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Binary Fission and Encystation of *Opalina* sp. in Axenic Medium

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ABSTRACT—*Opalina* sp. were cultivated under an anaerobic condition in the complex medium containing DME/F-12 HAM mixture (Sigma) as a basic medium, and fetal bovine serum and autoclaved rumen fluid as additional components. In the medium, the opalinid cells reproduced by binary fission, and in the longest case they were maintained for more than one month, following successive transfers. When cells collected from the host in breeding season were cultured in the same medium, encystation occurred after rapid cell divisions. In this case, the population obtained from the frog had contained a sub-population of cells with small number of nuclei and small cell size. The sub-population might have been in the phase of palintomy that is a rapid sequence of binary fissions without cell growth and finally produces small cysts. Our successful maintenance of the opalinid cells can offer the first step for the establishment of the in-vitro culture method.

INTRODUCTION

The opalinids are multinuclear protists living widely in the hindgut of the cold-blooded vertebrates, mainly anurans, as endosymbionts. They are presently classified into four genera, *Opalina*, *Protoopalina, Cepedea* and *Zelleriella*, based on both their shape of cell body in cross-section and the number of nuclei (reviewed by Corliss, 1990).

The life cycle of the opalinids has been well investigated in the genus *Opalina* (Wessenberg, 1961; 1978). In adult frogs, the opalinid cells asexually reproduce by binary fission in any seasons. In breeding season of the host, their cells successively divide without cell growth. As a result, the fission products become smaller in size and their nuclei decrease in number until they finally encyst. This entire stage including such successive divisions is termed palintomy. After encystation, cysts are expelled with feces of their host and fed by tadpoles. In the intestine of tadpoles, the ingested cysts hatch and give rise to micro- and macro-gametes after meiosis. The gametes fuse to zygotes, and then the zygotes grow and increase their number of nuclei. In this way, the general outline of the opalinid life cycle has been described in details. However, the description seems not to be complete (Wessenberg, 1978).

The opalinids have long been one of the controversial protists with respect to their systematic position (Cavalier-Smith, 1993; Corliss, 1955, 1994; Margulis and Schwartz, 1982; Metcalf, 1923; Patterson, 1985; Sandon, 1976). At present, no information as to molecular phylogeny of the opalinids is available. A lack of valid methods for in-vitro cultivation of the opalinids makes it difficult to carry out their molecular biology; practically it is nearly impossible to get cells in large quantity and in high purity without contamination of other protozoa, fungi, and metazoa such as nematodes.

In order to clarify the complete life cycle of the opalinids, to know the switching mechanism from the asexual to sexual cycle and to progress molecular biology of the opalinids, in-vitro cultivation might be indispensable. So far, several attempts in in-vitro culture have been reported in *Opalina* (Yang 1960; Yang and Bamberger, 1953) and in *Cepedea* (Lwoff and Valentini, 1948). However, no confirmation in other laboratories has been obtained and thus no successful culture method has been established: The researchers of the field seem to have an agreement that the axenic culture of the opalinids is still a real obstacle (Corliss, 1990; Wessenberg, 1978).

Under this situation, it is worthwhile to try to establish in-vitro culture method. In this report, a new attempt for in-vitro culture of *Opalina* sp. is reported: We show that cells increased in number by binary fission in a new axenic medium. We also report palintomy and encystation in the same medium. Some further problems for the axenic culture will be discussed.

MATERIALS AND METHODS

Source and preparation of opalinid cells

Cells used in this study were obtained from three different species of frogs, *Hyla japonica*, *Rana japonica* and *Rhacophorus schlegelli*, which inhabited around Kanazawa in Japan. The dates when the hosts were collected are listed in Table 1. Each experiment was carried out within two days after the dates. In order to obtain the

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Table 1. Host species of *Opalina*

Fig. No.	Date*	Host species
1, 2, 3, 4	Jun. 11. 1998	*Rana japonica*
5, 6, 7	Oct. 16. 1998	*Hyla japonica*
8, 9	Apr. 10. 1999	*Hyla japonica*
10, 11, 12, 13	Apr. 24. 1999	*Rhacophorus schlegelli*

