Growth factor and somatic cell regulation of mouse gonocyte-derived colony formation in vitro

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At birth, the mouse gonocyte does not resume mitotic activity for several days in vivo but, in an in vitro clonogenic system, cell division commences soon after culture. Somatic testis cell underlays had potent inhibitory activity on gonocyte-derived colony formation (23 ± 15% compared with 84 ± 1% in controls; *P* = 0.0001) when added to cultures of gonocytes in vitro. A Sertoli cell line, TM4B, had an even more pronounced effect on gonocyte clonogenic capacity, with 1 ± 1% compared with 72 ± 17% colony formation in controls (*P* = 0.0003). Testis cells appeared to have a direct inhibitory effect since testis-conditioned medium did not show a significant reduction in the number of colonies. The observed reduction in colony formation with the testis cell underlay was not accounted for by decreased attachment of gonocytes as simultaneous addition of a single cell suspension of testis cells was still effective in significantly reducing colony number when compared with controls (*P* = 0.01). Therefore, the observed inhibition exerted by testis cells appears to be a consequence of decreased proliferation of gonocytes. Growth factors belonging to the transforming growth factor β superfamily which are known to be expressed in testis, such as transforming growth factor β and epidermal growth factor, did not exert any inhibitory action on gonocyte-derived colony formation when added together or alone. However, a shift to a smaller colony size occurred in the presence of transforming growth factor β and transforming growth factor β plus epidermal growth factor, indicating a reduction in colony cell proliferation. Evidence for the expression of the Müllerian inhibiting substance receptor on newborn gonocytes using in situ hybridization was inconclusive. This finding was in agreement with the lack of a direct action of Müllerian inhibiting substance on the formation of gonocyte-derived colonies in vitro. Leukaemia inhibitory factor, alone or in combination with forskolin, had neither an inhibitory nor an enhancing effect on gonocyte-derived colony formation. An in vitro clonogenic method to assay for the proliferation of gonocytes in the presence of specific growth factors, cell lines, testis cell underlays and cell suspensions was used to identify a somatic cell-mediated inhibitor which may be responsible for the inhibitory action on gonocyte proliferation in vivo shortly after birth.

Introduction

In the male gonad of the developing mouse, gonocytes undergo mitotic arrest in the fetal testis at 13.5 days after mating and this is thought to be autonomously regulated. Primordial germ cells (PGCs) are the immediate precursors of gonocytes and, in the newborn mouse, resumption of mitosis in gonocytes occurs in the first few days after birth. Initiation of cell division may be regulated by autonomous events or by interaction with somatic cells of the testis. The triggers for gonocyte mitotic arrest and subsequent proliferation are not known. The onset of gonocyte proliferation is independent of interaction or contact with the basement membrane of the seminiferous tubule (McGuiness and Orth, 1992). However, co-culture experiments using gonocytes and Sertoli cells have shown that laminin-containing matrix does enhance the proliferation of gonocytes that are already cycling (Orth and McGuiness, 1991). Co-cultures have also shown the positive action of basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) on the survival of Sertoli cells and gonocytes (De Miguel et al., 1996).

The male germ cell compartment constitutes a stem cell population capable of producing cells that undergo mitosis and are able to produce germ cells that differentiate into spermatooza throughout life. Although regulation of male germ cell development is not fully understood, it is presumably controlled by an interaction of intrinsic genetic expression and regulation of signal molecules in the local testis environment. Data from studies on Caenorhabditis elegans have shown that the *glp-3* gene encodes a gene product that is required for the mitotic and meiotic cycling of germ line cells (Kadyk et al., 1997). Asymmetric division of
germ line stem cells appears to be determined by the piwi gene in Drosophila and is expressed by adjacent somatic cells but is not essential for further differentiation of the committed daughter cells (Cox et al., 1998). Piwi homologues have also been isolated in C. elegans and humans and may retain this function in diverse organisms.

