RESCUE OF ARCHENTERON FORMATION IN VEGETAL-POLEDELETED EMBRYOS BY Li+, AND INDUCTION OF SECONDARY INVAGINATION BY INTRACELLULAR INJECTION OF Li+ INTO AN ANIMAL-HALF BLASTOMERE OF STARFISH EMBRYOS

RESCUE OF ARCHENTERON FORMATION IN VEGETAL-POLE-DELETED EMBRYOS BY Li⁺, AND INDUCTION OF SECONDARY INVAGINATION BY INTRACELLULAR INJECTION OF Li⁺ INTO AN ANIMAL-HALF BLASTOMERE OF STARFISH EMBRYOS[1]

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Two experiments were carried out to examine whether Li⁺, a vegetalizing agent, induces the archenteron forming potency of the area that lack the maternal factor responsible for archenteron formation by promoting the first (maternal factor-dependent) step of the archenteron specification in the starfish, *Asterina pectinifera*. They were that in which the embryos were treated with Li⁺ after their vegetal pole cytoplasm was removed, and that in which Li⁺ was co-injected with a lineage tracer to one of the eight-cell-stage blastomeres, and the blastomere descendants were traced. The archenteron formation and the expression of alkaline phosphatase (AP) activity were examined later in the development.

In the first group of experiments, the eggs whose vegetal pole cytoplasm was removed by a micromanipulation at the prophase of meiosis were fertilized and allowed to develop with 15 or 30 mM LiCl. In normal sea water, the archenteron formation and AP activity expression were strongly suppressed as more than 7% of the whole volume of the oocyte was removed from the vegetal pole region, though based upon the previous observation (KURAISHI and OSANAI, 1994) the presumptive archenteron area shares at least 15% of the vegetal cytoplasm of the oocyte. On the other hand, more than 15 and 25% of the vegetal cytoplasmic deletion was required to suppress the archenteron formation and the AP activity expression in about 50% of the larvae incubated with 15 and 30 mM LiCl, respectively.

In the second group of experiments, one of the animal-half blastomeres in the eight-cell-stage embryos was injected with 250-500 mM LiCl with FITC-dextran as a lineage tracer. Some of the descendants of the Li⁺-injected blastomeres formed a secondary archenteron which expressed the alkaline phosphatase activity as well. Then they apparently formed a part of the digestive tract including the esophagus and the anterior end of the stomach, whose specification is shown to rely on the maternal factors that eccentrically distribute around the vegetal pole in normal development.

These results indicate that Li⁺ promotes the first step of archenteron specification in the area that lacks (or has insufficient amount of) maternal cytoplasmic factors responsible for the first step of archenteron specification.

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INTRODUCTION

Because of the simple shape and the transparent body, it is very easy to observe the invaginated archenteron of echinoderm larvae even in the living state. Thus, they have long been used as the material for investigating the mechanism of archenteron (i.e., endoderm and mesoderm) specification (reviewed in HÖRSTADIUS, 1973). It has been suggested the archenteron formation is generally committed by the act of the cytoplasmic determinant and then it is determined finally by the cellular interaction (MARUYAMA, et al., 1985; HENRY, et al., 1989; KHANER and WILT, 1990, 1991; ZHANG, et al., 1990; MARUYAMA and SHINODA, 1990; RANSICK and DAVIDSON, 1993, 1995).

Among the echinoderms, the oocytes and the embryos of starfish are considered to be good materials for analyzing the role of the cytoplasmic determinants and the cellular interaction during archenteron specification, because they have endogeneous marker for animal-vegetal axis (SCHROEDER, 1985). Recently, KIYOMOTO and SHIRAI (1993a, b), and KURAISHI and OSANAI (1994) precisely investigated the role of these factors by removing and transplanting the vegetal pole cytoplasm. The results of these studies strongly suggest that the archenteron of the starfish, *Asterina pectinifera*, is specified as follows. The anterior mesodermal area (AMA; the presumptive cytoplasmic area of the anterior coelom and mesenchyme cells) and the alkaline phosphatase-positive area 1 (APA 1) in which alkaline phosphatase (AP) activity begins to show up at the mesenchyme migration stage are first specified to form archenteron by the maternal cytoplasmic factor that is distributed exccentrically around the vegetal pole. Later, the capacity of APA 1 to give rise to the anterior mesodermal tissues is suppressed by AMA. Then APA 1 expresses the AP activity and form the esophagus and the anterior end of the stomach in bipinnaria larvae. Afterwards, the APA 1 induces the surrounding area to become the AP-positive area 2 (APA2). This area is added to the posterior end of the archenteron after the mesenchyme migration stage, starts to express the AP activity at the late gastrula stage, then forms the middle-to-posterior part of the stomach and the intestine of the bipinnaria larvae.

