Stage of regeneration 30 days after amputation

	Males	Females	Males and females together
Group 1: untreated controls	4.7±2.8 (7)	6.4±2.8 (6)	5.5±2.8 (13)
Group 2: gonadectomized	4.0±3.2 (4)	3.7±2.9 (6)	3.8±2.9 (10)
Group 3: oil injected controls	8.0±0.6 (5)	3.1±2.9 (5)	5.3±3.3 (11)
Group 4: testosterone injected	5.7±1.5 (3)	5.0±1.4 (2)	5.4±1.3 (5)
Group 5: estradiol injected	4.3±2.9 (7)	6.6±2.9 (5)	5.3±3.0 (12)

Each figure is the mean regeneration stage (±SD) reached 30 days after amputation. Number of cases is given in brackets. Analysis of variance indicated no significance differences ($p > 0.05$) in the results whether the sexes were considered separately ($F = 1.74$) or together ($F = 0.60$). Brief description of regeneration stages follows[10]: stage 1, stump flattened on end; stage 2, tip of stump becomes rounded; stage 3, central protusion of blastema apparent; stage 4, blastema becomes conical; stage 5, well defined cone; stage 6, elongated cone; stage 7, cone begins to flatten dorsoventrally; stage 8, paddle stage; stage 9, blood vessels apparent; stage 10, notch seen on edge of paddle.

each group had ½ males and ½ females. Each animal was anaesthetized in 0.1% tricaine methane sulphonate and had the right forelimb amputated through the humerus. Treatments were given as follows. Group 1: no further treatment. Group 2: these were gonadectomized at time of amputation by methods previously described[8,9]. Group 3: injected i.p. 3 times weekly with 0.05 ml olive oil. Group 4: injected with 500 µg of testosterone in 0.05 ml olive oil 3 times weekly. Group 5: injected with 500 µg 17-β estradiol in 0.05 ml olive oil 3 times weekly. The animals were maintained in the laboratory as previously described[8,9].

A few limbs were fixed at intervals early during the course of regeneration, but most animals were reanaesthetized and their limbs removed and fixed in Bouin's fluid 30 days after amputation. The limbs were decalcified in 5% trichloroacetic acid for several days, embedded in paraffin, sectioned serially at 5 µm, stained, and examined histologically. The stage of regeneration was then recorded following the stage descriptions of Pritchett and Dent[10].

Results. The histological study indicated no apparent differences in regeneration among the 5 groups. Although there was considerable variation, especially in size and extent of regeneration among individual limb regenerates, there were no observable differences between the 5 groups. Further, when the stage of regeneration was compared between groups (table), there were no statistically significant differences in stage reached by 30 days whether the sexes were considered independently or together. The results then indicate no detectable difference in the rate of regeneration.

Conclusion and discussion. The sex steroids seem to have no apparent influence on limb regeneration. Injection of large non-physiological doses of testosterone or estradiol appears to have no effect on limb regeneration. On the other hand, reduction of these hormones by castration or ovariectomy also had no observable effect on limb regeneration. The possible effect at physiological levels is not known, but these results suggest that the sex steroids are not of great importance for limb regeneration. The present observations on *Notophthalmus viridescens* differ from those of Durand on *Triturus alpestris,* who reported an acceleration of regeneration by gonadectomy and an inhibition of regeneration by norethandrolone (an anabolic and androgenic steroid)[5]. Since estradiol appears to have no influence on limb regeneration in *Notophthalmus viridescens,* its accumulation in regenerating limbs[4] is puzzling.

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Intraperitoneal growth pattern of murine teratocarcinomas

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Summary. Ascitic teratocarcinomas showed a unique growth pattern, suggesting size regulation in the peritoneal cavity of syngeneic 129/Sv mice.

Ascitic teratocarcinomas are called as embryoid bodies (EBs) from the resemblance to the early mouse embryo[1]. These aggregates are composed of an outer endoderm cell layer and a core of embryonal carcinoma (EC) cells. They can differentiate to various types of tissues after attachment to the substrata in vitro and in vivo, but in suspension they are in an undifferentiated state[2]. In suspension, they can maintain their multipotency in forming EBs for many years. How do they hold the capacity of multiple differentiation for a long time? This report deals with the growth pattern of EBs in the peritoneal cavity of syngeneic mice.

Materials and methods. Murine teratocarcinomas OTT6050 were used in this study. These tumors were maintained as ascitic EBs in syngeneic 129/Sv male mice by transplantation every 3 weeks. Although this tumor line was established in 1967 and transferred to the ascitic form[3], EBs can now express multiple differentiation in vivo and in vitro. EBs were fractinated with nylon mesh according to the size.

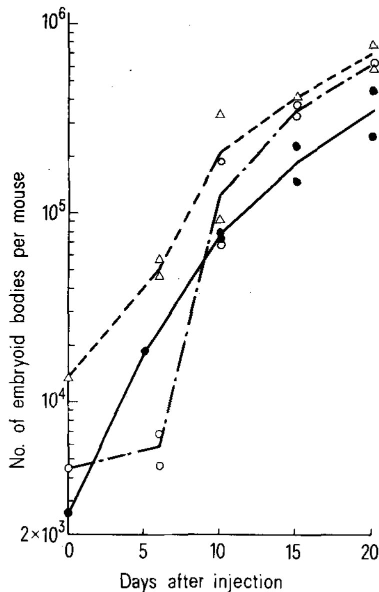

Fig. 1. Growth curves of EBs in the peritoneal cavity of syngeneic 129/Sv mice. Curves were obtained by EBs serially transplanted every 3 weeks (●——●), small EBs with a median of 40 µm in diameter (△---△) and large EBs with a median of 90 µm in diameter (○-··-○).

