Cell differentiation in *Dictyostelium* under submerged conditions

(oxygen/polarity/pattern formation)

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ABSTRACT

Hitherto it has not been possible to obtain spore and stalk cell differentiation of the cellular slime molds in submerged cultures. It is shown here that cells, when placed in roller tubes under an atmosphere of oxygen, will form clumps and differentiate in 48–72 hr into mature spores and stalk cells. Although differentiation occurs without the normal morphogenetic movements, there is the appearance of an anteroposterior polarity of the cells in the clump. In addition to oxygen we examined a number of other factors that affect differentiation.

It has always been assumed that differentiation in the cellular slime molds could not occur under water, for development would invariably stop at aggregation (1), and that an air–water or an oil–water interface was required. The success of an oil–water interface, which was first demonstrated by Potts (2), has been ascribed to the fact that mineral oil can be saturated with 10 times more oxygen than can water. As will be shown, if oxygen is added to roller tubes containing *Dictyostelium discoideum* cells suspended in a physiological salt solution, then clumps of cells will form that show both mature spore and stalk cell differentiation. This work was first presented at a meeting in May, 1976 at Cold Spring Harbor; at the same meeting I Takeuchi reported similar results in roller tubes using different methods. Ultimately, the findings of both these studies will increase our understanding of the mechanism of cell differentiation in the cellular slime molds.

MATERIALS AND METHODS

All the experiments reported here were done with *Dictyostelium discoideum* NC-4 grown on *Escherichia coli* B/r on 1% peptone–dextrose buffered agar. The aemae were washed three times by centrifugation at 75 × g in 10−2 M salt solution (1). Two milliliters of a cell suspension (5 × 10^6 cells per ml) were put into 15-ml test tubes with ground-glass stoppers. The tubes were flushed out with 20 volumes of 100% oxygen (Liquid Carbonic Co.) before they were sealed with silicone grease. (Earlier experiments were done with screw-cap test tubes sealed with Parafilm.) The tubes were placed on a Multi-Purpose Rotator (Scientific Industries, Inc.) and rotated at 18 rpm at a low angle to the horizontal at 21° in the light.

Two fixation methods were used: (1) 2% glutaraldehyde followed by 1% OsO₄ (both in 10−2 M salt solution), and (2) 2% glutaraldehyde plus 1% OsO₄ in 0.1 M cacodylate buffer at pH 7.2. For light microscopy, specimens were embedded in methacrylate and thick-sectioned with a Porter-Blum microtome. The sections were stained with the periodic acid-Schiff (PAS) stain for non-starch polysaccharides (3). For electron microscopy Epon was used for embedding and the thin-sectioning was done on an LKB Ultramicrotome III. The electron microscope used was a JEOL JEM-100C.

RESULTS

Time course description of differentiation

One of the most interesting aspects of this study has been the morphological events that accompany the differentiation of the clumps in the roller tubes. The following description, which is also shown in diagrammatic sequence in Fig. 1, is based on clumps that have been cultured in 10−2 M salt solution under an atmosphere of 100% oxygen. The observations described below were made on living material stained with a vital dye (neutral red) and fixed preparations stained by the PAS method for nonstarch polysaccharides. These stains allow one to distinguish between the prestalk and prespore regions (4, 5).

Small clumps of less than 100 cells form in minutes after the roller tubes are started, and by 2 hr they have coalesced into larger clumps of about 100–200 μm in diameter. At this time the individual cells show differences in their PAS staining, but the dark and light cells are randomly distributed. By approximately 8 hr, the outermost cells form a shell that is exclusively prestalk. (This would be the terminal state if the clumps were under air rather then oxygen.)

By 10–12 hr many clumps become somewhat elongated, and at the same time there is an accumulation of debris, which first appears in the center of the clump. If Sephadex beads (40 μm) are added to the tubes, then these are also incorporated into the center of the clumps and later extruded along with all other debris in a sticky matrix at one end of the clump. The matrix has the general appearance of slime sheath material. Both the vital dye-stained and PAS-stained material show that the prespore cells lie at the end of the clump that secretes the debris and the prestalk cells are at the opposite end. At this time, while the entire prestalk region is pure prestalk, a few prestalk cells still remain intermixed in the prespore region.

This situation persists until about 18 hr, by which time the outer shell of prestalk cells becomes quite rigid. (This can be easily demonstrated by using a fixative that causes shrinkage; there will be a clear separation between the rigid prestalk shell and the interior.) Also by this time the prespore region is made up of exclusively prespore cells. It is clear that an anterior-posterior polarity is established just as in the normal migrating slug. There is an anterior prestalk region and a posterior prespore region with attached slime sheath material. The only difference from normal slugs is that there is a cortical layer of prestalk cells that surrounds the entire clump.

