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The Developmental Capacity of Various Stages of a Macrocyst-Forming Strain of the Cellular Slime Mold, *Dictyostelium mucoroides*[1]

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Vegetative cells of certain strains of *Dictyostelium mucoroides* form fruiting bodies on an agar surface and macrocysts when placed under saline. This study sought to determine whether the aggregation and pseudoplasmodial stages of fruiting body formation could be induced to form macrocysts when placed under saline. Likewise, different stages in macrocyst formation were put on an agar surface to determine their potential to switch to fruiting body formation. It was found that 78% of the aggregates and 21% of the pseudoplasmodia placed under saline formed macrocysts indicating that as fruiting body development proceeds, there is a restriction of the capability of cells to respond to environmental conditions favoring macrocyst formation. Stages in macrocyst development prior to the formation of precysts always formed fruiting bodies when put on agar. Once precysts had formed, surrounded by their acellular sheath, they always developed as macrocysts on agar. Peripheral cells isolated from precysts and put on agar quickly aggregated; the aggregates became surounded by a sheath and developed as macrocysts. If isolated peripheral cells were allowed to proliferate on the agar surface, the resulting cells aggregated and formed fruiting bodies.

INTRODUCTION

When placed on an agar surface, amoebae of the cellular slime mold *Dictyostelium mucoroides* aggregate to form multicellular pseudoplasmodia. These pseudoplasmodia undergo morphogenesis and are transformed into fruiting bodies consisting of a cellular stalk surmounted by a mass of spores. Approximately 50% of the strains of *D. mucoroides* isolated from soil, in addition to forming fruiting bodies, have an alternative mode of development in which the end result is the formation of macrocysts (Filosa and Chan, 1972). The developmental sequence leading to macrocyst formation does not usually occur on an agar surface, but when amoebae of these strains are placed in liquid medium macrocysts are formed exclusively. (Filosa and Dengler, 1972).

Blaskovics and Raper (1957) described the gross morphological features of macrocyst formation but were unable to determine the cytological details of the process. These details were first worked out by Filosa and Dengler (1972) in an ultrastructural study. Briefly, what these authors found was that shortly after amoebae are put into a liquid medium, they form irregular clumps. Each clump becomes divided into a number of submasses each of which is surrounded by an acellular sheath. At the center of each submass or precyst, there appears a cell that they called a cytophagic cell. The remainder of the cells in the precyst are referred to as peripheral cells. As macrocyst formation proceeds, the cytophagic cell begins to engulf the peripheral cells which become enclosed in vacuoles in its cytoplasm. Such engulfed peripheral cells are termed endocytes. Eventually all of the peripheral cells in a precyst become phagocytosed, and the cytophagic cell has become so enlarged that its limiting membrane is now adjacent to the sheath that originally surrounded the pre-

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cyst. By this time the cytophagic cell is multinucleate and, after all the peripheral cells are engulfed, a thick cellulose wall is formed around the large multinucleate cell. The endocytes exhibit pycnotic nuclei, their cytoplasm appears degenerate and, finally, they are fragmented into granules that fill the cytoplasm of the cytophagic cell. Other investigators have shown that such mature granular macrocysts will, in time, germinate to release amoebae (Erdos *et al.*, 1973).

This present work is concerned with the developmental potential of cells in various stages of fruiting body and macrocyst formation. We wanted to see whether cells embarked on one of these two developmental courses could be made to switch to the alternative mode of development by placing them under environmental conditions that usually favor that mode. Thus, cells that had reached the aggregation or the pseudoplasmodial stage of fruiting body formation might be made to switch to the macrocyst mode by immersing them in liquid medium. Likewise, various stages in macrocyst formation might alter their course of development and give rise to fruiting bodies when removed from liquid medium and placed on an agar surface.

