CYTASTERS INDUCED WITHIN UNFERTILIZED SEA-URCHIN EGGS

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SUMMARY

Conditions that induce the formation of asters in unfertilized sea-urchin eggs have been investigated. Monasters were formed by treatment of eggs with acidic or basic sea-water, or procaine- or thymol-containing sea-water. A second treatment step, incubation with D$_2$O-containing, ethanol-containing or hypertonic sea-water induced multiple cytasters. The number and size of cytasters varied according to the concentration of agents and duration of the first and second treatments, and also upon the species of eggs and the season in which the eggs were obtained. Generally, a longer second treatment or a higher concentration of the second medium resulted in a higher number of cytasters per egg.

Asters were isolated and then examined by light and electron microscopy. Isolated monasters apparently lacked centrioles, whereas cytasters obtained from eggs undergoing the two-step treatment contained one or more centrioles. Up to eight centrioles were seen in a single aster; the centrioles appeared to have been produced during the second incubation. Centrospheres prepared from isolated asters retained the capacity to nucleate the formation of microtubules in vitro as assayed by light and electron microscopy.

Many microtubules radiated from the centre of isolated asters, whether they contained centrioles or not. This observation is consistent with many other reports that microtubule-organizing centres need not contain centrioles.

INTRODUCTION

There is a long history of studies of artificial parthenogenesis in sea-urchin eggs, and some of the conditions that activate unfertilized eggs were already identified as early as the beginning of this century (see Harvey, 1956, for a review).

Eggs give various responses to activating agents. From a cytological point of view, it has been noted that activated eggs develop along one of four different pathways, which have been categorized as monaster formation, cytaster formation, haploid mitosis and intranuclear chromosome replication (Von Ledebur-Villiger, 1972). However, it has been reported that under some conditions, 15–40 cytasters have been formed within unfertilized activated eggs (Miki-Noumura, 1977).

The ability to induce large numbers of asters within a cell offers a number of possibilities for experimental investigation of mechanisms controlling the activity of microtubule-organizing centres (MTOCs) (Pickett-Heaps, 1969).

One of the most intriguing problems to be investigated using artificial parthenogenesis, and one on which many investigators have been concentrating their interest, is the origin of centrioles. We have found no record in the literature that centrioles have been found in unfertilized eggs. However, using asters induced by the
double method of Loeb (1913), Dirksen (1961) showed the presence of centrioles in artificially activated sea-urchin eggs. This was subsequently confirmed by several groups (Sachs & Anderson, 1970; Kato & Sugiyama, 1971; Mazia, 1977; Miki-Noumura, 1977), providing strong evidence for de novo formation of centrioles. On the other hand, no centrioles were found in eggs activated with sea-water containing procaine (Moy, Brandriff & Vacquier, 1977) or ammonia (Paweletz & Mazia, 1979). Rather, the microtubules that were displayed in an astral arrangement were found to be centred on clusters of osmiophilic bodies.

Taken together these reports raise several important issues. They suggest that centriole formation may indeed be de novo but is not a necessary concomitant of activation. They also suggest that aster formation does not require centrioles. Since the reported findings were obtained by different workers using different conditions and species we considered it worthwhile to attempt to reproduce the findings within a single laboratory, and to explore more fully the basis for the difference in results.

This report describes our procedures for the induction of asters in unfertilized sea-urchin eggs and characterizes them in terms of the presence of centrioles and the ability to nucleate the formation of microtubules in vitro.

A brief account of this work has appeared elsewhere (Kuriyama & Borisy, 1978).

**MATERIALS AND METHODS**

**Formation of asters by parthenogenic activation**

Two species of sea urchins, Strongylocentrotus purpuratus and Lytechinus pictus, obtained from Pacific Bio-Marine Co., Venice, California, were used. Gametes were obtained by injection of 0.5 M KCl into the body cavities. The jelly coats of the eggs were removed by passage through 100-μm nylon mesh (Tobler Ernst & Traber Inc., New York). Dejellied eggs were washed by sedimentation at 1 g with artificial sea-water (ASW: 27 g NaCl, 0.7 g KCl, 4 g MgCl₂·6H₂O, 11 g MgSO₄·7H₂O, 0.4 g NaHCO₃, 1.5 g CaCl₂·2H₂O in 1 litre adjusted to pH 8.2–8.3 with Na₂CO₃) or when used for isolation of asters, with calcium-free artificial sea-water (CFSW). Washed eggs were then treated with various reagents as listed in Table 1. For studies of aster development in vivo, the eggs were exposed to reagents in ASW, whereas for isolation of asters, they were treated with reagents in CFSW.