All host species were collected in the vicinity of Kanazawa in Japan.
* Dates when the host frogs were captured. Culturing experiments were carried out within two days after the capture.

opalinid cells, the surface of the host frog body was disinfected with 70% ethanol. Then the opalinid cells were collected from the intestine of the host and were washed with the salt buffer solution (80 mM NaCl, 3.6 mM KCl, 1.1 mM $MgSO_4$, 0.049 mM KH_2PO_4, 0.12 mM $CaCl_2$) as described in Wessenberg (1961). In order to remove materials derived from the host intestine as completely as possible, cells were washed twice by centrifugation (300 x g), and then the cells were laid on the top in a long (11 cm) tube filled with the buffer and naturally precipitated for 1–10 min. The latter step was repeated 6–10 times.

Culture conditions

Culture medium consisted of 7.625% (w/v) DME/F-12 HAM mixture (Sigma), 5 or 10% (v/v) Fetal Bovine Serum (FBS: Nichirei, Japan) and 5 or 10% autoclaved rumen fluid (ARF) obtained from bovine. FBS was added without being inactivated. The rumen fluid was kindly supplied by the Kanazawa city meat inspection center and was used after filteration with gauze folded in eight and then autoclaved. Washed cells (30–50 µl) were transferred into a petri dish (35 mm× 10 mm) containing 2 ml culture medium and incubated anaerobically at 26°C. To avoid contamination of fungi, Aureobasidin A (Nacalai Tesque) was added (0.5 µg/ml). Penicillin-Streptomycin mixture (Sigma) was also added to establish a bacteria-free condition (200 units/ml and 200 µg/ml, respectively).

Anaerobic condition was prepared utilizing BBL gas pack anaerobic system which is hydrogen and carbon dioxide generator (Becton Dickinson, USA). The anaerobic condition reaches a redox potential of approximately –200 mV within 2 hr in the culture medium.

Observation of culturing cells

The cell number in a petri dish was directly counted everyday under a microscope equipped with a digital camera. Although culturing cells were transiently placed in an aerobic condition this time, they were transferred again into the anaerobic condition soon after taking photogarphs and counting the cell number. When the cultures finished, cells were fixed with formaldehyde and stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in order to measure their cell length and the number of nuclei.

RESULTS

The opalinids used in this study had a flat body in cross-section and a lot of nuclei. These characteristics show that they belong to the genus *Opalina*. Judging from the previous descriptions (Wessenberg, 1961; 1978), they seem to be *O. ranarum*-type cells, although they were obtained from three different species of frogs. However, it is unknown whether all opalinids used belong to the same species.

In preliminary experiments, several factors effective for longer maintenance of the opalinid cells were surveyed. Anaerobic condition, and addition of 5-10% FBS and autoclaved rumen fluid (ARF) proved to be effective (data not shown). Addition of ARF has already been reported to be effective in an axenic culture of the hypermastigote *Trichomitopsis* (Yamin, 1978). In particular, anaerobic condition was indispensable in our culture system. Under these conditions, the following experiments were carried out.

Binary fission in axenic medium

Opalina sp. obtained from the frog *Rana japonica* was cultured in the medium containing 5% FBS and 5% ARF (Fig. 1, 2). In this culture, growth of bacteria and fungi was completely inhibited by adding antibiotics. *Opalina* cells did not grow for the first four days after starting cultivation. Then the number of cells gradually increased and reached approximately 2.3 times as many as the initial number. Subsequently the number of cells fluctuated everyday, reflecting cell division and death of abnormal division products. At this point, two anomalies were observed: Cells were observed whose nuclei were irregularly arranged and whose shape was abnormal, when compared with the cells before cultivation (Fig. 3, 4). After culturing for 14 days, cells were fixed for observation. The two populations before and after cultivation were compared as to the number of nuclei and the cell size (Fig. 2): Cells before cultivation had 290 nuclei on average, whereas the average in cells after cultivation decreased by approximately a half, 150. Cells were observed to have miniaturized from 503 to 415 µm on average when cultivation finished. However, the distributions of the number of nuclei and the cell size were observed to roughly overlap before and after cultivation. In this experiment, the culture medium without antibiotics was also prepared as a control. In this culture, bacteria vigorously grew and *Opalina* cells died in six days (Fig. 1).

