MUTUAL COHESION AND CELL SORTING-OUT AMONG FOUR SPECIES OF CELLULAR SLIME MOULDS

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SUMMARY

Interspecific cell cohesion among 4 species of cellular slime moulds, *Dictyostelium discoideum*, *D. mucoroides*, *D. purpureum* and *Polysphondylium violaceum* has been studied. Binary mixtures of aggregation-stage cells of the 4 species were shaken in suspension, one species of each pair being labelled with [\(^3\)H]thymidine. Cell aggregates were sampled at intervals over 24 h and their composition examined by autoradiography. The following results were obtained: (i) Cells of each species were capable of cohesion with those of the other 3 species. (ii) In general cells of both species in any mixture were present in aggregates after 1 h, but were not localized according to species. (iii) Within 8-h aggregates cells of different species were regionally localized, i.e. sorting-out appeared to have taken place. (iv) 24-h aggregates were more varied: in mixtures of *Dictyostelium* species, the different species were localized within the aggregates; in mixtures of *Dictyostelium* species with *Polysphondylium*, there was a tendency for cells of the different species to become segregated into completely separate aggregates.

The significance of these results in relation to both previous descriptive work and recent biochemical studies on the mechanism of slime mould cell cohesion is discussed.

INTRODUCTION

The specificity of cell surfaces and selective cell adhesion are of central importance in relation to morphogenetic movements in embryonic development (Weiss, 1947; Townes & Holtfreter, 1955). The cellular slime moulds seem to provide an excellent system for the study of selective adhesion (Garrod, 1974). Although much work has been done on cell adhesion in *Dictyostelium discoideum* (Gerisch et al. 1974), little attention has been given to the adhesive interactions of cells of different slime mould species.

Descriptive work on the interactions of slime mould species has been reported in only 4 papers, Raper & Thom (1941), Shaffer (1957a, b), and Bonner & Adams (1958). All of these early experiments were done in situations which make interpretation in terms of cellular adhesiveness difficult, since the results could equally well be due to other morphogenetic mechanisms such as chemotaxis. To illustrate this, an experiment of Raper and Thom will be used.

When the species *Dictyostelium discoideum* and *Polysphondylium violaceum* were grown together in the same culture, it was found that they did not co-aggregate, and that aggregation streams of the 2 species would actually cross each other without the cells intermixing. This experiment clearly demonstrates species-specific cellular
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recognition but throws little light on the recognition mechanism. That the cells of the 2 species failed to intermix does not necessarily mean that they are not mutually cohesive. Instead it might be suggested that formation of separate aggregates occurs because the 2 species have different chemotactic mechanisms. There is now evidence that they do (Konijn, Chang & Bonner, 1969).

Recent biochemical work has revealed the presence of lectin-like carbohydrate-binding proteins on the surfaces of slime mould cells. These may be involved in cell adhesion. Distinct lectins have been obtained from 6 different species of slime moulds and it has been suggested that these lectins may be responsible for specific adhesion (Rosen, Reitherman & Barondes, 1975).

Because the descriptive basis of adhesive selectivity in the slime moulds has not been thoroughly worked out, it is difficult to assess the significance of this biochemical work. In this paper, therefore, we present an initial series of experiments aimed at describing the selective behaviour of slime mould cells of 4 different species, Dictyostelium discoideum (Dd), D. mucoroides (Dm), D. purpureum (Dp) and Polysphondylium violaceum (Pv).

Aggregation stage cells have been mixed in binary combinations in shaken suspension, one species of each pair being labelled with [3H]thymidine. In shaken suspension chemotactic mechanisms cannot operate so a simple test for mutual cell cohesion is obtained. We have shown that cells of each of the 4 species cohere to form mixed aggregates with those of the other three. Further, sorting-out of cells according to species appears to take place within these mixed aggregates.

MATERIALS AND METHODS

Growth of cells

All species were grown at 22 °C on SM agar (Sussman, 1966) in 90-mm Petri dishes in association with Escherichia coli B/r. For radioactively labelled cells, the medium was supplemented with [3H]thymidine at 5 μCi/ml (100 μCi per plate). All cells were harvested at the feeding stage in cold distilled water (4 °C) and washed free of bacteria by centrifugation.