On the other hand, LiCl, a vegetalizing agent, exaggerates the archenteron, and sometimes causes the exogastrulation in sea urchins (reviewed in HÖRSTADIUS, 1973) and starfish (CRAWFORD and CHIA, 1980; KOMINAMI, 1984) embryos. KOMINAMI (1984) showed that the Li⁺ treatment increased the relative volume and the cell number of the archenteron using the starfish larvae, though the volume and the cell number of the whole embryo had not been changed. This result suggests that Li⁺ treatment changed the developmental fate of the posterior ectoderm to that of the endodermal. Since microinjection of *myo* IP₃ canceled the effect of Li⁺ treatment (LIVINGSTON and WILT, 1995), Li⁺ seems to exert its vegetalizing effect by inhibiting the synthesis of IP₃, a key molecule for intracellular signaling system, in sea urchin embryos. Though, Li⁺ inhibits the IP₃ synthesis, it is not clear whether Li⁺ treatment enhances the maternal factor-dependent step of the archenteron specification.

To clarify whether LiCl affects the first step of the archenteron specification, the Li⁺ treatment on the vegetal pole cytoplasm-deleted (VP-deleted) embryos and the intracellular injection of LiCl into an animal-half blastomere at the eight-cell stage were carried out using starfish embryos, and the archenteron formation and the AP activity expression of these embryos were examined.

MATERIALS AND METHODS

Materials

Adult *Asterina pectinifera* were collected by SCUBA at Asamushi, Aomori prefecture and at Akiya, Kanagawa prefecture during their breeding season (September and May, respectively) They were then maintained in running sea water. Follicle-cell-free oocytes and spermatozoa were prepared as described before (KURAISHI and OSANAI, 1988). The oocyte maturation was induced by treatment with 1 μM 1-methyladenine (1-MeAde) in filtered sea water (FSW, pore size 10 μm) according to KANATANI (1969). The oocytes were inseminated by adding diluted suspension of spermatozoa about 15 min after germinal vesicle breakdown, washed several times with FSW and allowed to develop at 19°C.

Deletion of vegetal fragment

The oocyte fragment that includes the vegetal pole was sagitally severed using a fine glass needle according to the method described by KURAISHI and OSANAI (1994). The volumes of the animal and the vegetal fragments were calculated assuming that they are spheroids. Then the intensity of deletion was evaluated by calculating the relative volume of the deleted vegetal fragment to the sum of the volume of the both fragments. The animal fragments were then treated with 1-MeAde, fertilized, and transferred individually to each well of the microculture plates that was filled with FSW containing 100 units/ml penicillin G and 50 μg/ml streptomycin sulfate. Archenteron formation was morphologically examined under a stereomicroscope at 48-55 h after the initiation of 1-MeAde treatment. Then the embryos were fixed for histochemical observation of AP activity as described below.

Histochemistry for alkaline phosphatase activity

Expression of the AP activity was used as a marker for the endodermal differentiation. The activity was histochemically detected according to the method described by NISHIDA (1993). Stained larvae were observed and photographed as the whole mounted specimens. Aliquots of specimens were cleared and mounted in glycerol for observations with higher resolutions.

Vital stalning

To clarify whether the animal-vegetal axis is retained after the treatment with LiCl following the deletion of the vegetal pole cytoplasm, the animal pole of the immature oocyte was vitally stained with Nile blue sulfate according to the method described by MARUYAMA and SHINODA (1990) before the deletion of the vegetal pole cytoplasm. The stained oocytes were washed with FSW several times and used for the VP-deletion experiment.