METHODS AND MATERIALS

All experiments were done with clone 52 of an isolate of *Dictyostelium mucoroides*. Vegetative amoebae, aggregation stages, and pseudoplasmodia were obtained from cultures grown on Bonner's nutrient agar (Bonner, 1967) containing 1 g of $MgSO_4 \cdot 7 H_2O$/liter. These cultures were started by spreading 0.1 ml of a dense suspension of spores and *Escherichia coli* on the surface of the agar. The desired stage of development was removed from the culture with an insect pin and transferred to 3 ml of slime mold saline (Bonner 1967) in a 35 × 10-mm plastic petri dish. Observations and photographs were taken without removing the saline from the dishes.

For macrocyst cultures, the method of Filosa and Dengler (1972) was used. In brief, vegetative amoebae were washed from the surface of nutrient agar plates and freed from residual bacteria by low-speed centrifugation. The washed amoebae were suspended in 50 ml of slime mold saline in a 250-ml flask at a density of 1.7×10^7 cells/ml. The flasks were shaken in a reciprocal shaker at 20°C.

Peripheral cells were obtained from liquid cultures that had been shaken for 17–20 hr. By this time a cytophagic cell containing a few endocytes had formed in each precyst but most of the cells therein were peripheral cells. All the precysts from a single culture flask were washed in saline then broken in 2 ml of saline by means of a ground-glass tissue grinder with a loose-fitting pestle. This treatment resulted in the rupture of most precysts, but there were always some that had not been broken. The unbroken ones were allowed to settle by gravity, and the supernatant fluid was removed to a conical tube for settling of additional unbroken precysts. This procedure was repeated once more. In this way a suspension containing peripheral cells was obtained. This suspension also contained some sheath material. Cytophagic cells were only occasionally seen, apparently because they are easily ruptured. The peripheral cells were washed three times with saline then resuspended in 1 ml of saline. A droplet of this suspension was placed on the surface of 2% agar made up in saline. Observations and photographs at low magnification were made directly. When the 40× objective was used, a coverslip was placed over the cells on the agar surface.

RESULTS

Fruiting Body Stages Transferred to Liquid

Aggregation stages on nutrient agar plates were removed with an insect pin and placed under slime mold saline in plastic petri dishes. The dishes were kept at 22°C and examined every 24 hr for signs of macrocyst formation. By the end of the

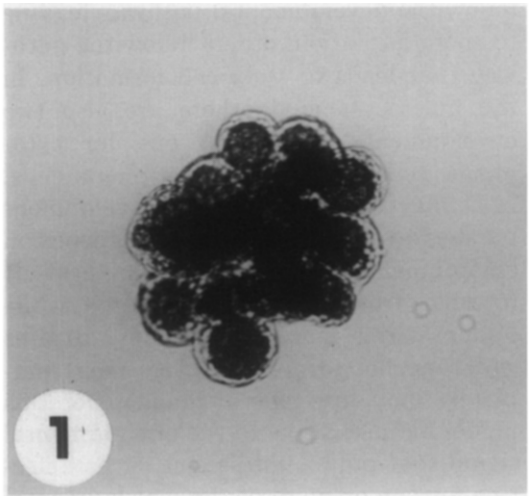
first 24 hr, 31 out of 40 (78%) of these aggregates had divided into numerous coherent precysts each of which had a cytophagic cell containing some endocytes. By 48 hr, a cluster of endocyte-filled macrocysts had been formed (Fig. 1).

When pseudoplasmodia were submerged in saline, the incidence of macrocyst formation was much lower than in the case of the aggregation stages. Of 116 pseudoplasmodia taken from a number of different cultures, only 24 (21%) produced macrocysts within 48 hr after being transferred to saline. In the successful cases, the pseudoplasmodial cells, which originally form an elongate mass surrounding part of the stalk, migrated off the stalk in a body and are no longer surrounded by the cellulose sheath that covered them in the pseudoplasmodium. Without disaggregating, these cells formed a rounded mass. As in the case of the aggregates, this mass subdivided into coherent precysts, each of which develops into a macrocyst (Fig. 2). In some cases where the pseudoplasmodia did not form macrocysts, the events described above occurred except that the cell mass did not subdivide and no precysts were formed. In other negative cases, the cells did not migrate off the stalk.

