A Rapid and Convenient Procedure for the Detection of Inhibitors of DNA Synthesis Using Starfish Oocytes and Sea Urchin Embryos

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A rapid and convenient procedure for the detection of inhibitors of DNA synthesis using starfish oocytes and sea urchin embryos at the 1-cell stage is described. The procedure is based on the findings that inhibitors of DNA synthesis block mitotic cell division of sea urchin embryos which requires DNA synthesis but do not affect meiotic maturational divisions of starfish oocytes which are independent of DNA synthesis. The efficacy of the method is verified by the isolation of aphidicolin, an inhibitor of DNA synthesis, from the culture filtrate of the fungus *Harziella entomophila* according to the bioassay procedure proposed in this study.

Knowledge of the mechanism governing DNA synthesis is central to our understanding of cell proliferation. The study of the control of DNA synthesis would be helped if we had a variety of chemicals which influenced DNA synthesis in various manners. Furthermore, inhibitors of DNA synthesis are of potential clinical use for cancer chemotherapy since many of the antitumor substances have been shown to inhibit DNA synthesis.

In this paper, we describe a rapid and convenient procedure for the detection of inhibitors of DNA synthesis using starfish oocytes and sea urchin embryos. The procedure is based on the findings that inhibitors of DNA synthesis block cell division of sea urchin embryos at the 1-cell stage which requires DNA synthesis¹⁾ but do not affect meiotic maturational divisions of starfish oocytes which are independent of DNA synthesis.²⁾ The efficacy of the method is verified by the isolation of aphidicolin, an inhibitor of DNA synthesis, from the culture filtrate of the fungus *Harziella entomophila* according to the bioassay procedure described here.

MATERIALS AND METHODS

The starfish mainly used in the present study was

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Asterina pectinifera. The sea urchin mainly used was *Clypeaster japonicus*. The sea urchins *Hemicentrotus pulcherrimus* and *Anthodidaris crassispina*, and the starfish *Asterias amurensis* were also used in some experiments. They were collected from the coastal waters of Kanagawa during their breeding seasons and kept in laboratory aquaria supplied with running sea water. Culture of oocytes and embryos was carried out at 18°~20°C.

Chemicals. The sea waters used in the present study were natural sea water (NSW) and calcium-free sea water (CaFSW), the latter being composed of 2.3% NaCl, 0.07% KCl, 0.34% MgCl₂ and 0.21% MgSO₄, and adjusted to pH 8.0~8.3 with NaHCO₃ (approximately 0.02%). 1-β-D-Arabinofuranosylcytosine was purchased from Yamasa, Chiba. 5-Fluorouracil was from Nakarai, Kyoto. N-Hydroxyurea, vinblastine sulfate and 1-methyladenine were from Sigma, St. Louis. Actinomycin D, cycloheximide and amphotericin B were gifts from Dr. N. Ōtake, Institute of Applied Microbiology, University of Tokyo, Tokyo. Aphidicolin was from Dr. B. Hesp, Imperial Chemical Industries, Macclesfield, U. K. Actinomycin D, amphotericin B and aphidicolin being insoluble in aqueous media were dissolved in dimethylsulfoxide and then dispersed in NSW so as not to exceed the concentration of dimethylsulfoxide 0.1% in the solution. Dimethylsulfoxide at this concentration did not affect biological assays described below. Other chemicals were dissolved in NSW.

Yeast extract was purchased from Difco Laboratories, Detroit.

The biological assay with starfish oocytes. Starfish oocytes were torn from a ripe ovary with forceps, immediately transferred to CaFSW to remove follicle

cells and sedimented with a hand centrifuge. They were washed four times by resuspension in CaFSW and allowed to settle for at least 1 hr. The oocytes were sedimented and suspended in NSW. The oocytes freed of the follicular envelope were at the germinal vesicle stage of meiosis. The addition of 1-methyladenine (45 ng/ml) to these oocytes induced synchronous meiotic maturation.^{3,4)} The first polar body was expelled 1 hr after the initiation of the treatment and the second one 70 min later at 18°C. Morphological changes of the oocytes placed in 0.4 ml of a test solution containing 1-methyladenine (45 ng/ml) and a sample of an appropriate concentration were observed at 150 min after the initiation of the treatment.