Microinjection

The embryo of *A. pectinifera* undergoes orthoradial cleavage producing almost equal blastomeres (KOMINAMI, 1983; MARUYAMA and SHINODA, 1990). At the eight-cell stage, the embryos are consisted of two tires, and each contains four blastomeres (See Fig. 4). One of the four animal half blastomeres which surrounds the polar bodies was injected with 500 mM LiCl, 5% FITC-dextran in distilled water as described below. Micro needles were pulled from micro capillary (GD-1, Narishige, Tokyo) using a micro capillary pullar (PG-1, Narishige). The mixed solution of LiCl and FITC-dextran was introduced to the tip of the micro needle using a fine stainless tubing (od=0.3 mm) attached to a 1-ml cyrindge. Then the micro needle was back filled with sillicon oil and distilled water, and attached to a microinjection equipment setup according to the method described by MARUYAMA, *et al.* (1986). The eight-cell-stage embryos were held in a wedge shaped holder (KISHIMOTO, 1986), and the solution was ejected into the blastomere by pressure. By calculating the diameter of the injected solution before it is mixed with the cytoplasm, the injected volume was estimated to be about 4% of the total volume of the blastomere. The embryos were then transferred to the microculture plates that were filled with FSW containing 100 units/ml penicillin G and 50 μg/ml streptomycin sulfate, and allowed to develop at 19°C.

RESULTS

Archenteron formation and expression of alkaline phosphatase activity after deletion of the vegetal pole cytoplasm and Li⁺ treatment

The deletion of VP fragment which was more than 7% of the total volume of the oocyte strongly suppressed the archenteron formation as previously reported by KURAISHI and OSANAI (1994). On the other hand, 15 and 25% cytoplasmic deletion was required to prohibit the archenteron formation in more than half of the larvae reared with 15 and 30 mM of LiCl, respectively (Fig. 1). According to the fate estimation of KURAISHI and OSANAI (1994), the 7% of the vegetal area give rise to AMA and APA 1, and at least 15% deletion is required to remove all the presump-
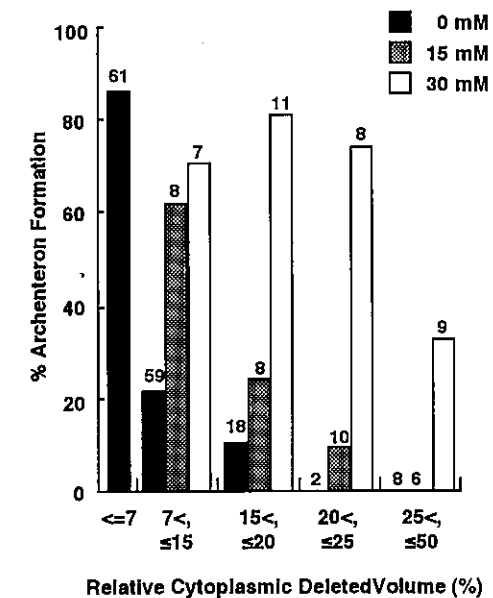

Fig. 1.   Corelation between the relative volume of the deleted vegetal cytoplasmic fragment and the rate of archenteron formation in the larvae incubated with different concentrations of Li⁺. In normal plain FSW (0 mM, solid), the archenteron formation was strongly suppressed after the deletion of vegetal cytoplasm with larger than 7% of the whole oocyte volume. Whereas, more than 15% and 25% deletion was required to suppress the archenteron formation in more than half of the larvae incubated with Li⁺ at the concentration of 15 (hatched) and 30 mM (open), respectively.

tive archenteron area. In the larvae that started archenteron formation after Li⁺-injection following the VP-deletion more than 7% of the vegetal cytoplasm, the anterior coelomic pouch was not observed, whereas some solitary cells that appeared to be the mesenchyme cells were detected in the blastocoel (Fig. 2c, d). Expression of the AP activity was detected in the archenteron of all the larvae that formed archenteron, whereas none of the larvae that lacked archenteron expressed the AP activity (Fig. 3). In most of the gastrulated embryos, expression of the AP activity was detected in the entire archenteron, unlike the normal embryos in which the anterior end of the archenteorn (AMA) does not express the AP activity.