Macrocyst Stages Transferred to Agar

Clumps of cells that were present 3 hr after vegetative amoebae had been put under saline were transferred to the surface of 2% agar. Such clumps had not yet subdivided and were not surrounded by a sheath. Within 10 min after being placed on the agar surface, cells began to migrate out of each clump, and in time almost all of the cells had dispersed from the original clumps. Twelve to fifteen hours later, these cells had produced numerous fruiting bodies of various sizes. Over 100 clumps from a number of different cultures were examined and fruiting bodies were always produced exclusively.

Next to be tested were clumps from liquid cultures that had been incubating for 10 hr. These clumps were already divided into precysts, each of which was surrounded by a sheath but lacked a cytophagic cell. When such clumps were removed from saline and transferred to an agar surface, each precyst always continued to develop into a macrocyst, i.e., a cytophagic cell appeared which engulfed all the peripheral cells and finally produced a thick cyst wall. This stage in macrocyst formation was reached approxi-



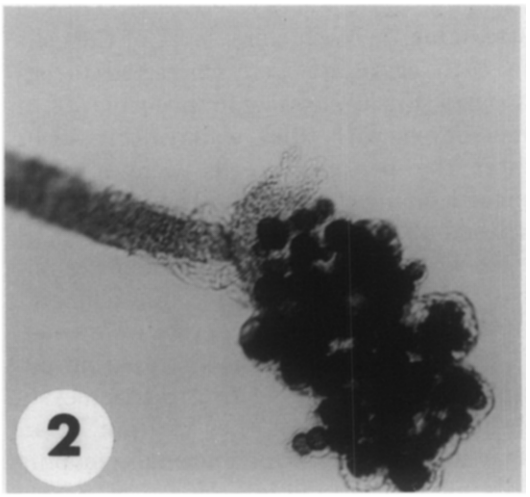


FIG. 1. A cluster of macrocysts formed by an aggregation stage that was removed from an agar surface and submerged in saline. × 220.

FIG. 2. A cluster of macrocysts formed by a pseudoplasmodium that was removed from an agar surface and submerged in saline. The original stalk is still present. × 140.

mately 36 hr after the clumps were first put on agar, making the total time 46 hr including the initial 10 hr in saline. This is about the length of time it would have taken for macrocysts to be formed if the clumps had remained in liquid culture continuously.

A most unexpected result was obtained with peripheral cells isolated from precysts. Within 10 min after being placed on an agar surface, the peripheral cells formed small clumps and, by 30 min, small, rounded aggregates were seen (Fig. 3). These aggregates increased in size as more cells moved into them and the larger aggregates became subdivided. While cells were still moving into the aggregates, endocytes appeared in each aggregate or its subdivisions, usually at a central location. In some aggregates, the endocytes appeared as early as 30 min after the peripheral cells were put on agar, and by 50–70 min almost every aggregate showed evidence of endocytes under low magnification (Fig. 4). These aggregates are thus equivalent to precysts formed under saline. Examination of these precysts with the 40× objective confirmed the presence of endocytes and also revealed a sheath covering each precyst (Fig. 5). The sheath appeared to cover each precyst completely except at those places where cells were still streaming in. With time, more of the cells in each aggregate were converted to endocytes (Fig. 6), leading to the formation of macrocysts with thick walls within 48 hr after the peripheral cells were initially placed on agar (Fig. 7). The events described above were observed in five repetitions of this experiment in which peripheral cells isolated from five different cultures of developing macrocysts were used.

These experiments demonstrate that peripheral cells will give rise to macrocysts despite the fact that they are put at an agar–air interface, which normally favours fruiting body formation. However, it was found that peripheral cells would produce fruiting bodies on agar after feeding and dividing. In these experiments, a loopful of *E. coli* was spread on 2% agar and a droplet of peripheral cell suspension was then placed on the surface. While the cells in the centre of the droplet went on to form macrocysts as described before, those at the perimeter fed on the bacteria and divided numerous times. These cells eventually formed aggregates which underwent morphogenesis to become fruiting bodies.