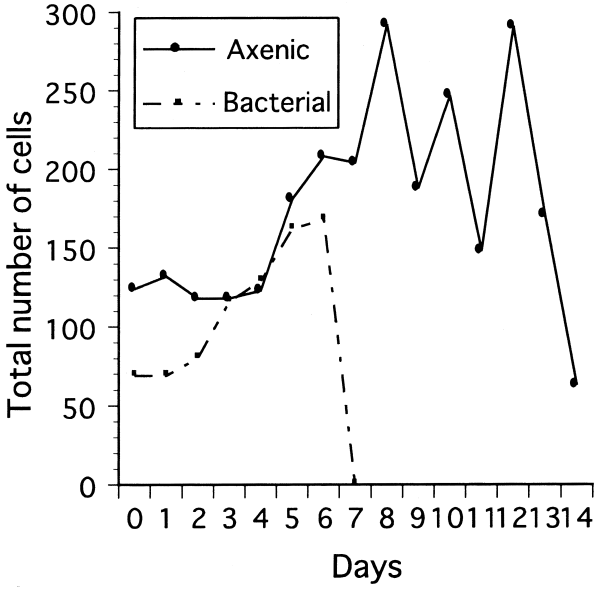

Fig. 1. Growth of *Opalina* sp. in axenic medium. The cells were obtained from the frog *Rana japonica*. The culture medium contained 5% fetal bovine serum (FBS) and 5% autoclaved rumen fluid (ARF) as additional components with (*solid line*) and without antibiotics (*broken line*).

These results suggest that the increase of cell number in this axenic medium is due to binary fission but not palintomy, as we show below.

In order to confirm the above conclusion, the next experiment was carried out using cells from the host *Hyla japonica*. We fixed cells 0, 8 and 13 days after cultivation and examined the number of nuclei and their cell size of the respective samples (Fig. 5). When we compared the cells after 0 days to those after 13 days, the similar result was obtained with that in the previous experiment. The average number of nuclei decreased and the cell size became smaller 13 days after cultivation. However, the cells 8 days after had approximately the same number of nuclei and the same size on average as the cells before cultivation. In particular, part of the cells in this population had more than a thousand nuclei and their sizes were bigger than those of the cells before cultivation. In addition, many dividing cells were observed although morphological anomalies appeared again (Fig. 6, 7). In this culture, the number of cells increased from 98 (0 day) to maximum 242 (12 days after). These results seem to show that at least part of the cell population increased their size and the number of nuclei before binary fission.

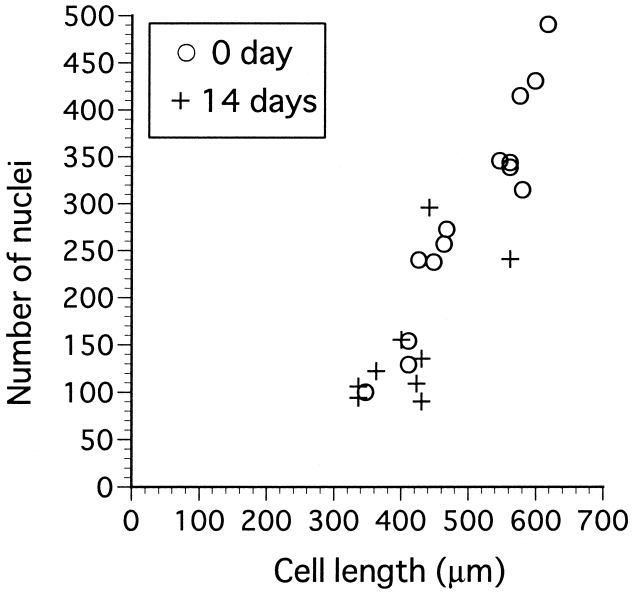

Fig. 2. Distribution of the number of nuclei and cell size (cell length) in two populations before (*open circle*) and 14 days after (*cross*) cultivation. The number of nuclei decreased from 290±115 to 150±72, and their cell size changed from 503±85 to 415±69 μm on average. The number of cells examined was 14 (0 day) and 9 (14 days), respectively. The limits of the distribution of the two populations fundamentally overlap.