Acidic ASW was prepared by bringing the pH to 5.0 with concentrated acetic or butyric acid. The eggs were suspended in those solutions for 1–3 min, then washed in ASW for 20–40 min. Basic ASW was prepared by adjustment of the pH to 9.3 with ammonium hydroxide (Mazia, 1974). Eggs were left in the solution for 20–40 min, then washed once with ASW. Treatment of eggs with

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* pH 4–7 adjusted with acetic acid or butyric acid.
† pH 9–10 adjusted with ammonium hydroxide or ethylamine.
† NaCl or KCl (0.25–0.45 M), sucrose (0.2–0.5 M) or glycerol (0.5–1.0 M) in sea-water.
Cytasters in sea-urchin eggs

Procaine or thymol was done according to the procedures developed by Moy et al. (1977) and Ishikawa (1962), respectively. The eggs were exposed to 2–10 mM-procaine for 30–90 min or to 20 % thymol for 20–70 s and then washed several times with fresh ASW.

For induction of cytasters, eggs had to be treated with a second solution, which contained hypertonic salt, D2O or ethanol. Hypertonic ASW was originally made by addition of 8 ml of 2·5 M NaCl or KCl to 50 ml ASW (Loeb, 1913), which gave 0·345 M as the final concentration of added salt. However, some eggs responded better to 0·25 or 0·45 M added salt. Glycerol or sucrose solution could be substituted for hypertonic salt and the solutions were prepared by addition of sucrose or glycerol to a final concentration of 0·2–0·5 M or 0·5–1·0 M, respectively. D2O/ASW was made by mixing 55 ml D2O with 45 ml of 2·2 times the normal concentration ASW (Mazia, 1977). Ethanol/ASW contained 2–5 % ethanol. Following treatment with the first solution, eggs were incubated with one of the second media for 40–90 min, and washed once with ASW. Asters appeared while the eggs were being cultured in fresh ASW with gentle stirring.

Isolation of asters

Asters were isolated by a glycerol/Mg2+/Triton X-100 method that was originally developed for isolating mitotic apparatus (Sakai, Shimoda & Hiramoto, 1977). Eggs were kept in CFSW at room temperature until many asters formed in most of the eggs. They were then sedimented and washed twice with 1 M-dextrose to remove CFSW. The packed pellet of eggs was resuspended in 10–30 vol. of isolation medium, which contained 1 M-glycerol, 5 mM-MES (2(N-morpholino)ethanesulphonic acid), 1 mM-EGTA (ethylene glycol bis(β-aminoethylether)tetraacetic acid), 2 mM-MgSO4, 0·05 % Triton X-100 at pH 6·15–6·2. After 1 min at 33 °C, the suspension was shaken to rupture the eggs. Isolated asters were collected by centrifugation at 1000 g for 5–10 min. Generally, asters were examined immediately after isolation, but for storage they were kept at −80 °C.

Isolation of centrospheres from mitotic apparatus and cytasters

Mitotic apparatuses were isolated from sperm-fertilized sea-urchin eggs allowed to develop to metaphase of first cleavage. The procedure and solution was the same as first used for isolating artificially induced asters. Centrospheres were prepared by extracting these metaphase spindles or cytasters by homogenization or with isolation solution containing 0·5 M KCl (Sakai et al. 1977).