DISCUSSION

For the purpose of discussing our results, we would like to distinguish between "mode of development" and "developmental pathway." By mode of development, we mean a total pattern of events (processes, reactions), both sequential and simultaneous, which, when realized, results in a particular developmental endpoint. Each mode of development includes a number of alternative routes by which cells may differentiate to produce the developmental endpoint. Each of these routes is a developmental pathway. Thus in the case of macrocyst-forming strains of *D. mucoroides*, there are two modes of development, viz., the fruiting body and macrocyst modes, each leading to a characteristic endpoint. Some of the cells that are engaged in the fruiting body mode will follow the developmental pathway leading to spore formation; others follow the pathway that leads to stalk cell formation. In the macrocyst mode there are also two developmental pathways: one for cytophagic cells, the other for peripheral cells. In *D. mucoroides* the mode of development pursued by vegetative amoebae depends on environmental conditions: at an agar-air interface fruiting bodies are formed, while under saline macrocysts result. Strains which do not form macrocysts may completely lack the genetic makeup for this mode, be insensitive to the environmental signal that puts it into effect, or be defective for one or more of the developmental pathways of this mode.

In terms of our distinction between

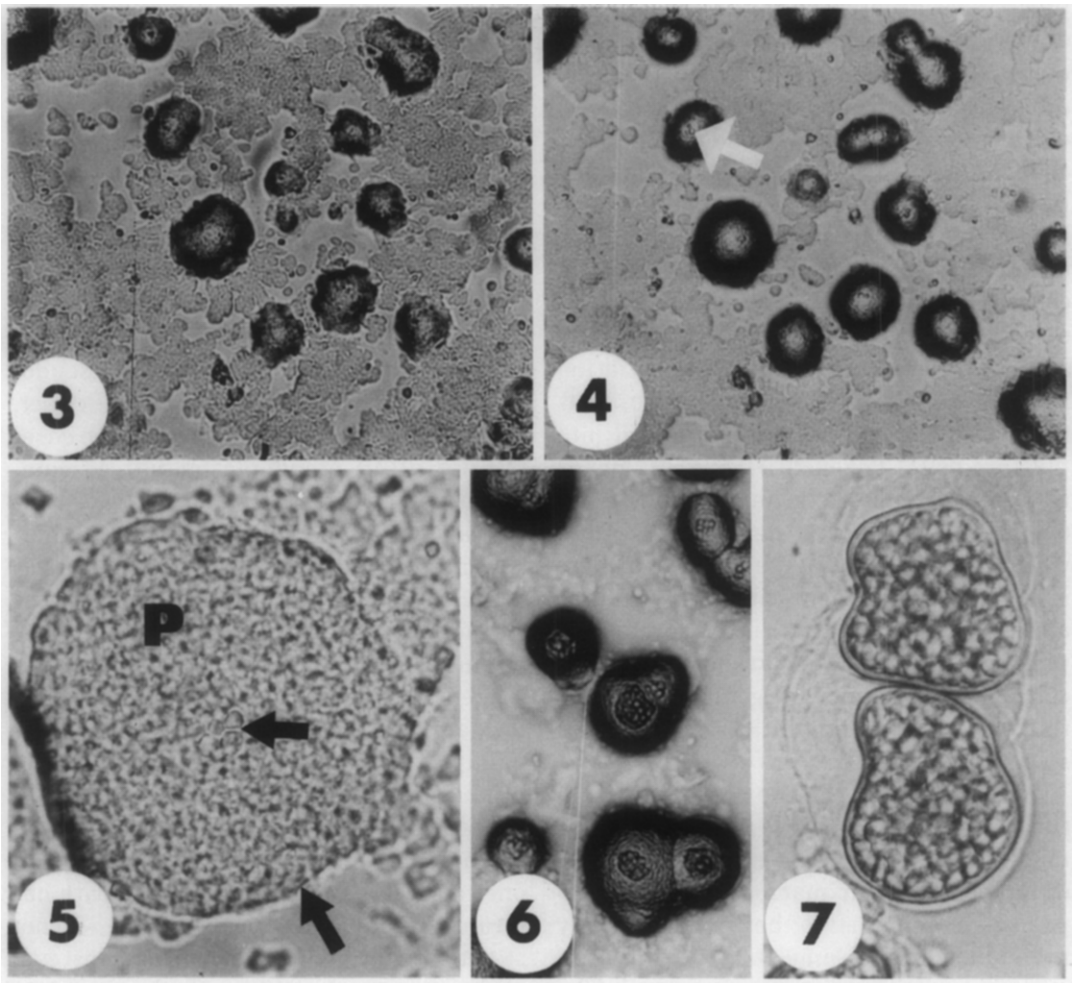


FIG. 3. Aggregates formed 30 min after isolated peripheral cells were placed on an agar surface. × 160.

FIG. 4. The same field as in Fig. 3 but 40 min later. Most of the aggregates have increased in size and their outlines have become smoother due to the sheath that surrounds them. A few endocytes (arrow) can be seen in most of the aggregates. × 160.

FIG. 5. One of the aggregates in Fig. 4 seen at higher magnification with a coverslip in place. The arrow at the periphery locates the sheath; the arrow in the center points to a small group of endocytes; the remainder of the cells are peripheral cells (P). × 750.

FIG. 6. Aggregates about 7.5 hr after peripheral cells were placed on agar. Each aggregate has many centrally located endocytes surrounded by peripheral cells. × 200.

FIG. 7. Two macrocysts that were formed about 46 hr after peripheral cells were put on agar. Each macrocyst is surrounded by a thick wall and is filled with endocytes. × 750.

modes and pathways of development, we wanted to find out whether it was possible by changing environmental conditions to switch from one mode to the other. More specifically, we wanted to know whether the switch could be accomplished if cells had already reached particular stages of development in a given mode.

In these experiments, 78% of the aggregates studied formed clusters of macrocysts when transferred to saline, whereas only 21% of the pseudoplasmodia were capable of responding in this way. The sharp decline in macrocyst formation by pseudoplasmodia as compared with aggregates may be interpreted as being a result of the