To examine if there is any relation between the site where invagination occurs and the original animal-vegetal axis, the animal pole of several eggs was vitally stained with Nile blue sulfate (Fig. 2a, arrowhead). In all these embryos, the invagination occurred at the opposite side of the staining (Fig. 2b), showing that the initial animal-vegetal axis was retained.
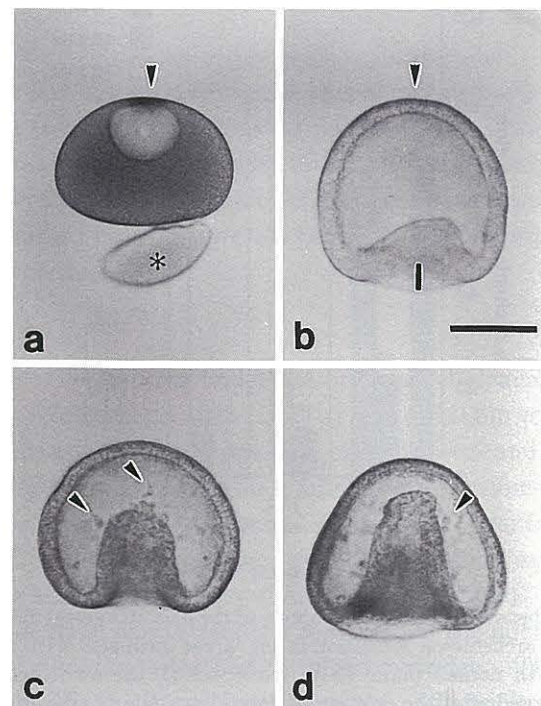
Fig. 2. (a) An immature oocyte immediately after deletion of the vegetal cytoplasmic fragment (asterisk). The deleted volume was about 9% of that of the whole oocyte. The animal pole was vitally stained with Nile blue sulfate (arrowhead). (b) a 50-h-old gastrula derived from the VP-deleted merogon shown in (a) reared with 15 mM LiCl. The invagination (I) took place at the opposite end of the stained area (arrowhead), suggesting that the most vegetal end of the merogon forms archenteron. (c) and (d) show three-day-old gastrulae derived from oocytes after more than 1 5% VP-deletion followed by the treatment with 15 mM (c), and 30 mM (d) Li⁺, respectively. Coelomic pouch was not apparently seen at the tip of the archenteron, whereas some mesenchyme-like cells were seen in the blastocoel (arrowheads). Scale bar＝100 μm

Fig. 3. About 50-h-old larvae after VP-deletion, Li⁺-treatment and histochemichal staining for alkaline phosphatase activity. The deleted volume was 8.1% (a), 21.7% (b), 12.0% (c) and 24.9% (d) of that of the whole oocyte, respectively. The VP-deleted oocytes were treated with 1-MeAde, inseminated, and allowed to develop with 15 (a and b) or 30 (c and d) mM Li⁺. The entire archenteron of the gastrulated larvae (a, c and d) expressed AP-activity, even at the tip. On the other hand, the larvae which had not gastrulated did not express AP-activity (b). Scale bar＝100 μm

Archenteron formation and AP activity expression after intracellular injection of LiCl into an animal blastomere at the eight-cell stage

It was examined if the cells in the animal hemisphere restore the capacity to form the archenteron by the Li⁺ treatment. One would think it could be examined easily by treating the embryoid that derived exclusively from the animal hemisphere with Li⁺ at the concentration higher than 30 mM. However, the deletion of vegetal cytoplasm as large as half of the whole oocyte's volume frequently caused the degeneration of embryos during early development. Moreover, the treatment with Li⁺ at the concentration higher than 30 mM often lead to the developmental delay even in the embryos with no cytoplasmic deletion, suggesting the occurrence of
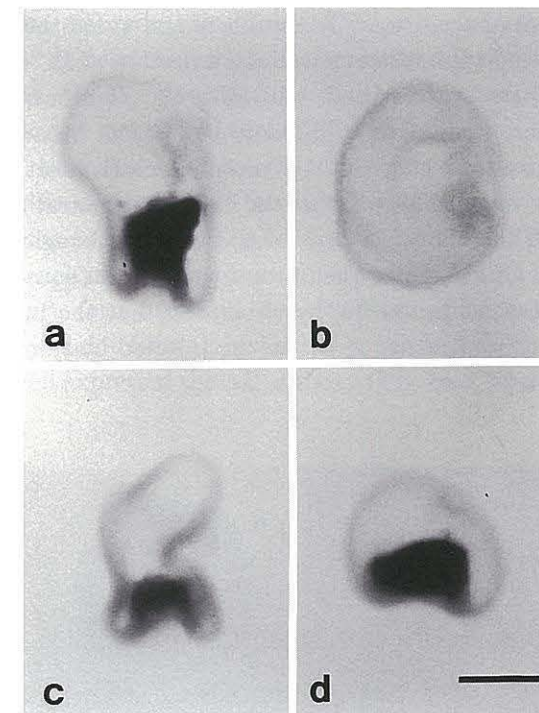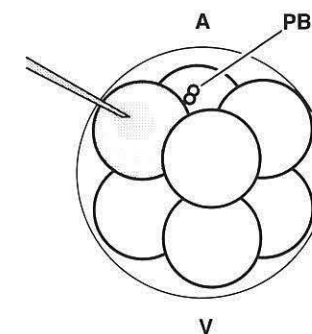

