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Short communication

Effects of thymulin on *in vitro* incorporation of ^{3}H-thymidine into gonocytes of newborn rat testes

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Summary — ^{3}H-Thymidine incorporation into gonocytes was investigated by means of quantitative autoradiography in organ cultures of testes from newborn rats between day 0 (day of birth) and day 7, in the presence or absence of thymulin. The data indicated that thymulin had no effect on gonocyte incorporation on day 0 or on days 1 and 7; in contrast, thymulin had a strong effect on day 2. At day 2 the percentage of labeled gonocytes was 10-fold higher in experimental animals than in controls. Between days 2 and 6, thymulin had moderate activity. Thymulin thus appeared to have an age-dependent effect upon incorporation of ^{3}H-thymidine into germ cells. On the other hand, in spite of a high incorporation rate of ^{3}H-thymidine into gonocytes after incubation with thymulin, the number of mitoses remained low, suggesting that thymulin may affect DNA duplication in gonocytes of newborn rat testes.

gonocytes / proliferation / thymulin / newborn / male rat

Résumé — Effets de la thymuline sur l'incorporation de ^{3}H-thymidine dans les gonocytes de testicules de rat nouveau-né *in vitro*. *L'incorporation de thymidine tritiée dans les gonocytes a été étudiée dans des testicules de rats nouveau-nés âgés de 0 à 7 j (J0 = jour de la naissance) placés en culture* in vitro *dans du milieu supplémenté ou non en thymuline. La thymuline n'a pas d'effet sur l'incorporation de thymidine à J0, J1 et J7. En revanche, la thymuline stimule fortement l'incorporation à J2 ; à ce stade, le pourcentage de gonocytes marqués est 10 fois plus élevé dans les explants cultivés en présence de thymuline. Entre J2 et J6, la thymuline exerce une action modérée. Il apparaît donc que l'action de la thymuline est dépendante de l'âge de l'explant testiculaire. Par ailleurs, malgré un fort pourcentage de gonocytes marqués, le nombre des figures de mitoses est très peu élevé, ce qui suggère que la thymuline agit, pour le moins, sur la stimulation de la réplication de l'ADN dans les gonocytes de rats nouveau-nés.*

gonocyte / prolifération / thymuline / rat nouveau-né / testicule

INTRODUCTION

In developing fetal and prepubertal rat testes, germ cells pass through 2 periods of extensive mitotic activity. The initial period takes place between the 13th and 18th day *post coitum* (pc) (Beaumont and Mandl, 1963), immediately after testicular differentiation (Jost, 1972). According to previous reports, the second period begins on the 7th day *post partum* (pp) (Huckins and Clermond, 1968). Between these 2 periods, *ie* between the 19th day pc and the 3rd day pp, the number of germ cells remains practically constant before sharply decreasing on the 3rd day pp (Beaumont and Mandl, 1963; Huckins and Clermont, 1968), and then increasing again from the 7th or 9th day pp (Huckins and Clermont, 1968). These classical data on the evolution of the number of male germ cells in the rat were obtained using the following methods: i) planimetry, *ie* evaluation of the total number of germ cells (Beaumont and Mandl, 1962) using the combined techniques of Chalkley (1943) and Dornfeld *et al,* (1942); ii) counting the germ cells per cross-section of seminiferous cords (Clermont and Perey, 1957); and iii) determination of the average number of gonocytes per external sex cords (Huckins and Clermont, 1968). However, ^{3}H-thymidine incorporation into germ cells was also investigated on the assumption that incorporation was always followed by mitosis (McGuinness and Orth, 1992). In that case, labeling of germ-cell nuclei was observed as early as the 2nd day, pp.

Earlier findings had emphasized the role of the thymus–gonad axis in mammalian reproduction (Nishizuka and Sakakura, 1969). Recently, we reported that thymulin, a well-defined nonapeptide hormone produced by thymic epithelial cells (Bach *et al,* 1975), stimulated *in vitro* proliferation of gonocytes within the fetal ovary and testis of the rat during the initial intense mitotic activity occurring on the 13th day pc (Prépin, 1991; 1993). Other results indicated that the number of germ cells in the ovaries was higher in fetal rabbits hypophysectomized by decapitation than in controls (Prépin and Jost, 1991). This suggests that hypophysectomy deprives the fetuses of ACTH, thereby preventing the adrenal gland from releasing the corticosterone known to inhibit thymic development (Bearn, 1960) or blocking the *in vitro* secretion of thymulin (Prépin, 1991).