By 22 hr final differentiation begins. The outside cells in the cortical shell start to differentiate into mature stalk cells, and this progresses inwardly, thickening the stalk cell shell. By about 36 hr mature spores start to appear in groups in and around the innermost stalk cells of the thickened cortex. Both spores and stalk cells continue to mature, but even by 72 hr the larger clumps still have a small slug-like structure with prespore and prestalk zones near the center of the fully differentiated mass of cells.
Evidence that stalk cells and spores are fully differentiated and mature

The evidence comes primarily from electron micrographs (Fig. 2). As can be seen, the stalk cells have characteristically thick walls, large vacuoles, and small residues of cytoplasm including debris and crystals. The spores have thick walls with the appropriately structured layers and a granular internal matrix containing crenulate mitochondria. It is also possible to recognize the characteristic prespore vesicles (6-8). A further test for the presence of mature spores was made by subjecting the clumps of cells to 50°C for 30 min, a regimen that kills undifferentiated cells or partially differentiated cells (9). If clumps were first squashed, treated with heat in this manner, and then spread on agar with E. coli, amebae emerged from the spores, proliferated, and gave rise to normal fruiting bodies.

Conditions affecting differentiation

First it was found that generally a greater number of cells differentiated in the submerged clumps if late interphase cells were used rather than vegetative cells. When such cells were used, if the atmosphere over the salt solution in the roller tubes was flushed daily with pure oxygen (and the oxygen concentration was checked at the conclusion of the experiment with an oxygen tension electrode), the stalk cell differentiation was extensive, while the differentiation of spores was variable, sometimes even absent. No spore or stalk cell differentiation occurred when the tubes were flushed with air. It soon became clear that in order to obtain higher amounts of differentiation, there were other factors in the medium besides oxygen that play a part.

Evidence in support of this possibility comes from experiments on conditioned medium. If cells were cultured in 10^{-2} M salt solution (under an oxygen atmosphere) that has previously contained cells (conditioned medium), then these cells showed far more spore differentiation. This was consistent in 10 experiments.

It is clear from preliminary studies that a variety of substances affect the degree of differentiation. As Takeuchi has found (personal communication), and we confirm, certain ions have a large effect. The point we wish to emphasize here is that in all the experiments done so far, either with conditioned medium or added substances, the amount of cell differentiation is significantly enhanced by adding oxygen. There are clearly a number of limiting factors, but a high oxygen tension is especially important.

Regulation of prespore and prespore regions in clumps

It is well known from the early work of Raper (10) that if a migrating slug is cut into segments, each segment is capable of producing a fruiting body with stalk cells and spores, even though one segment might come exclusively from a prespore (or a stalk) region. It has been possible to show the same phenomenon with differentiating submerged clumps using neutral red as a means of indicating prespore and prespore regions. If the clump showed a clear, dark stalk region and a light prespore region (16-24 hr), the two were separated, and then each was treated with heat in this manner, and then spread on agar with E. coli, amebae emerged from the spores, proliferated, and gave rise to normal fruiting bodies.

DISCUSSION

It is evident, that contrary to previous views, cell differentiation can occur under submerged conditions. Oxygen appears to be one of the significant limiting factors, although there are others. There are some obvious differences between normal develop-
Fig. 2. (A) A longitudinal section of a clump of cells that have been submerged in roller tubes under an O₂ atmosphere for 72 hr. Note that there are mature differentiated stalk cells (shown at higher magnification in B) and differentiated spores (shown at higher magnification in C). The circular dark mass lying above the spores in A is an undifferentiated mass of cells in which prestalk (left) and prespore cells (right) are visible. (D) An electron micrograph of a spore from a clump such as shown in A. (E) An electron micrograph of a mature stalk cell from the same clump. Note the large vacuoles and the thick cell wall. (F) A portion of a prespore cell in which the dark-edged prespore vesicles are clearly visible. (G) A similar view of a portion of a prestalk cell. Note that in these last two electron micrographs the density of the prespore cells is conspicuously greater than that of the prestalk cells. (Magnification: The length of the clump in A is 630 μm. The bar in B and C is 10 μm. In the remaining photographs the size is given by the width from edge to edge. For D the width is 3 μm; E, 6 μm; F, 4.4 μm; G, 5.3 μm.)
ment and that which takes place under water, but the same basic progression of stages still exists. At an early stage the clumps become polarized: a larger number of darkly stained cells are at one end compared to the other. Later, a rigid shell forms around the clump and at the same time the polar distribution of prestalk and prespore cells becomes even more obvious, resembling a normal slug. It is not known by what mechanism this pattern is established.

In order to produce mature stalk cells all that is needed is a rounded mass of cells. It is clear that differentiation can take place without aggregation, but this was known from earlier work as, for instance, in the elegant experiments of Gerisch (12). Also, differentiation can occur without the normal morphogenetic movements associated with culmination. But again this has been shown previously with the fruiting mutant described by Sonneborn et al. (13) and for stalk cell induction by cyclic AMP (14, 15). Very recently Town et al. (16) have found a mutant of D. discoideum that forms both stalk cells and spores without morphogenesis in the presence of cyclic AMP when the cells on the surface of the agar are covered with a layer of cellophane. It is conceivable that at least one factor responsible for the differentiation of these mutant cells is that oxygen can diffuse freely through the overlying cellophane.

With this method of obtaining differentiation in submerged cell masses, the cellular slime molds now provide a system in which it will be possible to do direct biochemical studies not only on cell differentiation, but also on pattern formation.

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