For all cohesion experiments, cells were allowed to develop to the aggregation stage, thus ensuring uniformity of developmental stage between different cell populations. Vegetative cells of Dd, Dm and Dp were placed on Millipore filters (0.5 ml of cell suspension at 2 x 10⁸ cells/ml in distilled water) and incubated at 22 °C (Sussman, 1966). At the onset of aggregation, about 8 h after plating, filters were placed at 4 °C until required. Vegetative Pv cells were plated on to 2% agar in 0.017 M phosphate buffer at pH 6.0 in 50-mm Petri dishes in 0.1-ml aliquots from a cell suspension at 2 x 10⁹ cells/ml in distilled water. Aggregation took place after 3-4 h of incubation at 22 °C. Then, the cells were either used immediately or stored at 4 °C until required.

Cell dissociation

Where cells had been stored at 4 °C, they were incubated at 22 °C for 30 min before use. The cells were then harvested in cold distilled water, collected by centrifugation and resuspended in 1 ml of cold distilled water. The suspension was triturated using a drawn-out Pasteur pipette until only single cells were present. The cell concentrations were counted using a haemocytometer.
Experimental procedure

Cells of 2 different species, one labelled with [3H]thymidine, were mixed in 1:1 ratios unless otherwise stated. The cell mixtures were set up at 1 x 10^6 cells/ml in 4-ml aliquots in 0.017 M phosphate buffer, pH 6.0, in siliconized 25-ml conical flasks. The flasks were shaken at 22 °C and 140 rev/min, on a New Brunswick G-86 water bath shaker, with a radius of rotation of 0.635 cm.

Histology

The aggregates were fixed in ice-cold absolute ethanol for 10 min. Thereafter routine paraffin wax histological procedure was employed, with the exception that the aggregates were embedded in square-pointed electron-microscopy embedding capsules. The blocks were sectioned at 5 μm using a rotary microtome.

Autoradiography

Slides were dipped in Ilford K4 nuclear emulsion diluted 50/50 with distilled water. The emulsion was exposed for 3 weeks at 4 °C and then developed in Kodak D19 developer for 5 min, followed by distilled water for 30 s and Kodafix for 8 min. After washing over-night in running tap water and dehydrating, slides were mounted in Canada balsam. Aggregates were examined unstained using phase-contrast microscopy.

Controls used were 100% labelled cells, 50/50 labelled/unlabelled cells of the same species and 100% unlabelled cells. No significant transfer of label from cell to cell was encountered either in 50/50 control mixtures or in experiments where cells of one species only were labelled.

RESULTS

Dm/Dd mixtures (Figs. 1-5)

For this combination, Dd cells were labelled with [3H]thymidine. Flasks of cells were taken at 1, 2, 4, 8, and 24 h after the beginning of shaking the mixed suspension, and the aggregates examined by autoradiography.

Mutual adhesion of cells of the 2 species was apparent in the aggregates at all stages. At times up to 4 h, there was no clear localization of cells within aggregates, but at 8 and 24 h cells were distinctly localized according to species. However, there appeared to be no consistent pattern of localization and no regular tendency for cells of one species to surround those of the other. Aggregates were generally variable in size and irregular in shape.

Pv/Dd mixtures (Fig. 6)

For this combination Dd cells were labelled with [3H]thymidine. Flasks of cells were taken at 1, 2, 4, 8, and 24 h after the beginning of shaking and the aggregates examined by autoradiography.