The biological assay with sea urchin embryos. Dry sperm of sea urchins obtained by intracoelomic injection of 0.5 M KCl was stored in the cold until use. Eggs were spawned into NSW by intracoelomic injection of 0.5 M KCl, sedimented with a hand centrifuge and washed three times by resuspension in NSW and allowed to settle until use. The egg divided around 90 min after insemination and the second division occurred 30 min later at 18°C. Five min after insemination when fertilization membranes were elevated, the eggs were treated with 0.4 ml of sample solutions. They were examined for morphological changes at 120 min later.

Culture of Harziella entomophila and isolation of aphidicolin. The fungus *Harziella entomophila* was cultured in the Czapek-Dox medium supplemented with 2% yeast extract at 26°C for 30 days. Then the filtrate (2 liters) was removed from the mycelia by filtration through a paper filter. Aliquots of the filtrate were diluted 10- to 40-fold with NSW and the biological activities were examined. The culture filtrate (pH 8.0) was extracted with 1.8 liters of ethyl acetate three times. Extracts were combined and washed with 900 ml of acidified water (pH 4.0 by HCl) three times followed by washing with 900 ml of distilled water. Extracts were dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to afford a powder (42 mg). Two mg of the powder was dissolved in 20 μ l of dimethylsulfoxide and dispersed in 20 ml of NSW. The solution was serially diluted with NSW and the biological activities were assayed as described above. The powder (40 mg) was applied to a Kieselgel HF₂₅₄ plate (20 $\times$ 20 cm). After developing with benzene-ethyl acetate-methanol (50:50:1, v/v), the sulfuric acid-reactive zones were scraped off and extracted with benzene-ethyl acetate-methanol (50:50:1, v/v). Evaporation of the solvents gave powders. Bioassays of an aliquot of each extract were carried out by dissolving it in dimethylsulfoxide and then dispersed in NSW. The biologically active fraction (PC fraction; 5 mg) was recrystallized from benzene-methanol to afford needles (2 mg). The crystal

(500 μ g) was bioassayed as described above.

Other methods. Melting points were recorded on a hot plate and were not corrected.

Gas-liquid chromatography was carried out with a Hitachi K-53 gas-chromatograph using a column (0.3 $\times$ 200 cm) of 3% Silicone OV-1 on Chromosorb W at 210°C. The flow rate of the carrier gas, nitrogen, was maintained at 35 ml/min. Samples were converted to acetanides as described by Dalziel *et al.*⁵⁾ The acetanides dissolved in acetone were injected into the column.

RESULTS

Effects of inhibitors of macromolecule syntheses and other chemicals on Echinoderm cell divisions

Effects of 1- β -D-arabinofuranosylcytosine, N-hydroxyurea, 5-fluorouracil, cycloheximide, vinblastine sulfate, actinomycin D and amphotericine B on the first cleavage of sea urchin (*Clypeaster japonicus*) embryos and 1-methyladenine-induced meiotic maturational divisions of starfish (*Asterina pectinifera*) oocytes were examined, and the results are summarized in Table I.

TABLE I. EFFECTS OF INHIBITORS OF MACROMOLECULE SYNTHESIS AND OTHER CHEMICALS ON CELL DIVISION OF *Clypeaster japonicus* EMBRYOS AT THE 1-CELL STAGE AND MATURATION OF *Asterina pectinifera* OOCYTES INDUCED BY 1-METHYLADENINE (45 ng/ml)

Chemicals	Range of minimum effective concentrations (μ g/ml) to inhibit cell divisions	
	Cleavage of embryos	Oocyte maturation
Arabinofuranosylcytosine	10-1	>100
N-Hydroxyurea	>100	>100
5-Fluorouracil	>100	>100
Cycloheximide	100-10	100-10
Actinomycin D	>10	>10
Vinblastine sulfate	1-0.1	1-0.1
Amphotericine B	1-0.1	1-0.1

Arabinofuranosylcytosine was shown to be a potent inhibitor of DNA synthesis in eukaryotic cells.⁶⁾ The chemical is converted to the triphosphate derivative *in vivo* and the phosphate inhibits the activity of DNA polymerase by competing with deoxycytidine triphosphate.

sphate.⁷⁾ Arabinofuranosylcytosine inhibited the first cleavage of the sea urchin embryo but did not affect meiotic maturational divisions in starfish oocytes (Table I). If arabinofuranosylcytosine had any harmful effects on cell functions other than DNA replication which were essential for karyokinesis and cytokinesis to occur, it would affect maturational process of starfish oocytes as well. Such selective inhibition of cell divisions by arabinofuranosylcytosine as revealed in the present study supports the view that the chemical is a selective inhibitor of DNA synthesis *in vivo*.