The aim of the present work was to use quantitative autoradiography to investigate the effects of thymulin on *in vitro* incorporation of tritiated thymidine into newborn rat germ cells within organ cultures of testes in the presence or absence of thymulin. The results indicated that thymulin has an age-dependent effect on ^{3}H-thymidine incorporation into gonocytes.

MATERIALS AND METHODS

Animals and culture procedure

Wistar CF rats (stock from the CNRS, France) were used. In this strain, females give birth during the night of the 21st day following mating. Males pups aged 0–7 days (date of birth = day 0) were killed by cervical dislocation at 2–3 h pm, then the testes were aseptically removed and immersed in RPMI 1640 medium (Eurobio, Paris). Four testes were used at each age and cultured in medium with or without a thymulin supplement. Each testis was then cut into fragments of approximately 1 mm^3 and maintained in culture for 24 h in 0.8 ml RPMI 2640 medium (Eurobio, Paris) containing 250 iu of benzylpenicillin/ml (Specia, France) and 100 μg streptomycinsulfate/ml (Diamant, France). The culture dishes (Falcon Plastics No 3037) were placed at 36 ± 1°C in contact with a mixure of 80% O_2 and 5% CO_2 in air. For experimental organ cultures, media were supplemented with 10 μg/ml thymulin (Bachem, Switzerland), while for controls, testis fragments were incubated in a thymulin-free medium.

^{3}H-Thymidine incorporation and autoradiography

After 20 h culture, the medium was replaced by an identical medium to which 1 μCi/ml ^{3}H-thymidine (specific radioactivity: 25 Ci/mmol) (Amersham, France) was added. After a 3-h pulse and chase in the medium enriched with unlabeled thymidine (4 nmol/ml), explants were fixed by immersion for 1 h at 4°C in 3% glutaraldehyde in 0.2 M collidine buffer, then post-fixed in 1% osmium tetroxide acid and embedded in epon. Sections (1-μm thick) were dipped in LM1 nuclear emulsion (Amersham, France). After 10 d exposure at 4°C, autoradiograms were developed in Kodak D19 B for 4 min at 18°C and fixed in 30% sodium thiosulfate for 10 min. The sections were then stained with toluidine blue. At each day between day 0 and day 7, quantitative autoradiographic analysis was performed on 500 or more gonocytes from 4 different organ cultures. The germ cells with at least 5 silver grains over the nucleus were considered to be labeled. Background noise of the order of 0.5 grains per 1 000 μm^2 was neglected. The labeling index at each time interval was expressed as the percentage of labeled nuclei. In all samples, labeled and unlabeled nuclei were identified by considering the usual cytological features of gonocytes.

Statistical significances were assessed using one-way analysis of variance.

RESULTS

Testes from pups aged 0–5 d and tissue maintained in organ culture only contained gonocytes with characteristic large, clear, round cells and several nucleoli. Sertoli cell nuclei varied in shape and had single nucleoli (fig 1). In contrast, testes from 6- and 7-day-old pups contained about 1–5% spermatogonia in the absence and in the presence of thymulin in the medium. These A-type spermatogonia were often paired and were located at the periphery of the seminiferous cords.

After ^{3}H-thymidine incorporation and whatever the age of the explants, different types of testicular cells were labeled: germ cells, Sertoli cells, and peritubular cells. Of the germ cells, only the gonocytes were labeled and mainly between days 1 and 7 pp. Nevertheless, on days 6 and 7, the few spermatogonia present were also labeled (fig 1).

In organ cultures without thymulin the percentages of labeled gonocytes increased with the age of the explants, from nearly 0% on day 0 to more than 47% on day 7.

After culture in a thymulin-containing medium, on days 0 and 1 pp, the percentages of labeled gonocytes remained low and were roughly similar to those in controls (fig 2). In contrast, on day 2 pp, the number of labeled cells was 10-fold higher in explants cultured in thymulin-containing medium than in controls (19.9 and 2%, respectively) ($P< 0.001$).

