peroxide in Tris-buffered saline. The slides were then washed in distilled water and, in some cases, counterstained.

Spermatogonia were classified into two stages: the primary spermatogonium and secondary spermatogonium. The number of cells in each stage was scored as labelled cells or unlabelled ones in alternate cross sections of each sample. About 1,500 primary spermatogonia and about 15,000 secondary spermatogonia were counted per frog. The frequency of labelled cells, i.e., the labelling index (L.I.), for each frog was calculated by the following formula: $\text{L.I. (\%)} = \frac{\text{number of labelled cells}}{\text{total number of cells}} \times 100$. All data in each group were expressed as the mean $\pm$ SEM.

Data obtained for each time were compared by Duncan's new multiple range test following the confirmation of homogeneity between the error variance at each time by Bartlett's test.

In the testes of the initial control frogs, all spermatogenic stages were seen (Fig. 1a). By counterstaining with hematoxylin, the nuclei of cells that had incorporated BrdU were seen as amber in color, while unincorporated nuclei were blue. Germ cells labelled with BrdU were only spermatogonia throughout the day irrespective of the time of the injection. Secondary spermatogonia were seen within the cysts (Fig. 1a), and with respect to all secondary spermatogonia in any given cyst, all were labelled or unlabelled (Fig. 1b). Spermatogonia-supporting Sertoli cells also incorporated BrdU irrespective of the type of supporting spermatogonia (Fig. 1b).

The labelling indices of spermatogonia are shown in Fig. 2. The index of primary spermatogonia was always less than about 5% 1 h after the injection, whereas the index of secondary spermatogonia was always more than about 20%. In the

Fig. 2 Rates of BrdU incorporation into primary and secondary spermatogonia. The vertical bars represent the mean $\pm$ SEM.

present experiment, no significant variation was recognized in the labelling index of spermatogonia throughout the day ($P < 0.05$).

In *Xenopus laevis*, the primary spermatogonia are characterized as the largest of various germ cell generations, often in excess of $20 \mu\text{m}$ in diameter, and have a highly convoluted, euchromatic nucleus containing prominent nucleoli. The secondary spermatogonia are about $10 \mu\text{m}$ in diameter, their nuclei are spherical, and, as more divisions occur, multiple nucleoli are no longer visible. The stage of spermatogonia is also identified by the fact that each primary spermatogonium is solitary and surrounded by a somatic sustentacular cell, the Sertoli cell; whereas more than two secondary spermatogonia are seen in one cyst (8, 9, 11). In the present study, all secondary spermatogonia in any given cyst were labelled or unlabelled, and this indicates that secondary spermatogonia divide synchronously within any given cyst.

In amphibians, each seminiferous tubule contains numerous 'cysts', each of which starts out as a stem cell or a primary spermatogonium surrounded by Sertoli cells. The spermatogenic change in germ cells in each cyst progresses synchronously (11). The present study revealed that spermatogonia-supporting Sertoli cells proliferate in the spermatogenic process. Although there are a few reports dealing with the proliferation of Sertoli cells in mammals (4), there is no information on amphibians as far as we know. In the present study, the timing of proliferation of spermatogonia was not

always consistent with that of spermatogonia-supporting Sertoli cells. Because of the proliferation of Sertoli cells, it is conceivable that enlarging the size of the cyst is necessary to keep proliferating spermatogonia within the cyst.

The examination of spermatogenic stages in *Xenopus laevis* revealed no cell population morphologically comparable to the preleptotene cell type found in mammals. In an autoradiographic study, Kalt (1976) reported that the premeiotic S cell must be morphologically indistinguishable from secondary spermatogonia in *Xenopus laevis* (9). After the last mitotic division, secondary spermatogonia enter the premeiotic DNA-synthesizing period, and then differentiate into spermatocytes. The present study also provided evidence that the germ cells labelled by BrdU were spermatogonia only in *Xenopus laevis*. These findings indicate that the DNA-synthetic period during spermatogenesis is limited only to the spermatogonial stage.

In the present study, no significant variation in the labelling indices of spermatogonia was recognized throughout the day. On the other hand, Minucci *et al.* (12) did a similar study on *Rana esculenta* by means of a colchicine method, and reported that the mitotic index of the primary spermatogonia was between 2 and 11%, and a daily rhythm was recognized in the appearance of mitotic cells. Although we cannot exactly determine the cause of the difference between their results and our observation mentioned above, it is possible that the labelling index was rather low (2–5%) in the present study, so no significant statistical difference was detected. Furthermore, in *Rana esculenta*, spermatogenesis undergoes seasonal variation, and the mitotic index of primary spermatogonia also varies with the season: the peak was 11% at 20 00 and the lowest value was 2% at 05 00 in March, 7.5% at 08 00 and 3.5% at 02 00 in June, and 4% at 11 00 and 2% at 20 00 in November (12). In *Xenopus laevis*, on the other hand, the spermatogenesis does not exhibit any definite seasonal variations, at least under laboratory conditions (9). Therefore, it seems rather probable that the difference between the two species in the rate of spermatogonial proliferation is due to the difference in the species-specific testicular cycle.

Rastogi *et al.* (13) reported that two morphologically distinct primary spermatogonial cell types, a pale type and a dark type, were observed in *Rana esculenta*, but such types could not be observed in *Xenopus laevis*. In the rat, spermatogonial cells are

classified into several types: types A₀, A₁, A₂, A₃, A₄, intermediate, and B spermatogonia (4), and type A₀ spermatogonia are classified into isolated, paired, and aligned A spermatogonia (7). The type A₀ spermatogonium corresponds to the primary spermatogonium in amphibians. Furthermore, in mammals, the length of the cell cycle in each population of spermatogonia is different (7). It is very likely, therefore, that the present data for *Xenopus laevis* are due to the difference in the timing of entering into the cell cycle in each population of primary spermatogonia because the classification of populations of primary spermatogonia could not be done. In the present study, the cell population treated as secondary spermatogonia included from the two-cell stage to premeiotic S spermatogonia. Huckins (7) reported that from the type A₂ spermatogonium to the type B one, the duration of the cell cycle was the same in rats, i.e., about 42 h, but the duration of S phase was different in type A₁ and B spermatogonia, 14 and 22 h respectively. In amphibians, the duration of the premeiotic S stage was 6–7 days at 18°C in *Rana esculenta* (14) and *Xenopus laevis* (8), and 9–10 days at 16°C in *Triturus vulgaris* (1). Therefore, the fact that no significant variation in the proliferative activity of secondary spermatogonia was recognized throughout the day may be interpreted as follows: (a) the difference between the length of a day, 24 h, and the length of the cell cycle causes the time lag in timing on entering the S phase in each population of secondary spermatogonia; or (b) the difference in the length of the S phase in each population varies the probability that cells are entering the S phase at the time of treatment with BrdU.

In conclusion, we used BrdU for labelling instead of [³H]Tdr, and determined the activity of spermatogonial proliferation. The labelling index was above 20% over a 24-h period in secondary spermatogonia, and spermatogonia-supporting Sertoli cells proliferated with the proliferation of spermatogonia.

Received 1 February 1991; and accepted 24 May 1991

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