Negative regulators may undergo changed expression and so be responsible for resumption of cell division in gonocytes after birth. The members of the transforming growth factor β (TGFβ) superfamily are candidate negative regulators of germ cells, and TGFβ is a potent inhibitor of PGC proliferation (Godin and Wylie, 1991) and is expressed in Sertoli and peritubular cells during spermatogenesis (Mullaney and Skinner, 1993). Mullerian inhibiting substance (MIS) is expressed by developing Sertoli cells in the embryo but whether it acts directly on PGCs or gonocytes has not yet been determined. A chalone-like inhibitor may be present in the testis (Irons and Clermont, 1979; De Rooij, 1986; De Rooij et al., 1985, 1989) which has an effect on the proliferative activity of undifferentiated spermatogonia, the immediate progeny of gonocytes. Experiments using testis extracts showed partial inhibition of undifferentiated spermatogonia both in vivo and in vitro and was apparently tissue-specific (De Rooij et al., 1989).

Leukaemia inhibitory factor (LIF) is involved in development of PGCs and also has pleiotrophic biological activities on a variety of cell types in vitro (Hilton, 1992). In the presence of bFGF and LIF, the PGCs proliferate extensively and produce pluripotent cells, termed embryonic germ cells (Matsui et al., 1992). The combination of LIF and forskolin, which acts to increase intracellular cAMP concentrations, also causes an enhancement of PGC proliferation and facilitates the propagation of embryonic germ cells (Koshimizu et al., 1996).

Experiments using isolated gonocytes have not been performed until now and an in vitro clonogenic assay for gonocytes (Hashtorpe et al., 1999) provides a new approach to the question of the regulation of mitotic arrest and the reinitiation of mitosis in gonocytes. The methodology for gonocyte-derived colony formation and the validation of this assay system have been described by Hashtorpe et al. (1999). Testis cells from newborn mice inhibited gonocyte proliferation markedly, raising the possibility of an active mechanism of cell cycle arrest in the newborn testis. The inhibitory molecules known to be present in the testis were tested to determine the identity of the proliferation inhibitor that may be responsible for holding gonocytes in growth arrest in the developing embryonic and post-birth stages.

**Materials and Methods**

**Mice**

In all experiments, the testes of HSD OLA:ICR Swiss male mice were used. The day of birth was denoted as day 1.

**Cell line underlays**

Embryonic STO (subline of SIM mouse fibroblasts) and TM4B Sertoli cell lines were cultured in Dulbecco’s modified Eagles medium (DMEM; Gibco BRL, Grand Island, NY) with 10% fetal calf serum (FCS; Trace Laboratories, Melbourne). When the cell lines were confluent, medium was aspirated and replaced with medium containing 10 μg mitomycin C ml⁻¹ (Sigma Chemical Co, St Louis, MO). The cells were incubated for 2 h, and then the medium was removed and the cells were washed three times with 5 ml PBS. The cells were then trypsinized, collected and centrifuged at 250 g for 5 min. The cells were resuspended in DMEM plus 10% FCS, and a 200 μl aliquot at a concentration of 5 × 10⁶ cells ml⁻¹ was pipetted into individual wells of a 0.1% (w/v) gelatin-coated 96-well microtitre plate. These cell underlays were incubated and used for gonocyte clonogenic assays within 4 days.

**Testis cells and underlays**

Adherent testis cell underlays were made by preparing a single cell suspension of testis in Iscove’s modified Dulbecco’s medium (IMDM) with the addition of non-essential amino acids, nucleosides (Robertson, 1985), 10% FCS and 1 × 10⁴ mol 2-mercaptopethanol l⁻¹ (Sigma). Microtitre wells were coated with collagen IV (1 μg per well; Collaborative Biomedical Products, Bedford, MA) and 1 × 10⁴ cells were plated into each well. Two days later, when confluent, the cells were treated with mitomycin C for 2 h at 37°C in the above medium. After incubation, the medium was aspirated; the cells were washed twice, and then fresh medium was added and the underlays were kept at 37°C in a humidified atmosphere until used for experiments, within 7 days.

Fresh testis cells were also plated simultaneously with individual gonocytes. In all cases, the gonocytes were transferred using micromanipulation and the testis cells depleted of germ cells were added at a concentration of 1 × 10⁵ and 1 × 10⁶ cells per well, which had been pre-coated with collagen IV. Control wells were also plated in parallel to assess any possible contamination of germ cells in the testis preparation. Testis cell-conditioned medium was prepared in the same culture medium with 2 × 10⁶ testis cells ml⁻¹ and incubated for 4 days. Conditioned medium was harvested by centrifugation at 250 g for 20 min and then used immediately and added at 50% of culture volume.