From day 3 to day 6, the percentages of labeled gonocytes remained significantly higher in explants cultured in medium supplemented with thymulin than in controls ($P < 0.001$), although the differences between experimental animals and controls were lower than on day 2. The percentage of labeled germ cells increased by 1.3 on days 3, 4, 5, by 1.17 on day 6, and by only 1.08 on day 7 (fig 2).

It must be noted that images of mitosis were rare in the seminiferous cords, in spite of the high percentages of labeled gonocytes between days 2 and 7. Thus, no gonocytes mitosis was observed up to day 6. After this time, the percentages of mitotic gonocytes were always lower than 4.3, both in controls and experimental animals.

DISCUSSION

Our results demonstrate that thymulin stimulates the incorporation of ^{3}H-thymidine into male germ cells in testicular fragments maintained in organ culture for 24 h, between days 2 and 6 pp, with a maximal effect on

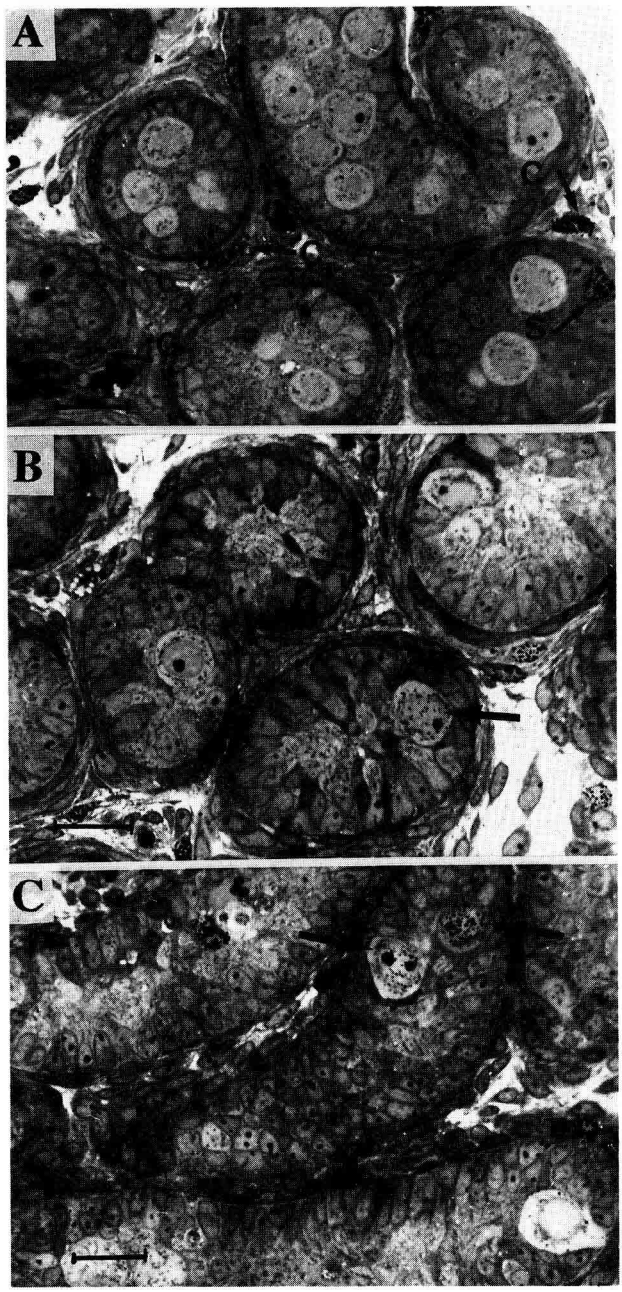

Fig 1. A Testicular explant from a 0 day-old newborn cultured in medium supplemented with thymulin. There are no labeled gonocytes. In contrast, cells located outside of the seminerous cords **(C)** and Sertoli cells are labeled. **B** and **C**: Testicular explants from a 3-day-old pups cultured in fresh medium **(B)** or in medium supplemented with thymulin **(C)**. Gonocytes (arrows) and some cells located outside of the seminiferous cords are labeled. Bar = approximately 20 μm.