progressive differentiation of cells that is occurring as fruiting body formation proceeds. There is ample evidence that cell differentiation is underway in *D. mucoroides* in late aggregation and that further changes take place in pseudoplasmodia. Bonner *et al.* (1955), for example, have shown that by late aggregation cells have differentiated into prespore and prestalk cells that are spatially organized into two distinct regions. In pseudoplasmodia, prestalk cells are continually differentiating into stalk cells, and, as they do so, cells from the prespore area are converted into prestalk cells. Takeuchi (1963), by means of immunochemical techniques, has demonstrated that during development from vegetative cells through aggregation there is a synthesis of new combining groups, and when pseudoplasmodia are formed the prespore cells begin to show an increase in certain cytoplasmic antigenic substances that become incorporated into mature spores.

While differences in the level of differentiation between aggregate and pseudoplasmodial stages may be responsible for the differences in their macrocyst-forming capacity, it remains to be explained why individuals of the same stage vary in their capacity to develop as macrocysts. The failure of some aggregates to switch to the macrocyst mode may not be indicative of variation in the level of differentiation of the aggregates. Since some pseudoplasmodia can still make the switch to macrocyst formation, cells at the earlier aggregation stage must not yet be irreversibly committed to the fruiting body mode. It is possible that the failure of some of the aggregates to form macrocysts is due to the absence or below-threshold level of a critical factor that is required for the switch to the macrocyst mode. The aggregates used in this study varied in size, and this could conceivably account for variations in the amount of such a factor. The failure to get 100% transformation of pseudoplasmodia, on the other hand, might be ascribed to either the absence or low level of the critical factor or the irreversible differentiation of pseudoplasmodial cells with time. The size and age of pseudoplasmodia varied, and these variations could account for both possibilities. In some preliminary experiments (Filosa and Kent, unpublished results) we have obtained evidence that some soluble factor may be required for pseudoplasmodia to develop in the macrocyst mode. Seventeen-hour-old precysts broken in saline were centrifuged and the supernatant fluid collected. When pseudoplasmodia were submerged in this extract, they formed macrocysts in 93% of the cases (42 out of 45 pseudoplasmodia) as compared with 21% under plain saline. Thus we can tentatively conclude that submersion in saline may be a necessary but not a sufficient cause for the switch from the fruiting body to the macrocyst mode.