**Gonocyte clonogenic cultures**

The method used for producing gonocyte clonogenic cultures has been reported by Hashtorpe et al. (1999). In brief, newborn mouse testes from at least 15 mice were dissected and placed in cold PBS and then enzyme-treated (5 μg DNase I ml⁻¹; 100 μg hyaluronidase ml⁻¹; 100 μg trypsin ml⁻¹; 100 μg collagenase ml⁻¹) at 37°C for 20 min. A single cell suspension was prepared by first washing the testes with PBS containing DNase I and adding 1 ml culture medium, consisting of IMDM, 20% FCS and 1 × 10⁴ mol 2-mercaptopethanol l⁻¹. Then testes were gently homogenized into a cell suspension with a small Dounce homogenizer. The cells were placed on ice and allowed to settle. A 500 μl aliquot of cells was placed in 5 ml culture medium in a 60 mm Petri dish and gonocytes were picked using a glass micropipette and transferred into a second dish of medium. This micromanipulation procedure
was repeated but the individual gonocytes were placed in a microtitre well with 150 μl culture medium and incubated at 37°C in a humid atmosphere for 4-5 days. Individual wells were observed under a microscope and the presence of gonocyte-derived colonies was recorded for each well. The colonies ranged from ≥ 4 to ≥ 256 cells per colony. Results are from between three and five experiments, with at least 18-36 replicates in each experiment. Means and standard deviations were calculated and statistical significance was determined using Student’s t-test.

In situ hybridization

A cDNA probe to rat MIS-receptor was made from RNA (3 μg) extracted from the urogenital ridge of a rat 14.5 days after mating using RNAzol™B (Biotex Laboratories Inc., Houston, TX). The RNA was reverse transcribed with AMV using the Reverse Transcription System kit (Promega, Madison, WI). The cDNA was amplified by the polymerase chain reaction (PCR) using a forward (TCCGTGACATCTTTAGT) and reverse primer (CACTCTACAGGCTTTTCGTT) producing a PCR product corresponding to the sequence between the 5' position of 35 bp and the upstream 5' position of 1362 bp. Partial DNA sequencing (AmpliCycle Sequencing kit, Perkin Elmer, Branchburg, NJ) was performed to confirm that the PCR was identical to the rat MIS-receptor sequence. The fragment was cloned into a pCR-Script vector using the pCR-Script™ SK(+) Cloning kit (Stratagene, La Jolla, CA). Riboprobes were generated from linearized vector which had been cut with EcoRI or NotI to give the antisense or sense probes, respectively. A Riboprobe II Core System kit (Promega, Madison, WI) was used to synthesize the riboprobes. These riboprobes were radiolabelled by incorporation of [35S]CTP (100 μCi, at 1200 Ci mmol⁻¹; Dupont, Boston, MA) using the method described by Russo et al. (1994) and Edmondson et al. (1995).

Tissues were fixed in 10% (v/v) formalin and sections prepared on aminomethylsilane-coated slides by conventional methods. Sections were dehydrated, dehydrated, and enzyme-treated with pronase E (125 μg ml⁻¹) for 10 min at 37°C and post-fixed in 4% (w/v) paraformaldehyde/PBS for 10 min. Labelled riboprobes, at a concentration of 5 × 10⁴ c.p.m. per probe contained in 25 μl hybridization buffer with 50% (v/v) formamide per slide, were heated to 85°C and applied to slides; coverslips were added and the slides were incubated at 55°C overnight. Post-hybridization washes in 50% (v/v) formamide with 2 × SSC (sodium tri-sodium citrate) at 55°C were followed by RNase digestion for 1 h at 37°C, a wash in 2 × SSC, dehydration and air drying. Slides were dipped in LM-2 emulsion (Amersham International plc, Amersham), developed in D19 developer (Eastman Kodak Co., Rochester, NY) after 3 weeks exposure at 4°C, and stained in Mayer’s haematoxylin and mounted in DPX (BDH, Poole).

