ショウジョウバエ胚細胞初代培養におけるSex Ratio Organismの増殖

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Transient proliferation of sex ratio organisms of *Drosophila* in a primary cell culture from infected embryos

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ABSTRACT

The Sex Ratio Organism (SRO), a kind of spiroplasma parasitic to the fruit fly *Drosophila*, proliferated transiently in a primary cell culture from SRO-infected fly embryos. The density of SRO increased more than a hundred times the initial density, reaching 6×10^5 per μ 1 in 3-4 weeks. The serum concentration in the culture media was found to be critical to the SRO proliferation. SRO proliferated *in vitro* were capable of killing male embryos following their injection into female adult hosts, indicating normal SRO activity *in vivo*.

1. INTRODUCTION

The sex ratio organism (SRO) is a parasitic spiroplasma found in four neotropical species of *Drosophila; D. nebulosa, D. willistoni, D. equinoxialis,* and *D. paulistorum.* This organism proliferates in the host hemolymph and distorts the sex ratio in the progenies of the infected female by selectively killing male embryos (Williamson and Poulson 1979). Since SRO could be effectively introduced into an uninfected fly by injection of SRO-laden hemolymph, analysis of the male-killing mechanisms has been made using *D. melanogaster* carrying SRO of *D. nebulosa* or *D. willistoni.* The genetic analysis indicated that embryos with two or more X chromosomes survived whereas those with a single X were killed by SRO infection regardless of the presence of a Y chromosome and phenotypic sex (Sakaguchi and Poulson 1963; Miyamoto and Oishi 1975). Any additional fragments of X chromosome produced by segmental aneuploidy did not rescue male embryos from killing by SRO (Yamada *et al.* 1985). The cause of the death of male embryos was also studied by mosaic analysis (Tsuchiyama *et al.* 1978), and the result indicated the primary target tissues of SRO to possibly be the mesoderm and/or nervous system of the embryos. Koana and Miyake (1983) found that, in a primary cell culture from single SRO-infected male embryo, nerve cells were severely affected but mesoderm-derived muscle cells and fat body cells were relatively normal. This result support the idea that the primary target site of SRO is in nervous system, rather than in mesoderm. Koana and Miyake

(1983) also noted that SRO sensitivity of the host is dependent on the absolute number of X chromosomes per cell, rather than the X : A ratio.

Compared with analyses of the target cells of SRO, little research has been directed to the characterization of SRO and molecular analysis of SRO-embryonic cell interactions since this organism could not be propagated *in vitro* (Williamson *et al.* 1983). Recently Hackett *et al.* (1986) reported the cultivation of SRO *in vitro*. SRO from *D. willistoni* was co-cultured with a lepidopteran cell line in H-2 medium, and after 9 passages, it adapted to grow in the cell-free medium. To apply *in vitro* culture system for elucidating the mechanism of cytocidal effect of SRO, however, conditions of SRO culture with *Drosophila* cells have to be established.

The present paper describes the transient proliferation of SRO (from *D. nebulosa*) in a primary cell culture from SRO-infected *Drosophila* embryos. This culture system may help clarify interactions between SRO and *Drosophila* embryonic cells *in vitro*.

2. MATERIALS AND METHODS

Fly stock

The *Drosophila melanogaster* wild type stock Oregon-R carrying SRO derived from *D. nebulosa* (NSRO) was kindly supplied by Dr. B. Sakaguchi. NSRO was further transferred by hemolymph injection to the *D. melanogaster* mutant stock, *y v f mal*, and the embryos from this stock were used for the present experiments (for genetic nomenclature, see Lindsley and Grell 1968). The mutant stock was used because development of primary embryonic cells of this mutant in culture is particularly successful (Miyake, unpublished observation). SRO infection was stably established and males were completely eliminated in the mutant stock. The stock was maintained on a standard cornmeal-agar-sugar-yeast medium at 25°C.

Mass collection of embryos

Newly emerged females were collected from the stock and fed in new vials for over 7 days for SRO growth in hemolymph. $4\text{–}5\times10^3$ females were then crossed with $3\text{–}5\times10^2$ males from uninfected *y v f mal* stock and used for collecting eggs in an egg collection cage. Flies in the cage were allowed to lay eggs on a nylon screen (150 mesh) placed over the grape plate (Elgin and Miller 1978) and spread with yeast paste (EBIOS, Tanabe). After 4 hr, the plate carrying eggs on the screen was removed from the cage and incubated in a moist chamber for several hours to get desired staged embryos. Egg collection and incubation were carried out at 25°C.