At 1 h the aggregates were small, loose, and contained both Pv and Dd cells. By 2 h many of the aggregates were larger and more compact. In some, cells were localized according to species. At 4 h and 8 h localization according to species was distinct, and, although the aggregates were irregularly shaped, there seemed to be a definite tendency for Dd to surround Pv. At this stage a few aggregates appeared to consist of one species only. By 24 h some mixed aggregates were present similar in size and appearance to those found at 8 h. However, there was also a considerable proportion of aggregates consisting entirely, or almost entirely, of cells of only one species.
Figs. 1-4. Autoradiographs of sections through aggregates of Dm/Dd mixtures, the Dd cells having been labelled with $^{3}H$thymidine. The aggregates had been maintained in suspension for 2, 4, 8, and 24 h (Figs. 1, 2, 3, 4 respectively).
Fig. 5. Drawings of autoradiographic sections through aggregates of Dm/Dd mixtures. The Dd cells were labelled with $[^3H]$thymidine and are represented in the drawing by black areas where there was complete labelling and by black dots where a few labelled cells occurred. The Dm cells were unlabelled and are represented by the white areas in the drawings. The aggregates had been maintained in suspension for 1, 2, 4, 8, and 24 h (A, B, C, D, E respectively).
Fig. 6. Drawings of autoradiographic sections through aggregates of Pv/Dd mixtures. The Dd cells were labelled with [3H]thymidine and are shown in black, while the unlabelled Pv cells are shown in white. The aggregates were maintained in suspension for 1, 2, 4, 8, and 24 h (A, B, C, D, E respectively).
Fig. 7A-C. Drawings of autoradiographic sections through aggregates of Dp/Dd mixtures. The Dd cells were labelled with [3H]thymidine and are shown in black, while the Dp cells were unlabelled and are shown white. The aggregates were maintained in suspension for 1, 8, and 24 h (A, B, C respectively).

D-F. Drawing of autoradiographic sections through aggregates of Pv/Dm mixtures. The Dm cells were labelled with [3H]thymidine and are shown in black, while the Pv cells were unlabelled and are shown white. The aggregates were maintained in suspension for 1, 8, and 24 h (D, E, F respectively).
Dd/Dp mixtures (Fig. 7A-C)

One experiment was done with this combination, the Dd cells being labelled with [3H]thymidine. One-, 8- and 24-h aggregates showed mutual adhesion of cells of the 2 species and distinct localization of cell types. There appeared to be no definite tendency for one cell type to surround the other.

Dm/Pv mixtures (Fig. 7D–F)

For this combination, Dm cells were labelled with [3H]thymidine. Flasks of cells were taken at 1, 8, and 24 h after the beginning of shaking and the aggregates examined by autoradiography.

At 1 h, 3 types of aggregates were apparent, some consisting of one species only and some mixed. In mixed aggregates there already seemed to be localization according to species but no regular spatial pattern could be discerned. The aggregates were irregularly shaped.

By 8 h the aggregates appeared slightly larger and more regular in shape. Three types of aggregates were again present. The mixed aggregates showed distinct localization of cells according to species and there was a definite tendency for Dm to surround Pv.

By 24 h, few mixed aggregates remained. Some of the aggregates had become quite large, exceeding 100 μm across. The large aggregates were generally irregular in shape.

DISCUSSION

The most significant result of this study is that aggregation-stage cells of each of the 4 species, D. discoideum, D. mucoroides, D. purpureum and P. violaceum, can stick to cells of the other 3 species. That Pv cells stick to those of the 3 Dictyostelium species is particularly surprising in relation to earlier work in which these cells have been found not to co-aggregate when grown together on an agar surface. We suggest that lack of co-aggregation on agar surfaces is due to the possession of different chemotactic mechanisms by these species and not to lack of mutual cohesiveness, for when cohesion is tested in shaken suspension where chemotactic mechanisms cannot operate, they are found to stick to each other quite readily.

After cohering together the cells become localized according to species, within the aggregates. We believe that this localization occurred by a process of cell sorting-out, i.e. translocation of cells within the aggregates so that cells of the same species become grouped together. We have to be slightly cautious about this interpretation because the aggregates continued to increase in size and to become more tightly cohesive as the experiments progressed and as the cells became localized. Thus we did not obtain a clear situation where localization of cells in aggregates increased with time while the size of the aggregates remained constant, as it is possible to do in the case of tissue-specific sorting-out of vertebrate embryo cells (Townes & Holtfreter, 1955; Steinberg, 1964; Trinkaus & Lentz, 1964; Wiseman, 1977). Analysis of the composition of aggregates at the earliest times when we could reasonably fix and section
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them (usually 1 h after beginning an experiment), showed that in general these early aggregates contained cells of both species and that these cells were not localized according to species. Thus we prefer the interpretation that localization generally occurred by sorting-out to the alternative possibility that adhesion was occurring between small aggregates composed of cells of one species only.