N-Hydroxyurea and 5-fluorouracil are known to be inhibitors of deoxyribonucleotide biosynthesis.⁶⁾ Pools of deoxyribonucleoside triphosphates in sea urchin eggs are high and sufficient to support two or three cycles of DNA replication.⁸⁾ Therefore, N-hydroxyurea and 5-fluorouracil do not affect the first cleavage of sea urchin embryos (Table I). Since maturation of starfish oocytes is unaccompanied by DNA replication, N-hydroxyurea and 5-fluorouracil do not affect the divisions. Effects of N-hydroxyurea on early sea urchin embryos and starfish oocytes have been studied by other investigators,^{2,8)} and our study confirmed their results.

Cycloheximide is known to inhibit protein synthesis in eukaryotic cells by inhibiting chain initiation as well as chain elongation by acting on the 60-S subunit of the ribosome.⁹⁾ Protein synthesis is essential for both cleavage of sea urchin embryos¹⁰⁾ and meiotic maturation in starfish oocytes.²⁾ As expected, cycloheximide affected these cell divisions (Table I).

RNA synthesis is quite low during the early cleavage of sea urchin embryos.¹¹⁾ Maternal messenger RNA synthesized during oogenesis and stored in unfertilized eggs plays a central role in translation. Therefore, actinomycin D, which inhibits RNA-polymerase reaction,¹²⁾ was shown to be unable to inhibit cleavage of sea urchin embryos by Gross and Cousineau.¹³⁾ The present study confirmed this (Table I). Zampetti-Bosseler *et al.*²⁾ reported that the maturational process in *Asterias fobesi* oocytes is insensitive to the actinomycin D treatment.

A similar result was obtained here by using *Asterina pectinifera* oocytes (Table I).

Vinblastine sulfate, an inhibitor of the organization of spindle microtubules in eukaryotic cells,¹⁴⁾ was effective in inhibiting divisions in both of starfish oocytes and sea embryos (Table I). The microtubule assembly is fundamental for cells to divide either mitotically or meiotically.

Amphotericin B, a polyene antibiotic, has been shown to form an adduct with sterols in plasma membranes resulting in cytolysis in animal cells.¹⁵⁾ Sea urchin embryos and starfish oocytes suffered lysis in the presence of the antibiotic. The minimum effective concentrations to block cell divisions in these cells were between 0.1 ~ 1 µg/ml (Table I).

These results show that except for an inhibitor of DNA synthesis, chemicals which affect cell functions to support cell division in sea urchin embryos also affect meiotic maturation in starfish oocytes. Therefore, chemicals which block mitosis in sea urchin embryos without affecting maturation in starfish oocytes may be inhibitors of DNA synthesis.

Isolation of aphidicolin from the culture filtrate of Harziella entomophila as a selective inhibitor of mitotic division in sea urchin embryos

In order to exemplify the usefulness of the bioassay system for the detection of inhibitors of DNA synthesis as described in the preceding section, effects of culture filtrates of 50 different actinomycetes and fungi were examined on divisions of sea urchin (*Clypeaster japonicus*) embryos and starfish (*Asterina pectinifera*) oocytes. It was found that the culture filtrate obtained from the fungus *Harziella entomophila* inhibited cleavage of sea urchin embryos but did not affect 1-methyladenine-induced meiotic maturation of starfish oocytes (Table II). Culture filtrates of 11 different strains inhibited both of mitotic division in sea urchin embryos and meiotic maturation in starfish oocytes. Filtrates obtained from the other 38 strains did not block cell divisions of both forms.