Primary culture of embryonic cells

The method for initiating a primary culture of embryos was similar to that

described elsewhere (Miyake and Ueda 1984). Briefly, the embryos were washed off the screen with tap water, collected on another screen and rinsed with tap water. For sterilization, they were immersed in 1/10 diluted cresoli saponata solution (Japanese Pharmacopoeia) for 7 min and washed with sterilized water. The embryos were then reimmersed in 2.5% NaOCl solution for 3 min to remove chorion membranes followed by thorough washing. About 0.3 g embryos was introduced to a 5 ml Potter homogenizer with a teflon pestle (clearance, ca. 0.2 mm) and gently dissociated on ice in Cross and Sang's M3 (BF) medium (Cross and Sang 1978) supplemented with 10% heat inactivated fetal bovine serum (FBS). The antibiotics were not supplemented in the medium since SRO were highly sensitive to them (Yamada and Nawa 1975). The cell suspension was then filtered with a nylon screen (150 mesh) to remove undissociated embryos, large cell aggregates and fragments of vitellin membrane. Adjustment of cell density was made by measuring absorbance of an aliquot of suspension at 660 nm with a spectrophotometer (spectronic-20, Shimadzu-Bausch and Lomb). A660=1.0 was employed as the standard concentration empirically (at this concentration, the final cell density was roughly 10^6 per ml). The remaining cell suspension was centrifuged at 500 rpm for 3 min at 4°C and the supernatant containing yolk granules was discarded. Cells in the pellet were resuspended in the fresh culture medium with FBS at the desired concentration, inoculated into 35 mm plastic dishes (Nunclon), and incubated at 25°C in a humid atmosphere of 5% CO_2–95% air.

Titration of SRO

Six microliters of the SRO-containing culture medium were mixed with 3 μl of fixative (2% glutaraldehyde, 2% paraformaldehyde, 0.1 M cacodylate buffer, pH 6.8). Three microliters of the resulting mixture were dropped onto a siliconized slide and covered with a non-siliconized round cover slip 22 mm in diameter. The SRO were observed with a dark field microscope (Zeiss ICM 405) at $\times$1000 magnification. The concentration of SRO in the medium was calculated by counting the number of SRO in 5 randomly chosen fields. When one SRO was observed in a total of 5 random fields, the original concentration of SRO was estimated to be 6×10^3 per μ1.

Test for male-killing activity of SRO

About 0.5 μl of the culture medium containing 3×10^5 SRO per μl was injected into 1 to 2 day-old female flies. This SRO dose corresponds to about one tenth of that in the hemolymph of SRO-infected female fly. The same culture medium from which SRO had been eliminated by filtration through a membrane filter of 0.1 μm pore size, or fresh culture medium was injected as the control. Twenty to thirty flies received injections of each medium. Each injected female was crossed with 3 males, allowed to lay eggs in a food-containg vial and transferred to a new vial every 2 days. The number of result-

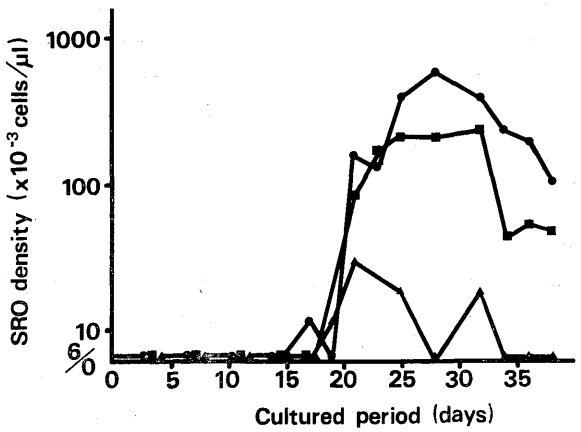

Fig. 1. The effect of serum concentration on SRO proliferation in the primary culture of SRO-infected embryos. Cells from dissociated embryos were cultured in the medium containing 10% (▲), 20% (■), or 30% (●) FBS (Gibco). Because of the method employed for SRO enumeration (see Materials and Methods), 0 on the ordinate means a density less than 6×10^3 SRO per μ1.

ing male and female progenies from each vial (brood) was scored.