Fig. 4. An illustration that shows the position of the Li⁺-injected blastomere (shadowed area). A: the animal pole. V: the vegetal pole. PB: polar bodies.

nonspecific toxic effect of the drug. To minimize and avoid the damage caused by VP-deletion and Li$^+$-injection, the intracellular injection of Li$^+$ exclusively into an animal half blastomere was carried out (Fig. 4). Concentration of the Li$^+$ introduced to the microneedle was adjusted to be 500 mM because injection of Li$^+$ at the lower concentrations caused little morphological effect, and most of the blastomeres that were injected with Li$^+$ at the higher concentrations ceased cleavage (data not shown). Thus above Li$^+$ concentration was thought to be optimal to induce morphological effect. Since the injected volume was equivalent to about 4% of the whole volume of a blastomere, by supposing that the injected Li$^+$ defused evenly the eventual concentration of Li$^+$ in the injected blastomere is estimated to be about 20 mM.
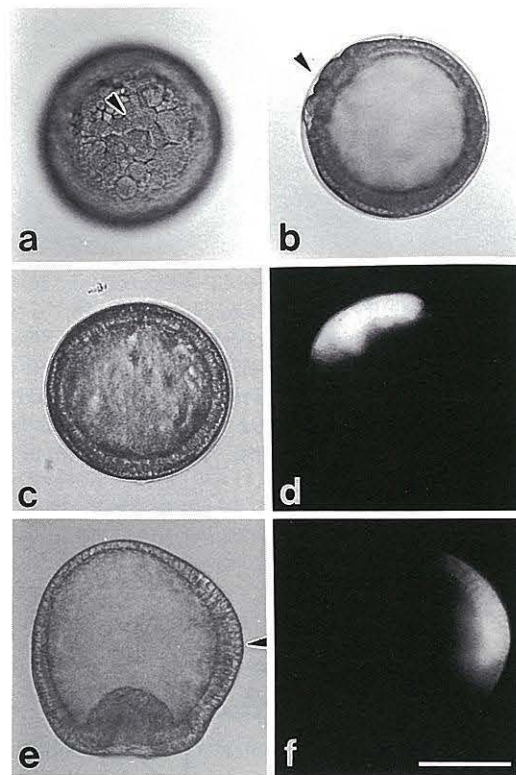

Fig. 5.   Early development of Li$^+$-injected embryo up to the mesenchyme-differentiation stage.   An abnormal blastula with the blastomeres that showed an arrest or a delay in cleavge (a and b, arrowheads).   A bright-field (c) and a fluorescence (d) microscopy of an apparently normal blastula after injection with 500 mM Li$^+$ and FITC-dextran at the eight-cell stage.   A bright-field (e) and a fluorescence (f) microscopy of an early gastrula derived from the apparently normal blastula after injection of Li$^+$ at the eight-cell-stage.   The progenies of the injected blastomere formed a thichened area (e, arrowhead) that resembled the vegetal plate.   Scale bar=100 μm.