Increase of the concentrations of FBS and ARF improved the culture condition to some extent. Cells obtained from the frog *Hyla japonica* were cultured in the medium containing 10% FBS, 10% ARF and antibiotics. In this case the number of cells increased up to more than seven times as many as the initial number in six days (Fig. 8). There was no remarkable decrease in the number of nuclei or change in cell size (Fig. 9), and no morphological anomaly was observed during the culture. Prolifecation of *Opalina* cells by binary fission was clearly demonstrated in this culture. Although it is still preliminary, in another batch of culture, cells were maintained for more than one month by transfers of them into a fresh medium (data not shown). In this case, morphological anomaly was observed 10 days after the beginning of the culture.

Encystation in axenic medium

In contrast to the above experiment, when cells from the frog *R. schlegelli* were cultured, the cells showed different behaviors. The cells rapidly divided and reached 25-fold of the initial cell number in six days (Fig. 10). At the same time, cyst formation was observed. This population contained the cells from the beginning which had relatively small cell size and fewer number of nuclei (Fig. 11). Each of the round shaped cysts had one to six nuclei, but most of them contained two to four nuclei (Fig. 12, 13). The cysts also differed in size (14.7 to 27.6 μm) depending upon the number of nuclei. The average diameter was 20.2 μm. The remaining cells consisted of

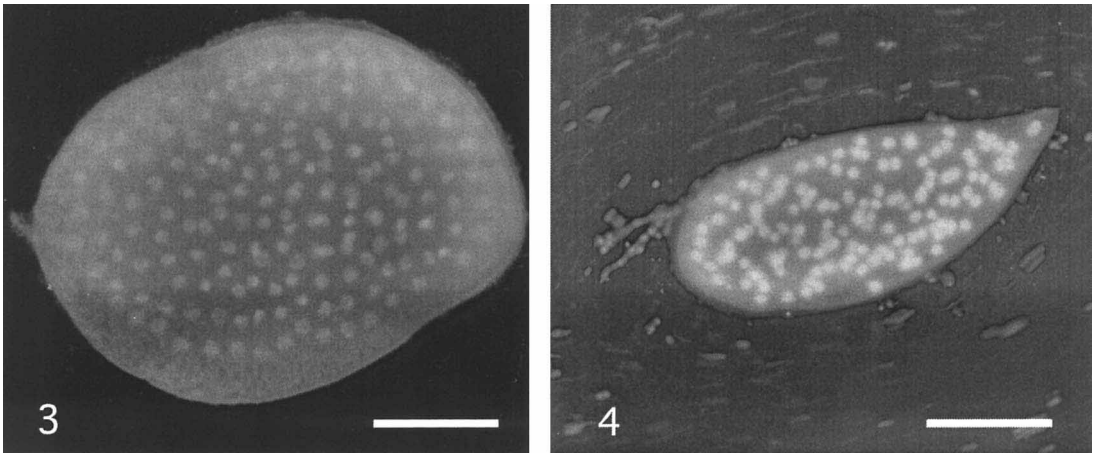

Fig. 3–4. Photographs of *Opalina* before and after cultivation. **3**. Before cultivation. **4**. 14 days after cultivation. Arrangement of nuclei in the cells 14 days after was irregular when compared with the cells 0 day. Bar=100 μm.

the following two types of population (Fig. 11). One population contained cells that had a small number of nuclei and were of small body size. The other contained cells that had many nuclei and were of relatively big body size. Judging from the appearance of the cysts, the former population would have been in the phase of palintomy and the latter in the phase of binary fission. In this experiment, no significant morphological anomaly of the cells in palintomy was observed in six days. These results indicate that palintomy and the subsequent encystation normally occurred in the axenic medium.