The experiments that tested the developmental capacity of stages in macrocyst formation indicate that cells that have clumped under saline but have not yet produced precysts surrounded by a sheath are still capable of forming fruiting bodies when placed on an agar surface. However, once a precyst surrounded by its sheath has formed, removal to an agar surface will not result in fruiting body formation; instead, such precysts continue to develop into macrocysts. The presence of the sheath seems critical for macrocyst development and, as has been suggested before (Filosa and Dengler, 1972), it may act as a diffusion barrier that is responsible for the buildup of substances elaborated by the cells in the precyst. Indeed, our preliminary observations mentioned above, indicated that precysts do contain a substance or substances that induce pseudoplasmodia to produce macrocysts.

Peripheral cells isolated from precysts and placed on agar immediately began to aggregate and, within 30 min, endocytes, which are indicative of the presence of a cytophagic cell, were found in the aggregates. Here again, it appears that the formation of the cytophagic cell is related to the presence of a sheath around the

periphery of the aggregate. The rapid appearance of cytophagic cells among the isolated peripheral cells contrasts with the approximately 10 hr required for them to appear once precysts have formed in liquid cultures. This result indicates that peripheral cells are still capable of producing cytophagic cells and, because of the rapidity with which they do so, are apparently primed for it. At the same time, the question is raised as to why only one cytophagic cell is produced in each precyst.

Our results show that the isolated peripheral cells will not engage in macrocyst formation on an agar surface if they undergo cell division. During the period of proliferation the peripheral cells evidently withdraw from the macrocyst mode and return to a status where they can now respond to their environmental situation, viz., the air–agar interface, by entering the fruiting body mode of development. However, caution must be exercised in ascribing the change of modes to cell division itself. In our experiment the cells were induced to divide by feeding them with *E. coli*. While cells are actively feeding and dividing, they forego any attempt to aggregate, so it is possible that it is the delay in forming contacts between cells that is significant in switching cells out of the macrocyst mode.

Recently, Maciness and Francis (1974) reported genetic evidence indicating that the macrocyst mode of development represents the sexual phase of the life cycle of *D. mucoroides*. Their investigation suggests that a macrocyst contains a zygote resulting from the fusion and karyogamy of two haploid cells. The zygote nucleus undergoes meiosis so that reassortment of genetic factors is possible. None of these events is known to occur during the fruiting body mode of development; thus the committment to macrocyst formation is a commitment to sexual reproduction.

Considering the genetic evidence in the light of the ultrastructural studies on macrocyst formation in *D. mucoroides* (Filosa and Dengler, 1972), it is reasonable to assume that the zygote is the newly formed cytophagic cell found in each precyst. This assumption requires that the cytophagic cell originates by fusion of two cells in a precyst. The present work, then, suggests that all of the peripheral cells in a developing macrocyst are potentially capable of engaging in fusion and subsequent karyogamy to form zygotes. The question of why a precyst contains only one cytophagic cell can now be rephrased in terms of what controls the fusion of cells that is prerequisite for zygote formation.

Now that the macrocyst has been shown to be the sexual phase of the life cycle of *Dictyostelium*, genetic analysis of the development of these organisms becomes feasible. Our method of disrupting developing macrocysts and plating them on agar could be a useful procedure for increasing the yield of zygotes required for genetic analysis.

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