In most of these embryos, the delay (8/56) or the arrest (41/56) in cleavage occurred among the descendants of the Li$^+$-injected blastomeres (Fig. 5a, b).   In seven embryos out of 56, descendants of the injected blastomeres cleaved synchronously with the other uninjected blastomeres and the embryos formed apparently normal blastulae (Fig. 5c, d).   In these embryos, the area that was composed of the cells derived from the injected blastomere thickened when the vegetal plate started invagination (Fig. 5e, f, arrowhead), and it also invaginated by the end of the mesenchyme differentiation stage (about 27 h after 1-MeAde treatment).   At the mouth forming stage, the secondary archenteron derived from the injected blastomere did not form the coelomic pouch and the mesenchyme cells, and the entire archenteron expressed the AP activity (Fig. 6).   These results suggest that
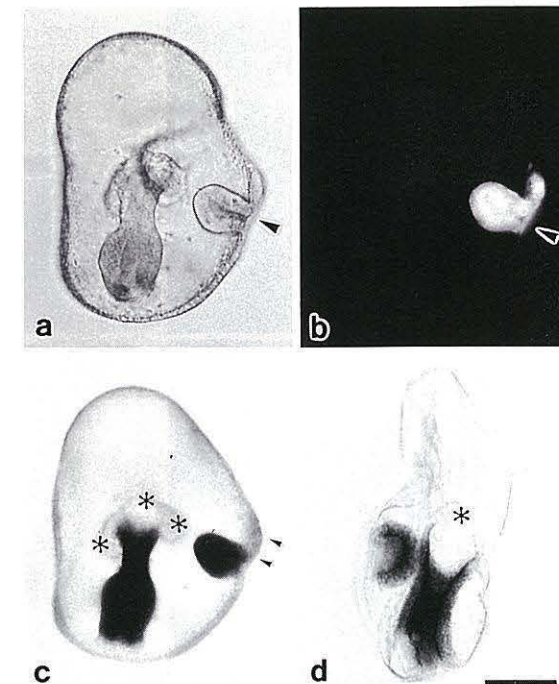

Fig. 6.   Mouth-formation-stage larva (about 50 h after 1-MeAde application) after Li$^+$-injection at the eight-cell stage.   A bright-field (a) and a fluorescence (b) microscopy of a larva in which the progenies of the injected blastomere formed the secondary archenteron at the equatorial region (arrowhead).   A mouth-formation-stage larva with the secondary archenteron after histochemical staining for the AP activity (c and d).   The larva shown in (c) was the same one as that shown in (a) and (b).   The cells in the secondary archenteron expressed the AP activity even at the tip, whereas the cells in at the tip of the intrinsic archenteron (cells in APA) did not express the AP activity (c and d, asterisks).   Note that a part of the progenies of the injected blastomere which had not gastrulated did not express alkaline phosphatase activity (c, arrowheads).   Scale bar=100 μm
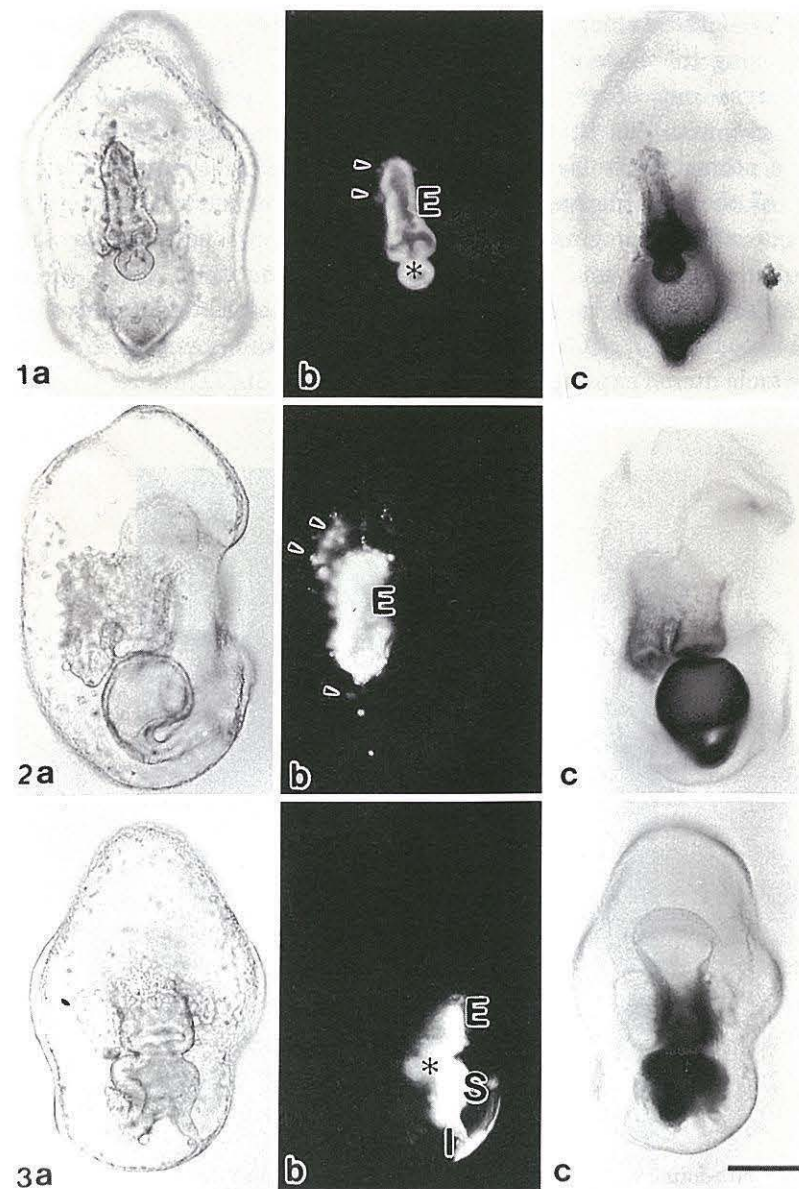
Fig. 7. Three-day-old bipinnaria larvae after Li⁺-injection at the eight-cell stage. Bright-field (a) and a fluorescence (b) microscopy of a living larva, and a bright-field microscopy of the same larva after fixation and histochemistry for AP activity (c). The progenies of the Li⁺-injected blastomere formed the esohpagus (1b, 2b and 3b, E), the anterior end of the stomach (1b and 3b, asterisk), middle-to-posterior part of the stomach and the intestine (3b, S and I) and expressed AP-activity. There are some labeled mesenchyme-like cells around the secondary esophagus in 1b and 2b (arrow-head). Scale bar=100 μm