Preparation of microtubule protein and polymerization onto isolated asters or centrospheres

Microtubule protein was purified from porcine brain (Kuriyama, 1975; Borisy et al. 1975) centrifuged at high speed to suppress self-nucleation and stored at −80 °C. Phosphocellulose-purified tubulin (PC-tubulin) was prepared as follows. The phosphocellulose column (1·4 cm × 3 cm) was equilibrated with elution buffer (10 mM-PIPES (piperazine-N',N'-bis(2-ethanesulfonic acid), 1 mM-EGTA, 0·5 mM-MgSO4, 1 mM-GTP at pH 6·8), and charged with the microtubule protein in the elution buffer (5–10 mg/ml bed volume). Tubulin (not bound) was eluted with the elution buffer and the final PIPES concentration of this unabsorbed tubulin fraction was raised to 0·1 M and stored at −80 °C until used.

Packed pellets of isolated cytasters or centrospheres, that had already been frozen or treated with high-salt solution to ensure the depolymerization of in situ microtubules, were resuspended with microtubule protein or purified tubulin at a final concentration of 0·5–2·0 mM. After incubation at 33 °C, polymerization was stopped and samples were fixed by addition of glutaraldehyde (to 2·5 %) in 10 mM-PIPES, 0·5 mM-MgSO4, 1·0 mM-EGTA at pH 6·8.

Microscopy

In order to observe microtubule formation by phase-contrast microscopy, one drop of sample containing brain tubulin and isolated asters was placed on a glass slide. Polymerization was initiated and maintained by warming the stage of the microscope with an air-stream incubator. Alternatively, a sample of the aster–tubulin mixture was pre-warmed and observed directly.

Whole mounts of samples for electron microscopy were prepared by dropping the samples onto Formvar-coated 400-mesh grids that had been coated with carbon. After fixing with 2·5 %
glutaraldehyde the grids were washed with distilled water, stained with 1% uranyl acetate, passed through an amyl acetate series and then air-dried.

Samples for thin-section electron microscopy were fixed with 2.5% glutaraldehyde, post-fixed in 1% OsO₄ and stained with 0.5% uranyl acetate for 2 h at room temperature. After dehydration through an ethanol series, they were infiltrated and embedded in Epon–Araldite plastic. Thin sections were stained with lead citrate and uranyl acetate and examined in a Philips 300 electron microscope operated at 80 kV.

RESULTS

Formation of asters within unfertilized sea-urchin eggs

When unfertilized sea-urchin eggs were treated with acidic sea-water as described in Materials and Methods, a single large aster, designated a monaster, appeared while the eggs were being cultured in fresh ASW. The monaster was detected by phase-contrast microscopy as a radial pattern in the egg (Fig. 1A). Fig. 1B shows the same kind of monasters induced in thymol-treated eggs, appearing as clear spots as observed with bright-field microscopy. Similar results were obtained when eggs were treated with ASW containing 2–10 mM-procaine or with ASW acidified to pH 5.0 with acetic acid or made basic to pH 9.3 with ammonia. In each case eggs developed only a monaster.

However, when eggs treated with any of the above solutions were incubated subsequently with certain other solutions, multiple asters were induced, the number depending upon the exact conditions of the second incubation. Fig. 2 shows a series of eggs that were activated with procaine followed by treatment with D₂O solution for different lengths of time. Monasters appeared in eggs cultured in ASW after 10 min incubation in D₂O medium (Fig. 2A). With longer incubation in the second medium, increasing numbers of cytasters per egg were detected (Fig. 2B–E). The

![Fig. 1. Light micrographs of monasters induced by treatment with butyric acid (a) in *L. pictus* and thymol (b) in *S. purpuratus* eggs. a, phase optics; b, bright-field optics. Bars, 10 µm (a), 50 µm (b).](image-url)
Cytasters in sea-urchin eggs

Fig. 2. Change in number of the cytasters depending upon the duration of second treat-
ment. The number of cytasters formed within one egg can be controlled to a certain extent
by changing the length of time of the second treatment. L. pictus eggs were activated by
procaine and then incubated with D₂O solution for: A, 10 min; B, 20 min; C, 45 min; D,
60 min; E, 90 min. The number of cytasters detectable in these photographs are: A, 1; B,
1; C, 1; D, 11; E, 20. ×300.

number of cytasters could also be regulated within a certain range by altering the
concentration of the second solution; that is, more asters appeared in eggs treated with
higher concentrations of the second medium (data not shown).