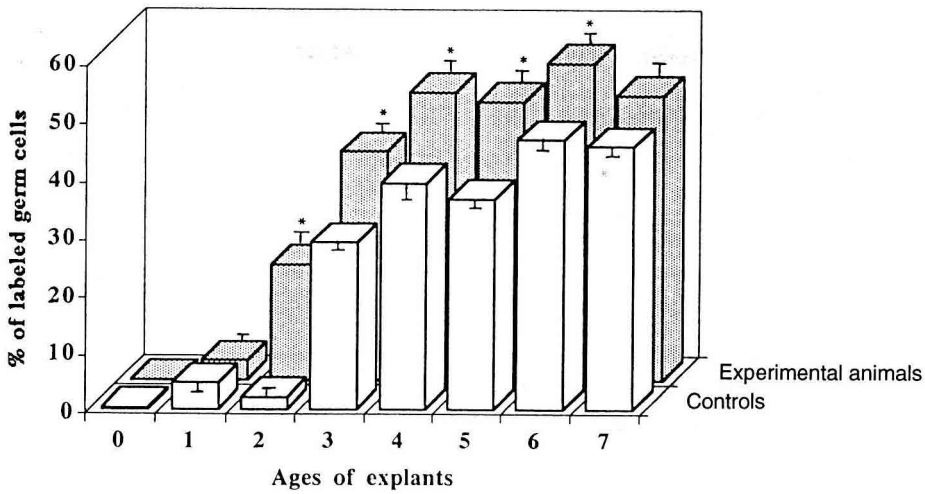

Fig 2. Comparison of percentages ± SEM of labeled germ cells after 24 h *in vitro* in experimental explants cultured in medium supplemented with thymulin and in controls, in testes explanted between day 0 (= day of birth) and day 7 of life. The effect of thymulin is non-existent or weak on days 0, 1 and 7; in contrast, the effect is strong on day 2 and the percentages increased significantly in the experimental animals between days 2 and 6 ($P < 0.001$ *vs* controls).

day 2. From day 7, however, it has no further effect on label incorporation. *In vitro* effects of thymulin therefore appear to be age-dependent. Moreover, thymulin is efficient at the beginning of the postnatal period, in addition to its capacity for stimulating *in vitro* proliferation of germ cells in fetal ovaries and testes, as revealed by previous work (Prépin, 1991; 1993). Our data are in line with other autoradiographic findings indicating that the *in vitro* incorporation of ^{3}H-thymidine into gonocytes in testes of newborn rats starts on day 2 pp without stimulation (McGuinness and Orth, 1992). The absence or the very low rate of ^{3}H-thymidine incorporation into gonocytes on days 0 and 1 is in agreement with a previous study stating that, in the rat, the number of germ cells does not increase during this period (Beaumont and Mandl, 1963). In contrast, it is intriguing to observe ^{3}H-thymidine incorporation into 45% of germ cells, even without thymulin, between days 4 and 7, whereas about 70% of germ cells disappear during that same period (Beaumont and Mandl, 1963; Franchi and Mandl, 1964; Huckins and Clermont, 1968). Furthermore, in this work, few images of mitosis were seen in seminiferous cords at the various stages. This is in agreement with earlier observations showing the occurrence of the first mitosis between days 5 and 7 (Franchi and Mandl, 1964) or only on day 7 (Huckins and Clermont, 1968).

The bromodeoxyuridine(BrdU)-labeling method, which has been shown to yield labeling indices comparable to those obtained by ^{3}H-thymidine radiolabeling, was used for measuring the proliferative activity of germ cells during prespermatogenesis in the developing testes of the golden hamster. Two hours after BrdU injection, some labeled prespermatogonia presented the morphological appearance of degeneration, indicating that apoptosis might affect pre-

spermatogonia during the terminal S-phase (Miething, 1993). Moreover, a recent study described prenatal germ-cell death occurring in the mouse as being apoptotic in nature based on flow cytometric and fluorescence microscopic investigations (Coucouvanis *et al,* 1993). Thus, the discrepancy between the high rate of ^{3}H-thymidine incorporation into gonocytes and the low rate of mitoses observed in the rat suggests that most of the gonocytes labeled from days 2 pp to day 7 pp degenerate before the M-phase or are delayed from entering the M-phase. Consequently, it can be reasonably assumed that thymulin acts by stimulating DNA duplication in gonocytes in the postnatal rat testis. Clearly, more research is needed to further explore an eventual relationship between thymulin and other factors secreted by Sertoli cells such as the Steel Factor (SLF) (Rossi *et al,* 1993), which is involved in germ-cell proliferation.

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