3. RESULTS

Culture conditions

SRO proliferation in the primary embryonic culture was strongly dependent on the serum concentration. Typical results on the effects of serum concentration are shown in Fig. 1. At initiation of the culture, the density of SRO was lower than 6×10^3 per μl, since no SRO could be found in 5 randomly chosen areas under the microscope (see SRO enumeration in Materials and Methods). In fact, only a few SRO were found in a thousand areas of a sample surveyed. In the medium with 20 or 30% FBS, SRO proliferation was as much as $3\text{–}6\times10^5$ cells per μl in about 4 weeks, this being more than 50–100 times the starting density. At these serum concentrations, the stages of embryos (4.5–6.5 hr), washing of embryonic cells (0–3000 rpm, 10 min), and inoculation cell density (0.5–4 times of the standard concentration) had no significant effect on SRO proliferation. Eventually, SRO proliferated in 21 out of 34 dishes (62%) at either of these concentrations. However, the results were quite different between serum lots. Of 6 lots from 4 makers, 677563 serum of Boehringer Mannheim and 29N3420 serum of Gibco showed remarkable stimulation of SRO proliferation when introduced into the culture medium at concentrations of 20 or 30%, while the other four were much less effective.

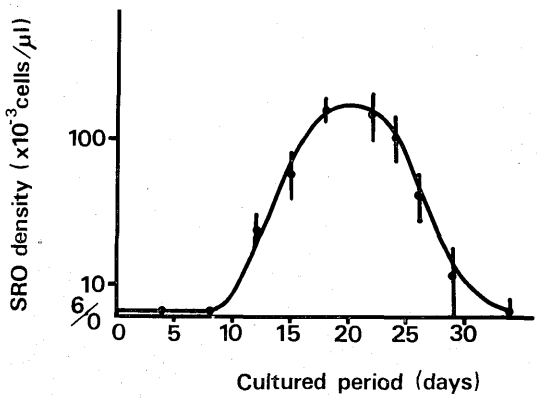

Fig. 2. SRO proliferation kinetics in the primary culture of SRO-infected embryos. Embryonic cells were cultured in the medium containing 20% FBS (Boehringer Mannhaim). Each point represents the average cell density from 4 dishes and the bar, standard error.

SRO proliferation kinetics

The growth kinetics of SRO are shown in Fig. 2. In the primary culture with 20% FBS, the number of SRO was counted every 2 or 4 days. Each point represents the average SRO density of 4 dishes and the bar indicates standard error. SRO began increasing at about 10 days, reaching a maximum (1.5×10^5 per μl) in about 20 days. This was followed by a decrease and after 40 days, SRO could no longer be seen. Change in the configuration of SRO was coordinated with their decline in density. They appeared to be bloated in width, the helical structure became loose and finally fragmented. Attempts to propagate SRO continuously proved unsuccessful. When the primary culture at about 20 days was subcultured with fresh culture medium at 1:2 to 1:4 dilution ratios, no sign of further proliferation could be observed. They fragmented and disappeared from the culture with degeneration of the embryonic cells (see below).

Evolution of the primary culture

When the primary culture was initiated from uninfected embryos, dissociated cells started to form large aggregates and several distinct cell types differentiated within a week (Koana and Miyake 1983; Shields *et al.* 1975) (Fig. 3A). Fat body cells appeared in the aggregates and muscle fibers connecting these aggregates frequently pulsated. Two types of hemocytes, macrophage-like lamellocytes and smaller roundish plasmatocytes, were spread out sporadically on the substratum. In the culture from SRO-infected embryos, cell aggregates were relatively small and muscle cell differentiation could hardly be seen, but fat body cells and hemocytes differentiated normally for the most part (Fig. 3B). The culture generally appeared to be