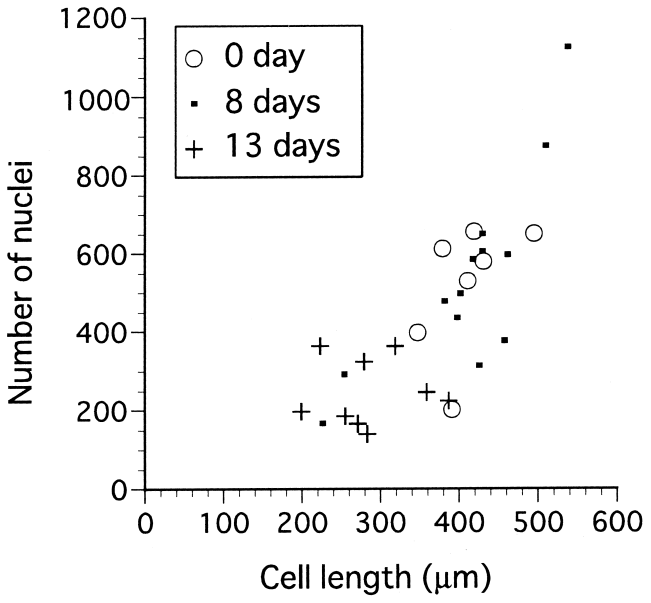

Fig. 5. Distribution of the number of nuclei and cell size in three populations 0 day (*open circle*), 8 days (*square*) and 13 days (*cross*) after cultivation. Cells from the host *Hyla japonica* was cultured. The number of nuclei changed as follows: 522±165 (0 day), 535±256 (8 days) and 245±85 (13 days). Similarly, cell size as follows: 411±47 μm (0 day), 412±87 μm (8 days) and 287±61 μm (13 days), respectively. The number of cells examined was 7 (0 day), 13 (8days) and 9 (13 days). The cells 8 days after cultivation had approximately the same number of nuclei and the same size on average as the cells before cultivation.

These last two experiments using *H. japonica* and *R. schlegelli* were carried out in April. The difference of the results, binary fission or encystation, may reflect on the difference of breeding season in the two host species. In regard to this, we discuss below in a little detail.

DISCUSSION

In the present paper, we demonstrated a cultivation of *Opalina* sp. in the axenic medium. An anaerobic condition was effective to maintain cells longer. It is known that intestinal environment in various animals is usually anaerobic. For example, in order to culture symbionts such as the hypermastigote *Trichomitopsis* (Yamin, 1978) and *Entamoeba* (Gillin and Diamond, 1978), an anaerobic condition is indispensable. In the case of *Opalina*, such a condition is quite important at least in our culture system. Once the in-vitro cultivation of *Cepedea dimidiata* (Lwoff and Valentini, 1948) and *Opalina ranarum* (Yang, 1960; Yang and Bamberger, 1953) were reported as successful in aerobic condition, although no one has succeeded in a double check of the findings (Wessenberg, 1978). However, the cultural conditions might have been anaerobic. In Lwoff and Valentini's culture, their complex medium contained vitamin C that is known to have a deoxidizing effect. The presence of the vitamin C could have produced a weak anaerobic condition that was tolerant of the maintenance of *Cepedea*. When their cultures were contaminated with a Gram-negative bacteria after 15 subcultures, the opalinid cells grew much better than before. It is possible that bacterial growth produced a more anaerobic condition in the medium. In Yang and Bamberger's experiment, bacteria and *Entamoeba* grew together with *Opalina*. As mentioned above, *Entamoeba* can be cultivated only in an anaerobic condition. The growth of *Entamoeba* in Yang and Bamberger's medium indirectly shows the establishment of an anaerobic condition; contamination and growth of bacteria might have been responsible for exhaustion of oxygen in the culture medium. The results of both Lwoff and Valentini's and Yang and Bam-