Raper & Thom (1941) and Bonner & Adams (1958) made grafts and mixtures between several species and strains of Dictyostelium. Such grafts and mixtures were generally successful but eventually partial or complete separation of the 2 species or strains occurred and separate fruiting bodies were constructed. Bonner & Adams reported a few cases in which a single sorus contained 2 separate patches of spores each belonging to one of the strains used, while Raper and Thom found occasional instances of the spores of Dp and Dd being present in the same sorus. We have found that there appears to be no tendency to complete separation of different species of Dictyostelium in mixed aggregates even after 24 h in shaken suspension. Instead cells of the different species remained mutually cohesive even though regionally localized within the aggregates. This is not because the development of cells in suspension is arrested at the aggregation stage. Several recent studies have shown that development and differentiation of Dd cells in suspension continues to a stage equivalent to terminal differentiation of the fruiting body (Garrod & Forman, 1977; Forman & Garrod, 1977; Sternfeld & Bonner, 1977; Takeuchi, Hayashi & Tasaka, 1977). We suggest that differences in cellular cohesiveness alone may be insufficient to explain separation of different species and strains from grafts and mixtures, but some additional factors which do not operate in shaken suspension may be required. We did, however, find complete separation into aggregates of one species only in mixtures of Pv with Dd and Dm after 24 h in shaken suspension. Both Bonner & Adams and Raper & Thom were unable to make successful grafts between Pv and Dictyostelium species. Our results suggest firstly that there is a greater difference between the cohesive mechanisms of Pv and Dictyostelium species than between different Dictyostelium species and, secondly, that the cohesive properties of cells change with time in aggregates in suspension since aggregation-stage cells of Pv and Dictyostelium species cohered readily with each other, but separated later.

Because in none of our combinations did a regular tendency for cells of one species to surround those of another emerge, it is difficult to decide whether an explanation of sorting out of different slime mould species should be attempted in the terms of the Differential Adhesion Hypothesis as applied to vertebrate cells by Steinberg (1964, 1970). Also, once the cells have cohered, chemotactic mechanisms could begin to operate within aggregates and thereby bring about cell localization according to species. Indeed, such a chemotactic mechanism of sorting-out might account for the variability in the pattern of distribution of cells within aggregates. Cells of a particular species might accumulate at regions within the aggregate where aggregation centres for that species arise, so that the distribution of cells within aggregates might be governed by chemotaxis rather than by their cohesive relationships. Further detailed study of the behaviour of cells within these aggregates will be necessary in order to decide upon these points.
The observation that cells of these 4 species are mutually cohesive seems compatible with recent biochemical studies on slime mould cohesion. Rosen et al. (1975) have isolated lectin-like carbohydrate-binding proteins from aggregation-stage cells of 6 species of cellular slime moulds, the same 4 species as have been used in our study plus *D. rosarium* and *P. pallidum*. There is evidence that these substances may be involved in cell cohesion. Inhibition by sugars of erythrocyte agglutination by these substances was tested and only slight differences between the lectins from different species were found. The differences were quantitative rather than qualitative, i.e. any one sugar inhibited all or most of the 6 lectins, but different concentrations of the sugar were required to produce a similar degree of inhibition. If these lectins are the adhesive molecules of slime mould cells, it is not surprising that cells of different species should stick to each other, because the adhesive molecules have similar binding properties.

Also Swan, Garrod & Morris (1977) have demonstrated a low-molecular-weight inhibitor of cell cohesion in the stationary phase medium of the axenic strain, Ax-2, of *D. discoideum*. This inhibitor is effective against cohesion of other species of slime moulds, again suggesting that the cohesive mechanisms of different species are similar.

We thank Mrs Sherilee Taylor for technical assistance. This work was supported by the Science Research Council.

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(Received 13 December 1977)