The mitosis-inhibitory activity in the filtrate

of *Harziella entomophila* was found in the ethyl acetate fraction obtained by extracting the

TABLE II. EFFECTS OF METABOLITES OF *Harziella entomophila* AT EACH PURIFICATION STEP ON CELL DIVISION OF *Clypeaster japonicus* EMBRYOS AT THE 1-CELL STAGE AND MATURATION OF *Asterina pectinifera* OOCYTES INDUCED BY 1-METHYLADENINE (45 ng/ml)^a

Purification steps	Cleavage of embryos	Oocyte maturation
Culture filtrate		
10-fold diluted	—	+
20-fold diluted	±	+
40-fold diluted	+	+
Ethyl acetate fraction		
100 µg/ml	—	+
50	—	+
25	—	+
13	+	+
PC fraction after recrystallization		
10 µg/ml	—	+
1	—	+
0.5	—	+
0.25	±	+
0.13	+	+
Aphidicolin (authentic)		
10 µg/ml	—	+
1	—	+
0.5	—	+
0.25	±	+
0.13	+	+

^a The degree of inhibition was expressed as follows: —, more than 90% of cells undivided; ±, 90~10% of cells divided; +, more than 90% of cells divided.

FIG. 1. Procedure of Purifying Aphidicolin from Culture Filtrate of *Harziella entomophila* as an Inhibitor of Cleavage of Sea Urchin Embryos at the 1-Cell Stage.

filtrate with ethyl acetate (Fig. 1). Table II shows that the ethyl acetate fraction at the concentration of 25 µg/ml inhibited mitotic division whereas starfish oocyte maturation proceeded normally in the presence of 4-times higher concentration of the fraction. The PC fraction responsible for the mitotic inhibition was obtained by plate chromatography (Fig. 1). The crystal obtained from the fraction carried the biological activity (Table II). Since the fungus has been shown to produce aphidicolin under similar culture conditions and the chromatographic behaviour is the same with that of aphidicolin as shown by three of the authors (K.K., Y.K. and A.S.),¹⁶⁾ the crystal was directly compared with authentic aphidicolin. Their acetanilides showed an identical retention time (25 min) on gas-liquid chromatographic analysis under the conditions described in MATERIALS AND METHODS. The crystal, authentic aphidicolin and their mixture showed the same melting point at 233°~236°C. These results demonstrate clearly that the crystal is aphidicolin. The biological activity of authentic aphidicolin is the same with that of the crystal obtained in the present study (Table II).

Aphidicolin was originally obtained from the culture filtrate of the fungus *Cephalosporium aphidicola* and its structure was convincingly determined by Dalziel *et al.*⁵⁾ Bucknall *et al.*¹⁷⁾ reported that aphidicolin inhibits the growth of herpes simplex virus cultured on human embryonic lung cells or on rabbit eyes. They also showed that aphidicolin inhibits the incorporation of thymidine into DNA of cultured human embryonic lung cells.

Effects of aphidicolin on cell divisions of other Echinoderm species than Clypeaster and Asterina

Clypeaster japonicus breeds during May, June and July at the coastal waters of Kanagawa. The breeding season of *Asterina pectinifera* there is May and June, and that at Mutsu Bay, Aomori, is August and September. Ripe ovaries can be obtained from the starfish collected at the vicinity of Uozu, Toyama, during

November and December. Although breeding periods of these animals can be prolonged for more than 3 months in some case by keeping them in aquaria supplied with cold running sea water, it is desirable to test the effectiveness of the bioassay with other sea urchin species to be able to work at different periods of a year.

Aphidicolin at 40 $\mu\text{g/ml}$ did not affect 1-methyladenine (45 ng/ml)-induced maturation in oocytes of *Asterias amurensis*. The breeding season of the starfish is January to April at the vicinity of Tokyo and May to July at Akkeshi, Hokkaido. The minimum effective concentrations of aphidicolin to block mitotic cell divisions of *Anthocardis crassispina* and *Hemicentrotus pulcherrimus* embryos were nearly the same with those found in *Clypeaster japonicus* embryos (Table III). *Anthocardis crassispina* spawns during July and August at Sagami Bay. *Hemicentrotus pulcherrimus* breeds during January to April at Sagami Bay and near Tamano, Okayama.

No species specificity of the aphidicolin action was observed in the present study.