It was clear from these experiments that multiple cytasters were induced in
unfertilized eggs that were treated with two-step procedures using various reagents
as summarized in Table 1. The first step of the procedure involved treatment of eggs
with acidic, basic, procaine or thymol solutions. The use of acidic conditions was
originally introduced by Loeb (1913) who prepared 5 mM-butyric acid in sea-water.
In our samples this procedure results in a pH of 5-8. ASW adjusted to pH 5-8 with
acetate had the same effect as butyric acid in terms of inducing egg activation.
Although the effective range of pH was relatively broad, from 4 to 7, the optimum
value, defined as the number of eggs with asters, was about 5 for both acetic acid
and butyric acid. HCl, H₂SO₄ and H₂PO₄, however, induced aster formation in less
than 5 % of the eggs. Basic sea-water prepared by addition of ammonium hydroxide
also causes activation of unfertilized eggs (Mazia, 1974). A range of pH (9–10) and
treatment time (80 s to 120 min) were effective. Ethylamine could substitute for
NH₄OH, but other bases such as NaOH, KOH or Na₂CO₃ failed to activate
eggs.

The second step of the procedure employed incubation of activated eggs with
hypertonic, D₂O or ethanol/sea-water. Hypertonic conditions were produced by
addition of salts (NaCl or KCl) to 0.25–0.45 M final concentration in ASW. Sucrose
(0.2–0.5 M) and glycerol (0.5–1.0 M) were able to substitute for salt and give the same
results.

Basically, most combinations of first and second treatments induced asters, except
for acid/ethanol, acid/D₂O, thymol/ethanol and thymol/D₂O. These exceptions
held for both species of urchins employed. Fig. 3 shows light micrographs of cytasters
formed by combination of: NH₄OH and D₂O (A); acetic acid and NaCl (B); thymol
and NaCl (C); procaine and NaCl (D); procaine and D₂O (E); and acetic acid and
glycerol (F).

It should be noted that the eggs of different species and different batches, or even
the eggs of the same batch, can react differently to the same concentrations of reagents
and duration of treatment. The optimal conditions for both first and second steps, therefore, have to be determined in each experiment. Generally, successful induction of cytasters in parthenogenically activated eggs appears to be mainly dependent upon
Cytasters in sea-urchin eggs

the maturity of the eggs. It was easy to form cytasters in a high percentage of eggs that were treated at the beginning of the spawning season. These eggs appeared to have a potential to react to all reagents and, as a result, cytasters could be formed in them by almost all combinations of first and second treatments. On the other hand, eggs obtained from sea urchins late in the season appeared more refractory, since the number of possible conditions capable of eliciting asters became restricted. As for the species of sea urchins used in this study, the induction of asters was much easier and more reproducible in eggs of *L. pictus* than in those of *S. purpuratus*.

Aster formation was inhibited or stopped completely by addition of $10^{-5}$ M-KCN in ASW at the beginning of either the first or the second treatment. This implies that aster induction depends on one or more respiration-dependent processes, as in normal fertilized eggs. It was also found that colchicine dissolved in ASW at concentrations above $10^{-4}$ M caused inhibition of aster formation when it was added at the beginning of either the first or the second treatment. On the other hand, cytochalasin B (10 µg/ml) showed no inhibitory effect on aster formation. Cycloheximide, a well-known inhibitor of protein synthesis, was examined for its effect on aster induction in the procaine/D$_2$O combination of first and second treatments. Only the continuous presence of this drug above 10 mM completely prevented formation of asters. However, monasters appeared when the drug was added in either the first or second treatment, while many cytasters were produced in control eggs.

Microscopic observations of isolated asters

Asters induced by various combinations of first and second treatment steps were isolated with the medium used for isolation of mitotic apparatus from sperm-fertilized eggs (Sakai *et al.* 1977). Fig. 4 shows a phase-contrast micrograph of mass-isolated cytasters. In Fig. 5 several kinds of asters are presented. Typical cytasters are shown

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Fig. 4. Phase-contrast micrograph of the mass-isolated cytasters. The cytasters were induced in *S. purpuratus* eggs by treatment with acetic acid/NaCl. Bar, 10 µm.
Fig. 5. Phase-contrast micrographs of the isolated cytasters induced by various combinations of first and second treatments. Bar, 10 μm.

in A and B and C–E represent various multipolar asters in which the central regions are clearly visible as dense dots. Some asters have condensed chromosomes (Fig. 5f–i); some of the eggs appeared to go through the chromosome cycle during the treatment, and as a result, nuclei were formed in the centres of the cytasters (Fig. 5j, k).