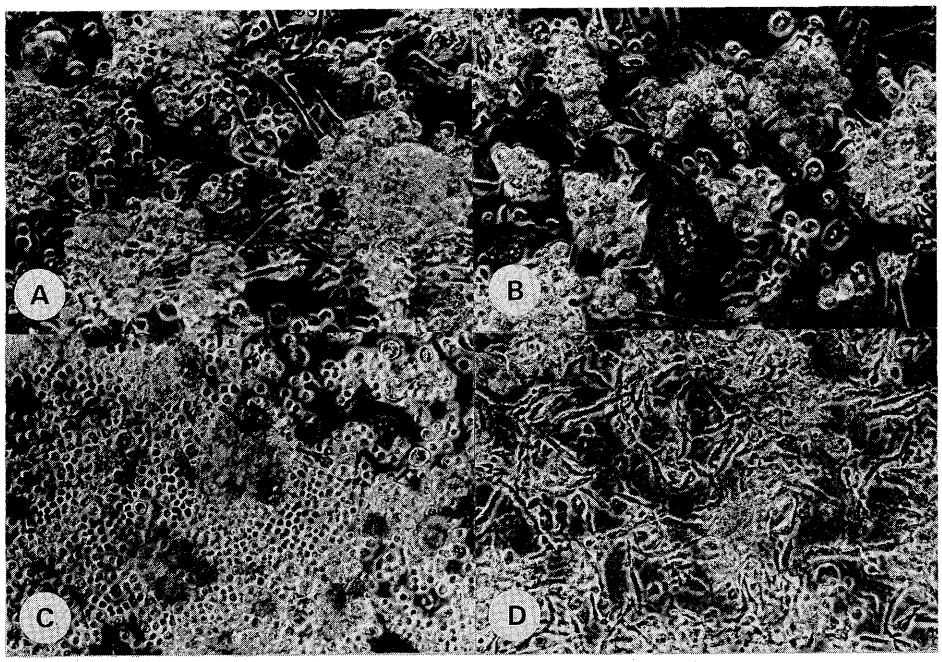

Fig. 3. Phase contrast micrographs of embryonic cells in the culture. The culture medium contains 20% FBS (Boehringer Mannhaim) ×115.
A, cells from normal embryos in 7 d culture.
B, cells from SRO-infected embryos in 7 d culture.
C, cells from normal embryos in 24 d culture (3 d after first subculture).
D, cells from SRO-infected embryos in 24 d culture (3 d after first subculture).

slightly unhealthy, judging from the presence of considerable cellular debris and elongated cells. Koana and Miyake (1983) found that many muscle cells differetiated and functioned in single embryo cultures from SRO-infected male embryos. The reason for the discrepancy between these results is not clear, but it may reflect the difference in culture conditions, e. g., a single or mass embryos cultured, serum concentration, hormone supplement, and initial cell density.

By 3 weeks, the small, round and rapidly proliferating cells appeared in the primary culture from the uninfected embryos. A sequential subculture of the cells at this stage stimulated cell division, leading to the establishment of continuous cell lines (Fig. 3C). The round cells also increased in the primary culture from SRO-infected embryos but ceased to proliferate after being transferred to new dishes. These cells transformed into bipolar-shaped cells, detached from the substratum and then degenerated during a period of several weeks (Fig. 3D). In the case of no transfer, nearly all the cells necrosed as SRO degeneration proceeded.

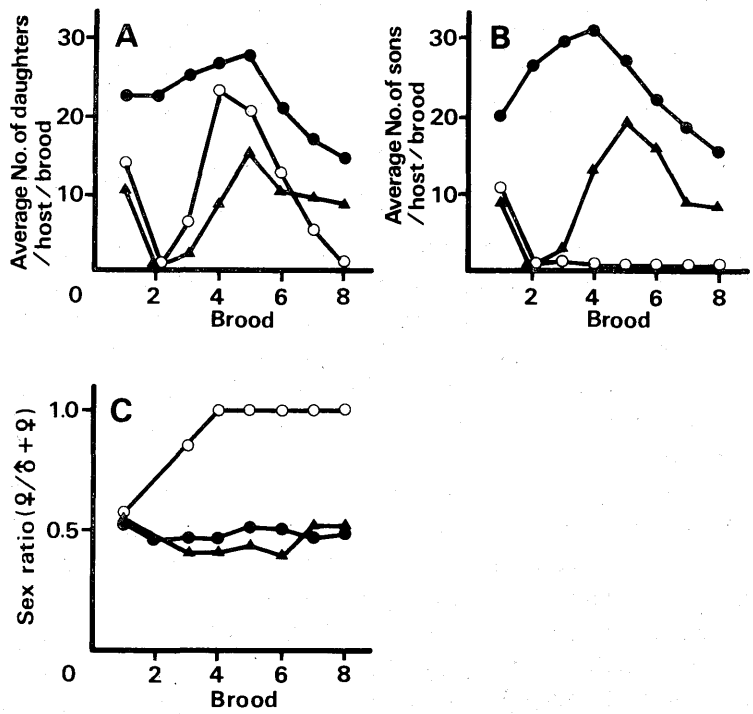

Fig. 4. Male-killing activity of SRO proliferated *in vitro*. Twenty to thirty female hosts in each group were received fresh culture medium (●), SRO-containing medium (○), or SRO-eliminated medium (▲). The hosts were transferred to new vial every 2 d. The progenies from these vials (brood) were scored for each sex.
A, B, average number of female (A) and male (B) progenies per host in each brood.
C, sex ratio of progenies (number of females/number of females plus males) calculated from the data in A and B.