Cytokines, inhibitors and agents

Human platelet-derived TGFβ (25 μg ml⁻¹) and mouse submaxillary gland epidermal growth factor (EGF; 2 ng ml⁻¹; ICN Pharmaceuticals, Costa Mesa, CA) were added at doses recommended by the manufacturer. Recombinant human TGFβ₁ (25 ng ml⁻¹; R andD Systems, Minneapolis, MN) was also tested at the optimal dose found for PGCs (Godin and Wylie, 1991). Recombinant human MIS (a gift from P. Donahue, Massachusetts General Hospital, Boston, MA) was used at the concentration of 0.5 μg ml⁻¹ used by Zhou et al. (1993). Forskolin (10 μmol l⁻¹; Sigma) was added to microtitre wells alone or in combination with LIF (1 × 10⁴ u ml⁻¹; Amrad Pharmacia Biotech, Melbourne). Both factors were used at the optimal concentrations for PGC cultures reported by Koshimizu et al. (1996).

Results

Inhibitory effects of cell lines and testis cells

When STO cells were cultured as an adherent cell underlay, they had an inhibitory effect on colony formation by newborn gonocytes with clones in 23 ± 4% of wells compared with 80 ± 14% in controls (P = 0.0007) (Fig. 1). The TM4B Sertoli cell line underlays had a more potent inhibitory activity with only 1 ± 1% (P = 0.0003) of microtitre wells containing colonies in the presence of the underlay (Fig. 1). Testis cell underlays also showed significant suppression of gonocyte-derived colony formation (P = 0.0001) (Fig. 2). In these experiments, underlays with no addition of micromanipulated gonocytes produced a small number of gonocyte-derived colonies formed probably by gonocytes from the input cell suspension. A single cell suspension of testis cells was added simultaneously to microtitre wells with a single gonocyte to determine whether inhibition of colony formation was a consequence of decreased attachment to the vessel surface. A reduction of colony formation of 43% resulted (1 × 10⁶ cells added per well), which was similar to the degree of inhibition with 1 × 10⁵ cells added per well, and a background contribution from the added cells occurred in some experiments (Table 1). A single cell

<table>
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<th>Number of testis cells</th>
<th>Types of cell in wells and percentage colony formation</th>
<th>Percentage reduction</th>
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<tr>
<td></td>
<td>Gonocytes only</td>
<td>Gonocytes plus testis cells</td>
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<tr>
<td>1 × 10⁴ per well</td>
<td>75 ± 6</td>
<td>49 ± 22</td>
</tr>
<tr>
<td>1 × 10⁵ per well</td>
<td>91 ± 2</td>
<td>52 ± 15</td>
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*The testis cells only background colony number was subtracted from the gonocyte plus testis cell group.*

Table 1. Effect of number of testis cells on the clonogenic growth of gonocytes in mice
suspension of testis cells caused a significant reduction in colony formation when compared with controls \( (P = 0.01) \) and the inhibition observed with testis seemed to be a direct effect of decreased cell proliferation. Testis-conditioned medium (from newborn testis) did not significantly reduce colony formation \( (P = 0.10) \) when added at 20-50\% of the culture volume (Fig. 2). Some examples of gonocyte-derived colonies demonstrating a range of colony sizes are shown (Fig. 3). There was a broad spread of colony sizes in all experimental groups despite the lower cloning capacity observed in some groups.

Fig. 1. Percentage of mouse gonocyte-derived colonies formed in the presence of STO and TM4B cell line underlays compared with control cultures without underlays. Means ± SD for four experiments are shown.

Fig. 2. The effect of a testis cell underlay (TUL) and testis cell-conditioned medium (TCM) on the formation of mouse gonocyte-derived colonies is shown. Control cultures contained one gonocyte per well, the TUL group consisted of gonocytes grown in the presence of underlays and the TUL-only group did not contain gonocytes added by micromanipulation. Means ± SD for four experiments are shown.