Electron micrographs of thin-sectioned isolated asters are illustrated in Fig. 6. Cytasters induced by the acetic acid/NaCl treatments were found to contain centrioles at the centre, from which microtubules emanated to the periphery of the aster (Fig. 6a). There was no exception to the existence of centrioles in the cytasters, and centrioles were always detected in cytasters that were induced by every combination of first and second treatments that we tested. Moreover, many cytasters seemed to include multiple centrioles, and up to eight centrioles were observed in one cytaster, as shown in Fig. 6b. Interphase and mitotic mammalian cells contain a pair of orthogonally oriented centrioles. Centrioles detected in isolated cytasters, however, lacked this characteristically perpendicular arrangement (Fig. 6b, c).

The typical cylindrical structure of nine triplet microtubules was found in these individual centrioles (Fig. 6c, d), and amorphous electron-dense material with
which astral microtubules seemed to be associated was noticeable around the centriole (Fig. 6D).

Unlike the cytasters induced by double treatment, monasters induced by single treatment or by only a brief treatment with second medium appeared to contain no centrioles (data not shown). A large number of sections were examined without any centrioles in monasters being found, while they were easily located in sections of cytasters. If there were centrioles in monasters, we should have come across them since much more effort was expended on these than on the cytasters.

The second treatment step is therefore indispensable for artificial induction of centrioles in eggs. On the other hand, microtubules in both cytasters and monasters were found to radiate from the central portion of asters whether centrioles were present or not, suggesting that the microtubule-organizing centres need not contain centrioles.
In vitro nucleation of microtubules from isolated asters or centrospheres

As is clearly shown in Fig. 6, astral microtubules radiated from the central portion of the isolated asters, implying the presence of microtubule-nucleating capacity in this area. In order to demonstrate the role of the central region in the asters as the microtubule-nucleating site more clearly, we incubated isolated cytasters with
microtubule protein purified from porcine brain. All samples used in these experiments were frozen at −80 °C or extracted with 0.5 M-KCl to ensure the depolymerization of in situ microtubules. Typical microtubules nucleated by whole isolated cytasters were revealed by phase-contrast microscopy (Fig. 7A). Many microtubules radiated from the centre in a spine-like configuration. This figure is quite similar to Fig. 7B, in which microtubule polymerization onto the central region of the isolated mitotic apparatus from inseminated eggs is demonstrated. It was also possible to detect restoration of astral birefringence with elongation of microtubules from these structures (data not shown). Fig. 8 shows electron micrographs of nucleated microtubules from isolated centrospheres of a cytaster (A) and mitotic apparatus (B) prepared as a whole mount and negatively stained with phosphotungstic acid. This figure corresponds to Fig. 7, showing microtubules originating from the central portion that appears as a strongly electron-dense body. Thin-section electron micrographs of isolated cytasters incubated with or without exogenous brain microtubule protein show more detailed ultrastructure around the centre. Very few microtubules were detected in cytasters incubated with only polymerization buffer (Fig. 9A). But after incubation with brain microtubule protein at 33 °C, many microtubules radiated from all sides of the centre where the centriole was found (Fig. 9B).

**DISCUSSION**

Unfertilized sea-urchin eggs exhibit various reactions to parthenogenic activating reagents, and one of the four pathways described by Von Ledebur-Villiger (1972) along which the artificially activated eggs develop is that of the formation of cytasters. However, the cytaster pathway was not fixed and the other three possibilities occurred with some frequency, making it difficult to set up definite conditions for cytaster formation. The occurrence of these problems was variable, depending on the stage of the spawning season at which the experiments were done.

Cytasters are formed by various combinations of first and second treatments, as summarized in Table 1. The number of asters formed per egg could also be regulated within a certain range by altering the conditions of the second treatment; that is, lower concentrations of reagents or shorter incubation in the second solution resulted in fewer cytasters being formed per egg. Consequently, only monasters were induced in eggs undergoing brief or no second treatment.

