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* To whom correspondence should be sent.
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Cultivation of Bacteria-Free *Hydra viridis*: Missing Budding Factor in Nonsymbiotic Hydra

Abstract. *Bacteria-free hydra, cultured in sterile media, were fed bacteria-free larvae of* Artemia salina. *Normal growth and budding were obtained in symbiotic and aposymbiotic* Hydra viridis. *Two nonsymbiotic hydra species did not form buds under bacteria-free conditions. When these hydra were fed nonsterile* Artemia, *or if the medium was reinoculated with bacteria isolated from budding stock cultures, normal budding was resumed. An exogenous budding factor, which can be provided by nonsterile* Artemia *larvae, or even by some bacteria, appears to be required by these nonsymbiotic hydra. This factor is endogenous in* Hydra viridis.

The simple freshwater hydra is a valuable animal for research in cell and developmental biology (*1*). The green symbiotic *Hydra viridis* has been used for research of nutritional aspects of the algae-hydra symbiosis (*2*). For many investigations the culture of hydra in the presence of bacteria does not cause problems. However, for critical nutritional and biochemical studies of hydra, the organisms grown in bacteria-free media are necessary to ensure that the measured parameters are those of the hydra and not of contaminants. We now report the successful maintenance and growth of bacteria-free *Hydra viridis* fed bacteria-free larvae of *Artemia salina*. Two nonsymbiotic hydra species were maintained, but did not bud under similar conditions.

Stock cultures and bacteria-free cultures of hydra were grown in dim light, at about 600 lux and 20°C, in M solution, a buffered mixture of inorganic salts resembling pond water (*3*). Phenol red, a nontoxic *p*H indicator, was added at 1 mg/liter to give continuous visual monitoring of *p*H changes caused by bacterial contamination. In contaminated cultures, the color of the medium changes from pink to yellow as the *p*H drops (*4*).

Bacteria-free hydra were obtained as follows. Several hydra were taken from a stock culture, starved for 48 hours, and washed three times in fresh M solution and once in a sterile solution. The hydra were then placed in a drop of the sterile M solution on a microscope slide, and the basal part, to which many microorganisms adhere, was cut off immediately below the budding zone. The upper parts were then washed twice in sterile M solution and transferred with a Pasteur pipette into standard test tubes (22 by 160 mm) containing M solution and penicillin, streptomycin, neomycin, and chloramphenicol or rifampicin (100 μg/ml of each). Excess light was avoided when chloramphenicol was used (*5*). After 48 hours, the hydra were transferred into sterile test tubes containing M solution only. For sterility tests, we transferred one hydra from each prepared batch into a test tube with a sterile 2 percent proteose peptone solution and incubated it at 33°C. We took the absence of bacterial growth in this medium, after 3 or 4 days, as an indication that the hydra were bacteria-free.

We continuously verified the absence of bacteria in our cultures by watching the pink color of the phenol red in the medium. We also occasionally tested the spent medium for sterility. A sterile proteose peptone solution was added until a final concentration of 2 percent was attained and the medium was then incubated for 3 to 4 days at 33°C. This incubation time was found to be sufficient since no bacterial growth occurred when such a medium was incubated for longer periods.

Bacteria-free larvae of *Artemia salina* were obtained by decapsulation (*6*) of commercially available cysts (*7*). The cysts (200 to 300 mg) were hydrated by soaking for 1 hour in a 3 percent sodium chloride solution and agitated by air bubbling. Floating cysts were discarded and the rest were placed for 15 minutes in a 20 percent solution of sodium hypochlorite (~ 2 percent available chlorine), in a small crystallizing dish. Decapsulation was monitored by observing a gradual change in the color of the cysts from brown through white to orange, indicating complete decapsulation. Routine aseptic techniques were then applied. The cysts were collected with a Pasteur pipette and washed three times in 10 ml of sterile M solution in standard test