Fig. 3. Mouse gonocyte colonies formed after 5 days of culture when one gonocyte per well was plated and a single cell suspension of testis cells was added. Colonies are shown for (a,b) a gonocyte only, and (c,d) a gonocyte with testis cells added \( (1 \times 10^4 \text{ per well}) \). Scale bars represent 50 μm.
Effect of defined factors and agents on gonocyte-derived colony formation

Environmental growth factors produced by the testis that could be responsible for the inhibition of gonocyte colony formation in vitro include the TGFβ family, as TGFβ1 is a known inhibitor of PGC proliferation. The two forms of TGFβ used were human platelet-derived and recombinant TGFβ1. Neither form of TGFβ inhibited gonocyte-derived colony formation (Fig. 4) and combining TGFβ with EGF also failed to produce a significant effect.

Although the clonogenic capacity of gonocytes was not significantly altered in the presence of TGFβ, TGFβ1 or EGF, there was some shift in the degree of proliferation of the colonies that had developed when compared with controls (Fig. 5). In all groups, the modal colony cell number was > 16–64 cells, and the other major colony size in controls was > 64–128 cells (Fig. 5d). This colony size was reduced in incidence in all growth factor groups (Fig. 5a,b,c) and was compensated for by a shift to more colonies with > 16–64 cells. There was also a slight increase in incidence of the > 4–16 colony size for TGFβ and TGFβ plus EGF groups (Fig. 5a,b), indicating that TGFβ does not effect clonogenicity but does reduce colony cell proliferation.

Müllerian inhibiting substance is a member of the TGFβ superfamily and its direct action on mouse gonocytes has not been demonstrated before. Therefore, one aim of the present study was to investigate whether the MIS receptor is expressed by gonocytes and whether gonocytes have a proliferative response to MIS in terms of either a reduction or an increase in gonocyte-derived colony formation in vitro.

In situ hybridization using rat MIS-receptor riboprobes on newborn mouse testis showed labelling of the Sertoli cells at the base of the seminiferous tubule but no conclusion could be made regarding expression of the MIS receptor on gonocytes (Fig. 6). In general, very few grains were localized to the gonocytes possibly as a result of scattering of the label from Sertoli cell-associated hybridization. Sertoli cells act as an internal control since Sertoli cell MIS receptor expression is well documented. However, responsiveness to recombinant MIS in vitro was not inhibitory and 80 ± 16% colony formation in the presence of MIS was almost equivalent to that of control cultures (73 ± 11%). MIS was also tested in combination with stem cell factor, LIF and platelet-derived growth factor BB and produced similar results to those for MIS alone and controls (data not shown).

Leukaemia inhibitory factor has both inhibitory and positive growth effects on different cell types and induces enhanced survival of PGCs. When LIF was added to gonocyte cultures, either alone or in combination with forskolin, there was no significant difference in colony formation compared with controls (Fig. 7).

Discussion

Potent inhibition of gonocyte proliferation is apparent with newborn testis underlays and with the TM4B Sertoli

![Fig. 4](https://example.com/fig4.jpg)  
**Fig. 4.** The effect of transforming growth factor beta (TGFβ; 25 μg ml⁻¹) and TGFβ1 (25 μg ml⁻¹) in combination with epidermal growth factor (EGF; 2 ng ml⁻¹) on the formation of mouse gonocyte-derived colonies. Means ± sd for three experiments are shown.
cell line. In control cultures in which gonocytes are isolated from all testis somatic cells, gonocyte proliferation appears to be unimpeded since a very high cloning efficiency of approximately 70-80% occurs in most experiments. The formation of gonocyte-derived colonies occurs by day 2 of culture (Hasthorpe et al., 1999), indicating that gonocytes are released from the effects of inhibitory agents expressed by testis somatic cells. The cell type in testis that produces an inhibitor has not been identified but is likely to be Sertoli cell-related since these make up about 90% of the cells in testis (De Miguel et al., 1996) and the TM4B Sertoli cell line exhibits strong inhibitory activity.

Partial inhibition of proliferation of undifferentiated spermatogonia has been demonstrated by various groups using testicular extracts (Clermont and Mauger, 1974; Thumann and Bustos-Obregon, 1978; Irons and Clermont, 1979; De Rooij, 1980; De Rooij et al., 1985). This inhibition was found to be tissue-specific and not species-specific but has yet to be identified. However, the inhibitory activity on undifferentiated spermatogonia has been attributed to production by intermediate and type B spermatogonia acting via a negative feedback loop (De Rooij et al., 1989). The inhibitory activity observed in the present in vitro clonogenic assay appears to have two distinctly different properties when compared with the testis extract-derived chalone. First, inhibition required the presence of whole testis cells, as testis-conditioned medium was not active. Second, in vitro clonogenic cultures contained gonocytes only and newborn testis does not contain type B or intermediate spermatogonia. The testis extract-derived chalone was produced in more differentiated germ cells than gonocytes.