Isolated monasters apparently lack centrioles, whereas cytasters obtained with doubly treated eggs contained one or more centrioles. This observation explains some apparent contradictions found in previous reports. Centrioles were detected in the asters formed by two-step treatment with butyric acid/NaCl (Loeb, 1913; Dirksen, 1961; Kato & Sugiyama, 1971; Miki-Noumura, 1977). The combination NH₄OH and D₂O, which was originally elaborated by Mazia (1977), also caused formation of cytasters with centrioles. On the other hand, centrioles could not be detected in the asters that were formed in activated unfertilized eggs with procaine-containing (Moy et al. 1977) or ammoniacal (Paweletz & Mazia, 1979) sea-water. Therefore, it can be
concluded that the second treatment is indispensable for de novo production of centrioles.

De novo formation of centrioles has already been demonstrated in mammalian tissue-culture cells (Zorn, Lucas & Kates, 1979) in addition to sea-urchin eggs, suggesting that this is a common feature among a wide variety of organisms. It is well recognized that the progression of centriolar events is normally tightly coordinated with other events in the cell cycle and that the number of centrioles in precisely controlled (Kuriyama & Borisy, 1981). However, the number of regenerated centrioles in karyoplasts or parthenogenically activated sea-urchin eggs is indefinite, and they never orient perpendicular to each other. A physical association between the centrosome and nucleus has been reported (Bornens, 1977; Nadezhdina, Fais & Chentsov, 1979; Havercroft, Quinlan & Gull, 1981; Kuriyama & Borisy, 1981) and a possibility that the nucleus influences the normal induction of new centrioles has been suggested (Kuriyama & Borisy, 1981). Therefore, one may speculate that abnormal de novo formation of centrioles in karyoplasts or in sea-urchin eggs results from disruption of a mechanism that, under normal conditions, controls the precise number and configuration of centrioles. In any case, it is possible to induce numerous cytasters in one egg. Because of the presence of one or more (up to eight in our work) centrioles in each cytaster, this system could be quite suitable for the purification and characterization of centrioles.

Many microtubules radiated from the centre of asters, whether they contained centrioles or not. This observation is consistent with many reports that MTOCs need not contain centrioles, and the capacity for organization of microtubules within asters must be a property of some other structure. Gould & Borisy (1977) have demonstrated that the pericentriolar material of Chinese hamster ovary cells is an initiating and anchorage site for microtubules both in vivo and in vitro. As for sea-urchins, Tilney & Goddard (1970) observed a satellite structure on which cytoplasmic microtubules converged in embryonic ectodermal cells. Aggregates of granular material seen in the vicinity of centrioles in the mitotic spindle in early developmental stages of sea-urchin eggs (Harris, 1975) have been suggested as acting as organizing sites for microtubules (Endo, 1979). More directly, Weisenberg & Rosenfeld (1975) demonstrated in vitro nucleation of microtubules from granular material that surrounded the centrioles in homogenates of surf-clam eggs. In the case of asters presented in this study, it was also possible to detect dense, but apparently featureless, material surrounding the centrioles. Astral microtubules appeared to be associated with this amorphous material. However, unequivocal identification of MTOCs in the asters can be made only from an analysis of their functional properties.

It is plausible to expect a specific interaction of centrioles with the material of MTOCs that is needed for microtubule nucleation. Isolated centrioles from starfish spermatozoa have been shown to polymerize microtubules in vitro in a templating manner, while they can initiate the formation of astral arrays of microtubules when they are introduced into eggs (Kuriyama & Kanatani, 1981). Moreover, these two distinct means of microtubule formation in vitro would be explained by the absence or presence of MTOC material around the centrioles. That is, stored or newly
synthesized MTOC components may be activated or accumulated around the centrioles in the cases in which asters form. Therefore, we speculate that the difference between monaster and cytaster formation in artificially activated eggs depends upon different distributions of MTOC material. When no centrioles are present, material may become organized so as to provide a single nucleation site. On the other hand, if centrioles are induced, as for instance by our second treatment, MTOC material may be redistributed around each centriole, resulting in the formation of multiple cytasters.

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