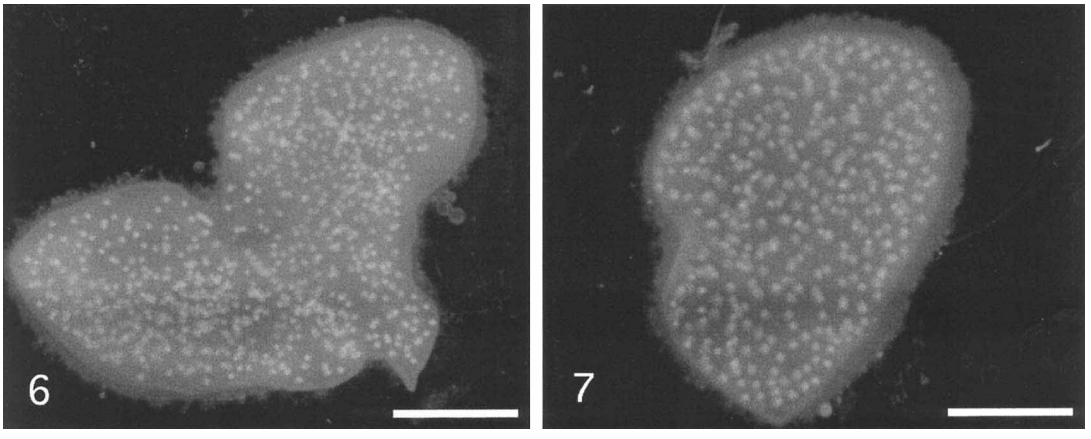

Fig. 6–7. Photographs of *Opalina* cells 13 days after cultivation. **6**. Dividing cell with morphological abnormality. Numerous nuclei are visible. **7**. Cell just begin to divide with slightly abnormal morphology. Bar=100 μm.

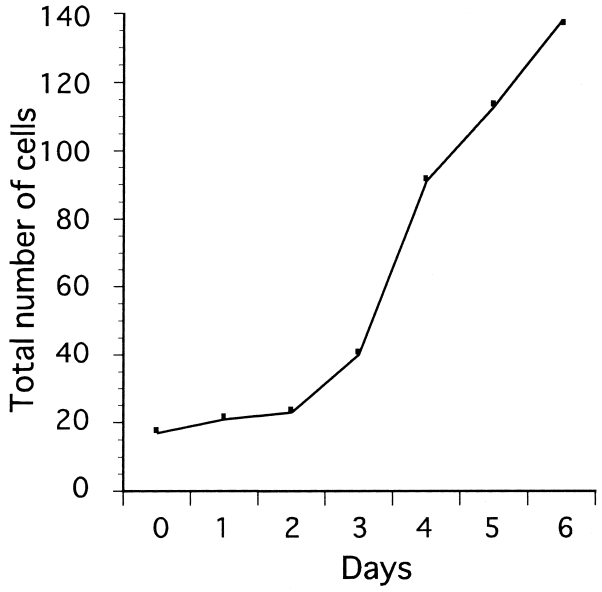

Fig. 8. Growth of *Opalina* sp. in axenic medium. The cells were obtained from the frog *Hyla japonica*.

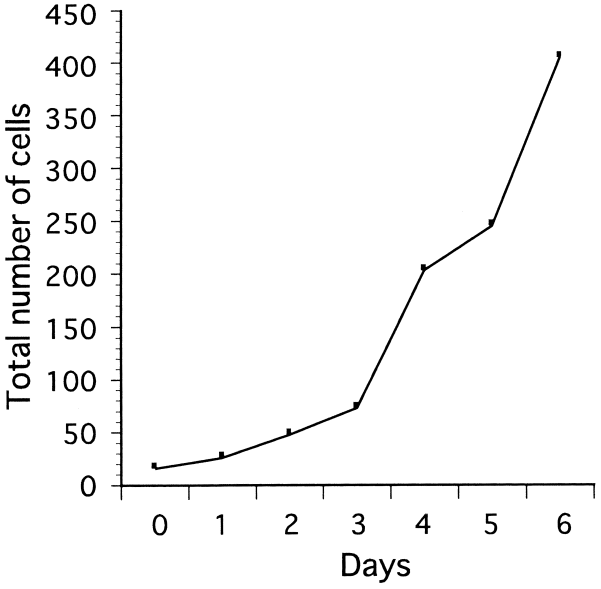

Fig. 10. Growth of *Opalina* sp. in axenic medium. The cells were obtained from the frog *Rhacophorus schlegelli*. Cells rapidly divided and reached 25-fold of the initial cell number in 6 days. Cyst formation was observed, but their cysts were not counted as cells.