Activity of SRO proliferated in vitro

SRO proliferated *in vitro* were examined for their ability to bring about SR-phenomenon following injection into the adult female abdomens. The average number of resulting male and female progenies from the flies received injection was plotted in Figs. 4A and 4B. The flies injected with old culture medium (SRO-containing or-eliminated) did not lay eggs at brood 2, suggesting the old culture medium (from 17 day culture) to possibly contain an unknown factor from either the unhealthy embryonic cells or proliferated SRO, which disturbed the homeostasis of the flies. Flies restored their fertility in a few days, but those which had received the SRO-containing medium produced almost only daughters. Those injected with the filtered medium also had their fertility restored by brood 4, and produced both daughters and sons in nearly the same ratio as that of fresh medium-injected flies.

In Fig. 4C, the sex ratios (♀/♂+♀) in the progenies are plotted. Those of

the fresh medium-injected and filtered medium-injected flies remained at substantially the same level (around 0.5) but that of flies injected with SRO-containing medium reached and continued to remain at 1.0 at brood 4. SRO proliferated *in vitro* was thus concluded to possess normal male killing activity.

4. DISCUSSION

A transient but prominent increase in number of *Drosophila* sex ratio organisms (SRO) were noted in a primary culture of embryonic cells from SRO-infected fly. The number of free-living SRO in the culture supernatant reached, at most, to 6×10^5 per μl. The total volume of SRO at this density was found to exceed one eighth that of the embryonic cells inoculated at the initiation of the primary culture. In consideration of this large volume, it is unlikely that all SRO would be hidden inside the embryonic cells at first and then appear later in the culture supernatant. In addition, aggregates of SRO approximating to this calculated volume were not observed under microscope at the initiation of the culture and consequently, the increase in SRO appears to result at least in part from actual proliferation *in vitro*.

In our primary culture system, why could SRO grow? The embryonic cells may possibly have suitably conditioned the medium for SRO growth. Ui *et al.* (*In Vitro*, in press) have recently obtained evidence supporting such a possibility. Using the supernatant of the primary embryonic cell culture, the imaginal disc cells of *Drosophila*, which grow in larval or adult hemolymph but not *in vitro*, have been noted to proliferate to become continuous cell lines. In view of the fact that embryonic cells in the primary culture differentiate into various types of larval cells and can be maintained in a healthy state for several weeks, they may possibly enable the medium to approximate *in vivo* conditions. There is also the possibility that the embryonic cells themselves can serve as the host for SRO. *In vivo*, SRO must penetrate into germ line cells in order to be transmitted to the next generation and thus, that SRO may infect particular cell type(s) in the primary culture is a possibility, but somewhat disqualified by the facts that (i) the spiroplasma from *D. willistoni* can grow without host cells *in vitro* (Hackett *et al.* 1986) and (ii) SRO proliferation does not induce histolysis of any internal organs or tissues in adults flies.

The transiency of SRO proliferation may reflect strict dependence of SRO growth on the state of embryonic cells in the primary culture. If SRO growth factor(s) are assumed to be produced by the cells at a certain stage of primary culture development, SRO beyond this stage may possibly become incapable of further proliferation and eventually degenerate. Even when the primary culture containing numerous SRO subcultured, the embryonic cells ceased to retain their healthy condition as a result of a too early dilution

of the primary culture, so that growth factor(s) do not become available in the culture.

To determine the roles of embryonic cells and finally establish a continuous culture of SRO, attempts are now being made to culture SRO in a cell-free conditioned medium prepared from primary embryonic culture, or in a primary cell culture of uninfected embryos. Such cultures should provide potential systems for examining SRO functions, particularly the selective male-killing action at the biochemical and molecular level. Additional research on SRO should also provide invaluable information regarding the genetic control of sex differentiation in *Drosophila*.

We wish to express our thanks to Dr. B. Sakaguchi (Kyushu Univ.) for supplying the fly strain containing SRO and to Dr. M. A. Yamada (National Institute of Genetics) for his helpful advice. Thanks are also due to Ms. N. Mae and S. Maruyama for their technical assistance and Ms. M. Suzuki for typing the manuscript.

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