TABLE III. INHIBITORY EFFECTS OF APHIDICOLIN ON CELL DIVISION OF *Clypeaster japonicus*, *Hemicentrotus pulcherrimus* AND *Anthocardis crassispina* EMBRYOS AT THE 1-CELL STAGE^a

Concentration of aphidicolin ($\mu\text{g/ml}$)	<i>Clypeaster japonicus</i>	<i>Hemicentrotus pulcherrimus</i>	<i>Anthocardis crassispina</i>
1	—	—	—
0.5	—	±	±
0.25	±	±	±
0.13	+	+	+

^a Degree of inhibition, see Table II.

DISCUSSION

Starfish oocytes are ideal cells for investigating meiotic maturational divisions since they can be obtained in great numbers, they are arrested at the dictyate stage of meiosis, and they divide rapidly and synchronously after 1-methyladenine addition.³⁾ Meiotic maturation as induced by 1-methyladenine is essentially a reduction in chromosome number. In other words, no chromosome replication

takes place during maturational divisions. We have demonstrated in this study that inhibitors of DNA synthesis have no effects on the maturational process.

Sea urchin eggs are ideal cells for studies of the control of DNA synthesis because they can be obtained in great numbers, they incorporate radioactive precursors into DNA, they are very synchronous, and the period of synthesis is short and very definable in time. DNA synthesis is essential for the successful cleavage and development. The dependence of cleavage on the synthesis of DNA may need some words since the famous experiment by Harvey¹⁸⁾ showed that parthenogenetically-activated annulate halves of sea urchin eggs can cleave in an orderly manner. In later studies,¹⁹⁾ however, she demonstrated that capabilities of the ooplasm to cleave were altered after fertilization, in that annulate halves derived from fertilized eggs did not cleave while the nucleated halves developed normally. The synthesis of proteins necessary for cleavage depends upon the synthesis of DNA according to the observations by Young *et al.*¹⁾ with the sand dollar *Echinarachnius parma* embryos.

The bioassay system for the detection of inhibitors of DNA synthesis as proposed in this study depends on the assumption that test samples are permeable equally to sea urchin embryos and starfish oocytes. The premise is not unreasonable in the case of lipophilic and non-ionic chemicals, aphidicolin being one of them, since a barrier to passive movements of such chemicals across plasma membranes is quite small.²⁰⁾

The experimental results described in this study suggest that aphidicolin is a selective inhibitor of DNA synthesis in sea urchin embryos. However, more detailed informations are obviously required to support this. In this context, one of the authors (S.I.) has recently obtained evidence supporting the procedure and verifying the usefulness of our procedure to detect inhibitors of DNA synthesis. The findings, which were reported in a brief preliminary form,²¹⁾ are as follows. The incorporation of thymidine into DNA of *Hemicentrotus*

pulcherrimus embryos was inhibited by 90% with a 2 μ g/ml concentration of aphidicolin which inhibited mitosis and cytokinesis of 90% of the embryos. A similar result was obtained with *Clypeaster japonicus* embryos (S.I., to be published). Aphidicolin at 10 μ g/ml did not affect leucine incorporation into protein fraction of *Hemicentrotus pulcherrimus* embryos at the 1-cell stage. Aphidicolin did not affect uridine incorporation into RNA of sea urchin embryos at the gastrula stage. Aphidicolin at 2 μ g/ml inhibited 90% of the activity of DNA polymerase α obtained from nuclei of *Hemicentrotus pulcherrimus* embryos without affecting the activity of DNA polymerases β and γ . This suggests that aphidicolin blocks mitotic cell division by interfering with the activity of DNA polymerase α and that DNA polymerase α is the enzyme responsible for replication of DNA in sea urchin embryos. Further, aphidicolin has been shown to inhibit the activity of DNA polymerase α obtained from regenerating rat liver without affecting the activity of DNA polymerase β and mitochondrial DNA polymerase.²²⁾ Aphidicolin had no effects on the activity of thymidine kinase obtained from rat liver. Activities of *Escherichia coli* DNA polymerase I and avian myeloblastosis virus reverse transcriptase remained unchanged by the treatment with aphidicolin.²²⁾ The remarkable biological activity of aphidicolin shown by these studies will be the basis for using the drug as a tool of dissecting the specific step in chromosomal replication, thereby better understanding of the mechanisms governing the complex process of replication can be achieved.

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