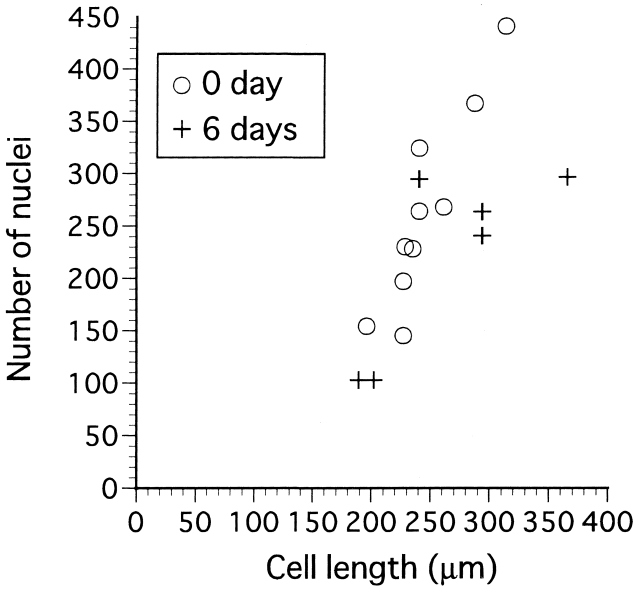

Fig. 9. Distribution of the number of nuclei and cell size in two populations before (*open circle*) and 6 days after (*cross*) cultivation. The number of nuclei slightly decreased from 264±95 to 217±91, whereas their cell size increased to some extent from 246±34 to 265±66 μm on average. The number of cells examined was 10 (0 day) and 6 (6 days), respectively. The limits of the distribution of the two population are also common each other.

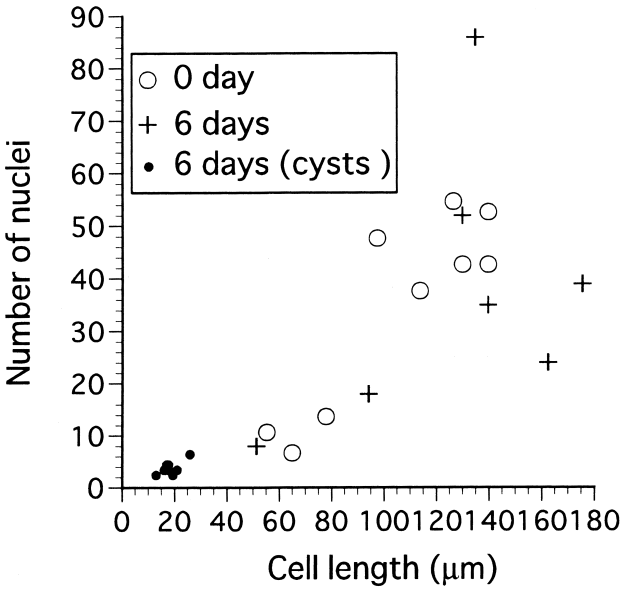

Fig. 11. Distribution of the number of nuclei and cell size in cells 0 day (*open circle*) and 6 days (*cross*), and cysts 6 days (*closed circle*) after cultivation. In cells except for cysts, the number of nuclei increased from 35±19 to 37±26, and their cell size also slightly increased from 105±32 to 127±42 μm on average. The number of cells examined was 9 (0 day) and 7 (6 days), respectively. When the culture finished in 6 days, three populations consisting of the cysts, the small cells with fewer number of nuclei, and the bigger cells with a large number of nuclei, respectively, can be distinguished.

berger's experiments do not necessarily contradict our conclusion that anaerobic condition is quite important for in-vitro culture of the opalinid cells. Alternatively, we can not deny the possibility that certain secretory materials from bacteria promoted the growth of opalinid cells

In the present experiments, cells grew by binary fission and were maintained for up to 14 days without successive transfer into fresh medium until the cultures were fixed for observation. It took 3–5 days until the number of cells began to increase. This period was thought to be necessary for adaptation of the opalinid cells to the new environment outside the host. As the culture progressed, cells began to die

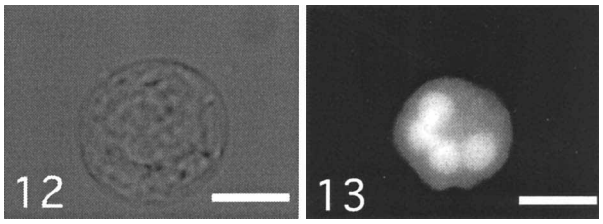

Fig. 12–13. Photographs of a typical cyst formed in axenic medium. **12**. Cyst observed under normal microscope. **13**. Cyst stained with DAPI. Four nuclei are visible. Bar=10 μm.