the entire secondary archenteron differentiated to the endoderm. LiCl injection into one of the vegetal blastomeres, on the other hand, enlarged the archenteron, especially in the basal part (Data not shown).

Five embryos that formed the secondary archenteron, which were obtained from another set of injections, were allowed to develop up to three days after 1-MeAde application. In one embryo, the secondary archenteron was lost and the FITC labeling was detected only in the ectoderm. In the rest four embryos the secondary archenteron elongated parallel to the intrinsic one and apparently formed the endodermal tissues. In these excess organs, a peristaltic movement of the esophagus and a constriction of the cardiac sphincter were observed. In one embryo among the four shown in the Fig. 7-1, the secondary archenteron formed a tubular structure that was aparently consisted of the esophagus and the anterior end of the stomach. In other two embryos out of the four, the secondary archenteron fused with the intrinsic one and formed a part of the esophagus (Fig. 7-2). In the last embryo, the descendants of the injected blastomere formed the middle-to-posterior part of the stomach, the intestine and the posterior end of the ectoderm surrounding the anus in addition to the esophagus (Fig. 7-3). No FITC-labeling was detected in the coelomic pouch of these four embryos. It was not possible to examine whether the muscle cells surrounding the secondary gut were labeled because of the strong FITC-labeling of the gut.   Unlike the normal mesenchyme cells, some labeled single cells that did not have pseudopodes were observed around the secondary gut (Fig. 6-1, 2). Since I had no access for the differentiation marker for mesenchyme differentiation, it is not clear whether they are the mesenchyme cells or the cell debris that had dropped out into the blastocoel.

DISCUSSION

Effect of Li⁺ on specification of APA 1

As discussed by KURAISHI and OSANAI (1994), the removal of 7% and 15% of the vegetal cytoplasm of the oocyte would take away the presumptive AMA plus APA 1 and the presumptive area of the whole archenteron from the oocyte, respectively.   In the present study, Li⁺ treatment rescued archenteron formation after the VP-deletion with more than 7% of the cytoplasmic volume. Moreover, more than 70% of the embryos whose 15% to 20% of the, vegetal polar cytoplasm were removed formed archenteron by the treatment with 30 mM LiCl. Additionaly, Li⁺ injection into an animal half blastomere at eight-cell stage caused secondary archenteron induction exclusively at the area that derived from the injected blastomere. At least a part of the secondary archenteron formed the esophagus and the anterior end of the stomach, which is normally derived from the APA1. These results strongly suggest that Li⁺ treatment can promote the first step of the archenteron specification, which relied on maternal factors in normal development,

in the presumptive APA2 area and that of the ectoderm. The result of Li⁺ injection also showed that the blastomeres in the animal hemisphere are sensitive to Li⁺-treatment.