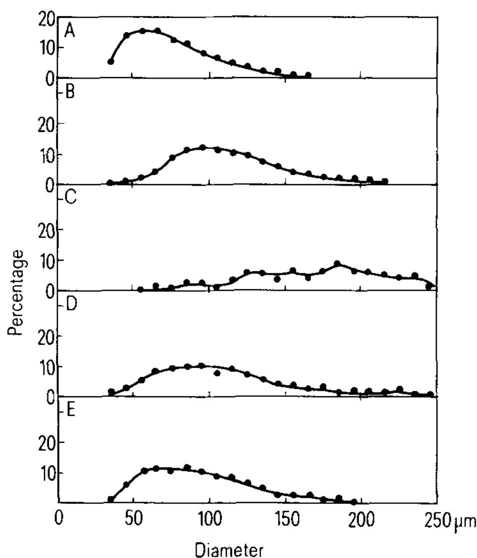

Fig. 2. I.p. growth pattern of EBs. *A* The size distribution of EBs serially transplanted every 3 weeks. *B–E* The transition of size distribution of EBs with a median of 90 µm in diameter in the peritoneal cavity of 129/SV mice; *B* 0 day, *C* 5 days, *D* 10 days and *E* 15 days. The number of EBs counted were *A* 837, *B* 419, *C* 126, *D* 501 and *E* 506.

1 fraction was obtained by EBs notfiltered through the nylon mesh of 94 µm and this population had a median of 90 µm in diameter (figure 2, B). For measuring the size of EBs, they were photographed and their diameters were measured on the photographs.

Results and discussion. EBs were injected into the peritoneal cavity of syngeneic 129/Sv mice and were taken out for counting the number of EBs at appropriate intervals. When EBs serially transplanted every 3 weeks were injected into mice, they showed a nearly constant proliferation rate (doubling time = 4.5 days at 10–20 days) from the initiation (figure 1). Small EBs which were filtrated through 40 µm nylon mesh and had a median of 40 µm in diameter, also constantly increased in number after injection. In contrast to the above 2 cases, the proliferation of large EBs, which had a median of 90 µm in diameter, showed the lag phase for about a week after injection and the constant proliferation rate was achieved in 2 weeks after injection. In the lag phase, EBs only grew and became large, thin and flat. Thereafter, the growth stopped and then giant EBs produced many pieces; the calculation according to the growth curve (figure 1) indicates that single giant EBs on average divide into about 20 smaller EBs. In vitro, cultured single EBs were reported to grow and to form often up to 30 cysts, in good agreement with our calculation[4]. Even small EBs also showed similar results, except for the timing of size reduction (figure 3, A–D).

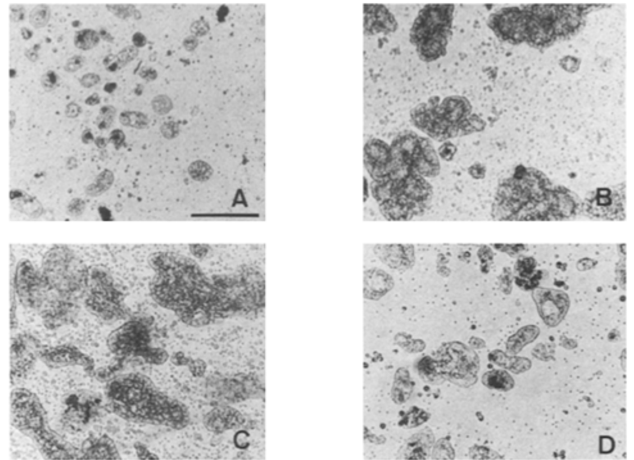

Fig. 3. I.p. growth of EBs with a median of 40 µm in diameter. *A* 0 day, *B* 5 days, *C* 10 days and *D* 15 days. Bar = 100 µm.

The size reduction of EBs possibly indicates the existence of size regulation in the growth of teratocarcinoma stem cells (EC cells). When giant EBs taken out 5 days after the initial transplantation were injected i.p. into another mouse, they did not show the same growth pattern as the initial transplantation (data not shown). These EBs from the 2nd mice had many projections indicating the local proliferation of EC cells. This observation presumably indicates the localization of EC cells in a giant embryoid body, and therefore many small EBs were produced from single EBs by fission, as previously suggested by Stevens[1].

In comparison to recent EBs, EBs soon after establishment had a different structure composed of an outer endoderm layer which enveloped ectoderm layer (see Stevens[3], figure 11–13). Undifferentiated cells become dominant in EBs in a large number of transplantations. The 2-dimensional growth of EBs to give rise to thin and flat EBs is probably essential to the maintenance of undifferentiated state for serial transplantations, because the 3-dimensional growth may induce the formation of ectoderm layer[4], and also the mesoderm formations which are found in the process of in vitro differentiation of clonal teratocarcinoma stem cells[5]. To maintain their undifferentiated state, the 2-dimensional growth of EBs observed by us seems to fit well in this situation.

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