after reaching the maximum number. In most of the cases, morphological abnormalities in binary fission products were observed. This kind of anomaly so far has never been reported in vivo. Although addition of 10% FBS and ARF somewhat improved the situation, such an abnormality of cell division is presently unavoidable. As the cause of these anomalies, the following reasons are available: 1) A certain specific component was exhausted or not contained in the complex medium, and 2) The complex medium contained some harmful component. Unlike free-living organisms, parasitic and symbiotic organisms inhabit in unique enviroments and tend to demand specific nutritions. Because of this, it is possible that the opalinids need additional components. One of such components may be supplied from some kinds of intestinal bacteria. With regard to the second possibility, the basic medium DME/F-12 HAM mixture is usually used for mammalian cell or tissue culture. It is implausible that this basic medium contain any harmful component. Therefore, FBS or ARF may contain some harmful component. In either case, palintomy proceeded normally without producing any anomaly. It is a subject of our further study to refrain from anomalies in our cultivation.

Artificial induction of encystation and palintomy in vivo and in vitro has been repeatedly reported. It was suggested by Lwoff and Valentini (1948) that induction of palintomy may be under the hormonal control of the host. Then it was demonstrated that palintomy can be induced by injection of pituitary glands into frog body cavity (Rugh, 1952; Wessenberg, 1961). Indeed, sexuality of hypermastigotes such as *Trichonympha* in the hindguts of wood-eating cockroaches is known to be triggered by the molting hormone, ecdysone, released by the host (Grell, 1967). Besides, it has been reported that palintomy can be induced when the hibernated frogs are placed in room temperature (Sukhanova, 1963). This fact suggests that both the effect of pituitary hormone and the resultant sexual activation are not responsible for induction of palintomy. However, Kaczanowski *et al.* (1972) used Yang's medium and cultured *O. ranarum* cells at 4°C and room temperature where the cells were isolated from frogs in hibernation. Induction of palintomy and the subsequent encystation were confirmed at both temperatures. Based on this result and others, the authors suggested that palintomy was induced by the transfer of the opalinid cells from the host into the nutrient-rich medium which served as the stimulus for switching the phase from binary fission to palintomy. Unfortunately, their cultures suffered from overgrowing of bacteria or fungi even in the presence of antibiotics. Such a situation might have limited their conclusion, as suggested below. Our results obtained in this study do not necessarily conform to these claims. Rather in many cases, we observed only binary fission when cells were collected from the frogs not in breeding season. In the course of our experiments, there was only one example in which palintomy-like behavior, e.g. miniaturization of cells, the decrease of the number of nuclei and rotation just before cyst formation, was observed although the cells were obtained from the host not in breeding season (unpublished data). In this case, the culture medium was contaminated by bacteria, and the cells were accompanied with several kinds of anomalies such as pyknosis of nuclei. Consequently, we assume that the palintomy-like phenomenon was induced by the secondary effect of bacterial contamination.

In the present study, the opalinids underwent encystation through palintomy within six days, when the cells were collected from the host *R. schlegelli* in April and was cultured. In Kanazawa, breeding season of the frog *H. japonica* is usually from late May to June, whereas that of *R. schlegelli* is from April to early May. In April when the experiments were performed, the host *R. schlegelli* had already entered the breeding season. Therefore the population of the opalinids included two sub-populations: One had already entered the phase of palintomy, and the other was still in the phase of binary fission. If the opalinid cells are once committed to enter the phase of palintomy during the breeding season of the host, they are probably accompanied with a physiological change which leads them to the cyst formation in any environment such as our axenic medium. The commitment may be due to a secondary effect from some physiological change of the host initiated by hormone, sexual activation, temperature shock or other stimuli. At present, it is difficult to distinguish which factor is a determinant. The present success of in-vitro cultivation of the opalinid cells will offer a definite clue towards the explication of the factor(s).

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