In sea urchin embryos, Li⁺ evokes the expression of vegetal-specific molecules such as endoderm-specific alkaline phosphatase and spicule matrix protein SM50 (LIVINGSTON and WILT, 1989). However, it is not clear whether Li⁺ exerts its effect by enhancing the maternal factor-dependent steps in normal development, because the expression of most of the vegetal-specific molecules are considered to require inductive interaction from the most vegetal area of the embryo called micromere (RANSICK and DAVIDSON, 1993; 1995). Although, the expression of some skeletogenic cell-specific genes such as SM50 and the differentiation of skeletogenic cells from the large micromere lineage undergoes autonomously (OKAZAKI, 1975; STEPHENS, et al., 1989), the lack of micromeres and skeletogenic cells can be regulated by the secondary mesenchyme cells long after the sensitive period to Li⁺ (FUKUSHI, 1962; ETTENSOHN and McCLAY, 1988; ETTENSOHN, 1990). On the other hand, Li⁺ decreases the expression of some animal region specific genes called *HE* and *BP10* which are expressed autonomously in normal development. The fact that the border of the domain expressing these genes shifts to the animal pole as the Li⁺ concentration increases suggests the presence of a gradient of sensitivity to Li⁺ along the animal-vegetal axis (GHIGLIONE, et al., 1993; GHIGLIONE, et al., 1996). In the present study, the higher concentration of Li⁺ is required to rescue archenteron formation when the larger cytoplasmic volume of vegetal pole is deleted. On the other hand, the deletion of cytoplasmic fragment from the equatorial region of the oocyte does not affect the archenteron formation (KURAISHI and OSANAI, 1994). These suggest that the difference of the capacity to promote some vegetal-specific genes responsible for the archenteron formation in respond to Li⁺ is already established along the animal-vegetal axis by the prophase of meiosis in the starfish oocyte.

Effect of Li⁺ on specification of mesoderm

In both experiments of the present study, Li⁺-treatment apparently did not evoke mesoderm differentiation. These results coincides with that by YOSHIKAWA (1996) that Li⁺-treatment increases the cell number of the esophagus, stomach and intestine but not the coelomic pouch in intact larvae. In sea urchin embryos, on the other hand, Li⁺-treatment induces the differentiation of mesodermal tissues (LIVINGSTON and WILT, 1989). In starfish larvae, KURAISHI and OSANAI (1994) suggested that the presumptive coelomic pouch area suppresses the potential of the surrounding area (APA1) to form the coelomic pouch. One could interpret the difference in the effect of Li⁺ between the two echinoderm groups on the specification of mesodermal tissues by supposing that the lateral suppression from the coelomic pouch is strong enough to prevent the increase in coelomic cells in

Li⁺-treated starfish larvae. However, both the present results and those by YOSHIKAWA's (1996) do not make it clear whether Li⁺-treatment increased the number of other mesodermal cells such as muscles and blastocoelar cells. Thus, for now, the possibility that Li⁺ affects the sequence of mesoderm differentiation can not be excluded.

Effect of Li⁺ on specification of APA2

JERKA-DZIANOSZ and FRANKEL (1995) reported the lack of a strong evidence for the effects of Li⁺ on specification of the body plan of ciliates, and claimed that the effects of Li⁺ on determination of the body plan in multicellular organisms might be mediated by the intercellular signal transmission pathways. In the present study, the effect of Li⁺ on the specification of APA2, which requires inductive interaction, was not directly examined. YOSHIKAWA (1996) showed that Li⁺-treatment increases the number of cells that construct both esophagus and the combined area of the stomach and the intestine, indicating that the treatment also increases APA2. Additionally the ratio of the cell number between the esohpagus and the combined area of the stomach and the intestine did not change under the presence of Li⁺. There are two possibilities to interpret these facts. They are that Li⁺ treatment also directly and positively affects the second step, i.e. induction of APA2, and that Li⁺-treatment indirectly increases APA2 by increasing the inducer of APA2 (APA1). Pulse treatment with Li⁺ during the time of induction may clarify which is the case.

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