The cloning of the rat MIS receptor and its expression in

![Fig. 6. In situ hybridization using rat Müllerian inhibiting substance (MIS) receptor antisense and sense riboprobes on mouse testis. (a) 1-day-old testis labelled with antisense MIS-receptor showing autoradiographic grains localized over Sertoli cell nuclei and adjacent to gonocyte nuclei (arrow indicates a gonocyte nucleus) and (b) the corresponding sense probe with relatively little labelling. (c) 7-day-old testis showing labelling of Sertoli cells with arrows indicating an unlabelled gonocyte and type A spermatogonium and (d) the corresponding sense probe. Scale bars represent 62.6 μm.](image)

![Fig. 7. The percentage of mouse gonocyte-derived colonies formed in the presence of forskolin (10 μmol l⁻¹) and leukaemia inhibitory factor (LIF; 1 × 10⁴ iu ml⁻¹). Means ± SD for three experiments are shown.](image)
Regulation of gonocyte proliferation

the gonads has been described by Baarends et al. (1994). Expression of the MIS receptor in the testis increases markedly with the onset of spermatogenesis. However, expression on neonatal male germ cells has not yet been fully elucidated and, therefore, the present study investigated this from two aspects: direct detection using in situ hybridization and the testing of responsiveness to MIS in an in vitro clonogenic assay. While Sertoli cells express the MIS receptor, labelling that was specific to gonocytes in newborn testis could not be distinguished. However, in agreement with this observation, the responsiveness of gonocyte proliferation to MIS was lacking in vitro and the results were indistinguishable from those from control cultures. Therefore, it is suggested the MIS receptor is not expressed on gonocytes and that MIS does not have a direct negative or positive proliferative effect at this stage of germ cell development.

Gonin and Wiley (1991) have shown that TGFβ is an inhibitor of PGC proliferation and may have an important role in control of germ stem cell proliferation. In rodents, TGFβ receptor expression appears to be restricted to type I and II TGFβ receptor in immature testis somatic cells, whereas the type I receptor is expressed predominantly by germ cells at the pachytene spermatocyte stage. The present findings do not show any direct inhibitory effect of TGFβ on gonocyte clonogenic capacity, although a reduction in the incidence of the > 64–128 cell colony size was observed and countered by a shift in colony size to the modal > 16–64 colony size and, to a lesser degree, the > 4–16 size. Transforming growth factor β appears to have some ability to limit the proliferation of gonocyte-derived colonies once they have been formed.

Co-culture of gonocytes and testis cells (predominantly Sertoli cells) in which structural changes and formation of gap junctions were observed have been used (Orth and Boehm, 1990; De Miguel et al. 1996) to show enhancement of Sertoli and gonocyte cell survival but could not show a proliferative effect under these conditions. These authors also demonstrated a lack of effect of LIF on gonocyte proliferation, which is in agreement with the results of Hashtorpe et al. (1999). The in vitro clonogenic method was used in the present study to assay for the proliferation of gonocytes and to determine the growth factors that regulate gonocyte proliferation and differentiation. The growth factors that have a positive effect on gonocyte proliferation in the first 5 days of culture have yet to be identified. It is likely that stem cell factor could be an important regulator of neonatal germ cell proliferation but not until after day 5 after birth (Tajima et al., 1994; Hashtorpe et al., 1999). Growth of gonocytes in vitro initially appears to be optimal in the presence of FCS and it is possible that no specific growth factors are needed. Moreover, isolation of gonocytes from testis cells results in optimal proliferation of gonocytes in culture. This observation in vitro indicates that gonocyte cell cycle regulation occurs via a negative mechanism. This negative effect mediated by cell–cell interaction may be the same regulator that induces mitotic arrest in gonocytes in the testis during early development of the embryo.

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