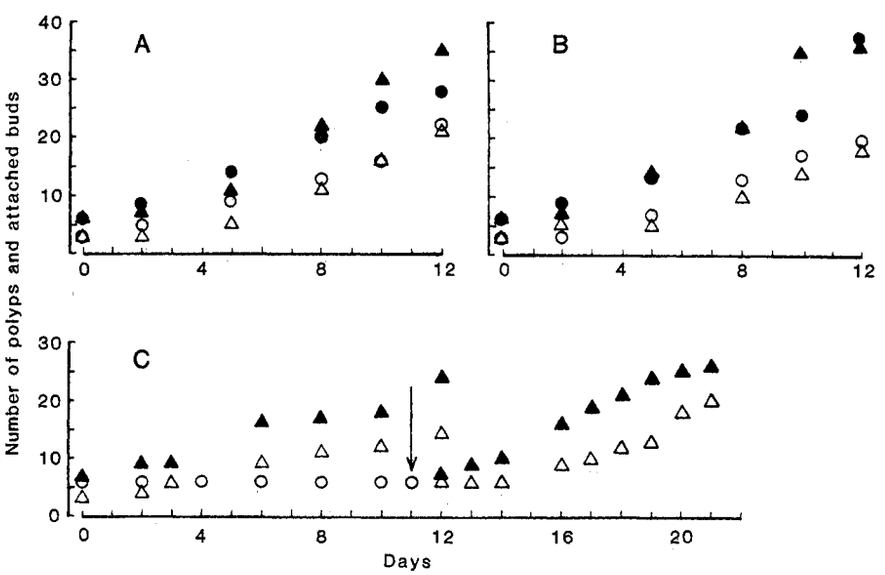

Fig. 1. Budding rates of nonsterile and bacteria-free hydra. (A) Green *H. viridis*, (B) aposymbiotic *H. viridis*, and (C) *H. vulgaris*. (○) Bacteria-free polyps, (●) bacteria-free polyps with their attached buds, (△) nonsterile polyps, and (▲) nonsterile polyps with their attached buds. Each experiment was done in three test tubes containing one budding polyp in 10 ml of M solution, except the bacteria-free *H. vulgaris* experiments (C), where each test tube contained two nonbudding polyps. Nonsterile animals were fed nonsterile *Artemia* five times a week and bacteria-free animals were similarly fed bacteria-free *Artemia*. In (C), no budding of bacteria-free *H. vulgaris* occurred up to the 11th day, at which time nonsterile *Artemia* were fed to these animals (arrow).

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tubes. Retention of the pink color (*p*H 7.5) by the phenol red in the M solution, indicates removal of excess sodium hypochlorite. The washed cysts were then divided into several test tubes with 3 ml of a 4 percent proteose peptone in M solution and placed on a rotating wheel (~ 25 rotations per minute) at 33°C in dim light. The proteose peptone solution served both to hatch the *Artemia* cysts and to test their sterility. Clarity of the peptone broth indicates that the hatched larvae are bacteria-free.

Normal hatching of the decapsulated cysts begins within 24 and 48 hours, and up to 75 percent of the cysts will hatch. Larvae were collected aseptically with a Pasteur pipette, washed three times in sterile M solution and offered to the hydra. Larvae of *Artemia* survive in the hypotonic M solution for several hours and are readily captured by the hydra. Dead larvae and unhatched cysts do not decay in the bacteria-free medium, but slowly undergo autolysis. Although we did not find any ill effects on the hydra from accumulated dead larvae, we transferred our hydra into fresh dishes about once a week.

Ten clonal cultures of bacteria-free symbiotic and five of bacteria-free aposymbiotic *H. viridis* (Swiss strain) were initiated from single hydra placed in 10 ml of M solution in standard test tubes. The feeding response of the hydra was normal, and their number doubled about every 4 days. Some hydra synchronously formed two or three buds. As the number of hydra in the test tubes increased (> 30), we transferred them into Erlenmeyer flasks (150 ml) or into test tubes (36 by 160 mm) containing 50 ml of M solution to prevent inhibitory effects of crowding (*8*), and to initiate mass cultures. Controls, consisting of 60 bacteria-free green hydra starved for 30 days, formed only six buds during this period. We concluded that our *H. viridis* obtained all of their nutritional requirements from the bacteria-free larvae of *Artemia* and that no additional factors or bacterial products are required for their vegetative reproduction.

We tried to initiate similar bacteria-free cultures from ten hydra specimens of each of two nonsymbiotic strains of *H. vulgaris*. These hydra, like *H. viridis*, were fed bacteria-free larvae five times a week, but in 3 to 4 weeks, no buds had formed. Some of these hydra were then fed nonsterile *Artemia*, and others were inoculated with bacteria isolated from budding stock cultures; in all of these hydra, normal budding was resumed after 1 to 3 days (Fig. 1).

We conclude that the nonsymbiotic species of hydra that we studied lack a budding factor that is endogenous to *H. viridis*. This missing budding factor, which may be a nutrient, a vitamin, or a hormone, can be provided exogenously by either nonsterile *Artemia* larvae or by some bacteria. Our method of producing an aquatic bacteria-free predator-prey system may be useful in providing similar bacteria-free systems for nutritional and developmental studies.

M. RAHAT
CH. DIMENTMAN
Department of Zoology,
Hebrew University of Jerusalem,
Jerusalem, Israel

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Cyclic Biological Expression in Mouse Mammary Tumors

Abstract. *Biological characteristics were assessed in GR mouse mammary tumors during 22 serial transplantations. Although unidirectional progression from hormone dependency to independency was observed, other biological markers such as progesterone receptors, polyploid frequency, and thymidine kinase activity demonstrated cyclic phenomena every fourth to sixth transplant generation, suggesting the continued presence of regulatory mechanisms among various cell subpopulations.*

Breast cancers, even of the same histologic type, show a wide spectrum of biological behavior and consist of heterogeneous cell populations. Treatment can provide temporary improvement in some patients, but nearly all patients with advanced breast cancers die as a result of the disease. Continuous changes in the biological behavior of tumors could account for the failure of many clinical therapies (*1*).

Most tumors show a gradual and unidirectional biological change toward a more undifferentiated state, a process usually referred to as tumor progression (*2*). Study of tumor biological markers has demonstrated a decrease in steroid receptor levels during the progression of human breast cancers (*3*) or after serial transplantations of rodent mammary tumors (*4*). Undifferentiated tumors also are associated with a polyploid chromosomal composition (*5*) and high thymidine kinase activity (*6*).

To gain a better understanding of the evolution of mammary tumors we assessed the changes of steroid receptor concentration, thymidine kinase activity, and chromosomal pattern in GR mouse mammary tumors during their transition from hormone-dependent to hormone-independent states.

Mammary tumors were induced with estrone and progesterone pellets in oophorectomized 3-month-old female GR mice, as described by Sluyser and Van Nie (*7*). Mammary tumors usually developed in 2 to 3 months. For transplantation, tumor cell suspensions were injected into the flank region of (BALB/c × GR)F_1 hybrid mice, which had been castrated 1 week previously. In transplant generations 1 and 2 (TG1 and TG2), 1.5×10^7 cells were used, and for the later generations (TG3 to TG22), 2×10^6 viable cells were used. The viability of the tumor cells was determined by trypan blue exclusion.

The transplant recipients were divided into two groups: group 1, those that underwent oophorectomy without hormonal maintenance, and group 2, those that were maintained with the estrone-progesterone supplement (*7*) after oophorectomy. Two of the tumors that developed in the mice in group 2 were used for the next transplantation into four or five mice each in groups 1 and 2. Four to five of the tumors that developed in each of groups 1 and 2 at various transplant generations were used for cytogenetic study, steroid receptor assay, and thymidine kinase assay.

The rate of tumor development was expressed as the number of days between tumor cell inoculation (day zero) and tumor diameter reaching approximately 1 cm. The difference in tumor development rate between groups 1 and 2 was used as a criterion for hormonal dependency or independency.

The tumor growth pattern (Fig. 1A)

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