Cell aggregation and sexual differentiation in pairs of aggregation-deficient mutants of Dictyostelium discoideum

By G. GERISCH¹ AND A. HUESGEN²

From the Biozentrum der Universität Basel, and Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tübingen

SUMMARY

A diffusible aggregation-stimulating factor (ASF) is released from a series of aggregation-deficient mutants. Biochemical markers indicate that these ASF-donor mutants are blocked at a later step of cell differentiation than an ASF-requiring mutant. ASF is able to bridge the initial block of differentiation in the latter mutant, such that development proceeds up to aggregation and even further. ASF is most probably neither identical with cyclic-AMP phosphodiesterase nor with an inhibitor of this enzyme which both are released from donor strains.

In certain combinations of aggregation-deficient mutants, macrocysts, the sexual stages of Dictyostelium, are formed. Also, motile giant cells believed to be zygotes are observed in these mutant combinations. The gamone known to be released from one mating type and to induce macrocysts in the other, is probably not identical with ASF since this factor is produced by mutants derived from either one of both mating types.

INTRODUCTION

One type of non-aggregating mutant in Dictyostelium discoideum is blocked in the initial step of cell differentiation from the growth-phase stage to aggregation-competence. Mutants of this type are characterized by the absence of any one of the membrane changes normally associated with the acquisition of aggregation-competence (Gerisch et al. 1975a). They also do not form a macromolecular inhibitor of cyclic-AMP phosphodiesterase which, in the wild-type, is released into the extracellular medium shortly after the end of exponential growth (Gerisch et al. 1972). This type of mutant is represented by aggr 50–2 (Malchow & Gerisch, 1974). Other aggregation-deficient mutants still form the inhibitor, indicating a later block of development (Riedel, Gerisch, Müller & Beug, 1973). Like certain other mutants of this type, Wag-11 releases a diffusible inducer of cell aggregation (ASF = aggregation-stimulating

¹ Author's address: Biozentrum der Universität Basel, 4056, Basel, Switzerland.
² Author's address: Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, 74 Tübingen, Germany.
factor) which can be assayed by the specific requirement of certain \textit{aggr} 50 clones for this factor (Gerisch, Huesgen & Malchow, 1975a). In this paper we report on the biochemical and morphogenetic phenotypes of ASF-donor mutants.

Macrocysts are considered to be the sexual stages of \textit{D. discoideum} and related species (Erdos, Nickerson & Raper, 1972; Clark, Francis, & Eisenberg, 1973; Erdos, Raper & Vogen, 1975). In combination with \textit{Wag}-11, certain clones of \textit{aggr} 50–2 form macrocysts rather than normal aggregates. \textit{Wag}-11 is a descendant of the wild-type strain \textit{NC}-4, and \textit{aggr} 50–2 is a descendant of strain \textit{v}-12. \textit{NC}-4 and \textit{v}-12 belong to complementary mating types (Erdos, Raper & Vogen, 1973). The occurrence of macrocysts in the mutants indicates the expression of the mating type characteristics in spite of a block in cell aggregation.

METHODS

Cells were cultivated together with \textit{E. coli} B/2 at 22–23 °C in 9 cm diameter petri dishes on 20 ml Difco agar 2 %, containing 0.1 % bacteriological peptone (Oxoid), 0.1 % glucose and 0.017 M phosphate buffer pH 6.0. For the determination of cyclic-AMP phosphodiesterase and inhibitor, both peptone and glucose were increased to 0.4 %. Macrocyst formation was obtained on wet agar surfaces either in darkness or at a yellow fluorescent lamp. Aggregation patterns were fixed and stained on the agar surface by placing the plates upside down and adding a few crystals of iodine to the cover.

For keeping mutant strains separate, a 0.15 \textmu m pore size membrane filter (Sartorius, Göttingen) was inserted into the agar in such a way that the filter extended from the bottom of the agar layer to 5 mm beyond its surface. Controls were run for all strains tested to ensure that within 8 days no cells would pass the filter.

\textit{Aggr} 50 is a particularly unstable mutant. After periods of serial transfer it was repeatedly re-cloned. First, \textit{aggr} 50–2 (Riedel \textit{et al}, 1973), was obtained from \textit{aggr} 50–4. Finally, \textit{aggr} 50–2 was cloned by picking up single cells under a dissection microscope. The primary cultures of these new clones were preserved in liquid nitrogen. \textit{Aggr} 50–2–5 was selected for its superb macrocyst formation and \textit{aggr} 50–2–8 for its ability to aggregate in response to \textit{Wag}-11. Another clone, 50–2–7, showed no significant response. Part of the experiments were done by Huesgen (1973) with the parent clone, \textit{aggr} 50–4, which was similar to \textit{aggr} 50–2–8.

Extracellular cyclic-AMP phosphodiesterase and its inhibitor were determined as described previously (Riedel \textit{et al}. 1973) in the fluid extracted from agar. To obtain the fluid, cells were stripped off the agar surface and pieces of the agar were put into bags of fine nylon gauze. The bags were fixed on top of a tube and centrifuged for about 5 min at 400 g. The clear extract was collected from the bottom of the tube. One phosphodiesterase unit was defined as the
Aggregation deficient mutants of Dictyostelium

amount of enzyme which hydrolysed 1 nmole cyclic AMP at 35 °C and pH 7.4 at saturating substrate concentrations.

The factor for converting these units into the activities at the pH and temperature of agar plates is 0.24 (Gerisch, 1976). One inhibitor unit inactivates under standard conditions two enzyme units by 50% (Riedel et al. 1973).

Contact sites A were assayed by absorption of $F_{ab}$ directed against membranes of aggregating cells. Freshly washed cells harvested from suspension cultures were used for absorption (Beug, Katz, Stein & Gerisch, 1973b). The aggregation-inhibiting activity of the absorbed $F_{ab}$ was re-titrated in the presence of 10 mM EDTA using aggregation-competent $v$-12/M2 cells (Beug, Katz & Gerisch, 1973a).

RESULTS

Induction of cell aggregation in aggr 50 lines

Pairs of aggregation-deficient mutants were inoculated about 3–5 cm apart on agar plates. Aggr 50–4 was the only mutant which, in combination with other mutants, gave rise to cell aggregates (Table 1). Also, in most combinations, rudiments of fruiting bodies were formed, in which no spores were detected, however. More recently, aggr 50–2–8 was selected as a clone that forms particularly long aggregation streams in combination with certain Wag-11 clones (Fig. 1).

When aggr 50–4 was separated from the other mutants by a membrane filter, aggregation was observed only in the compartment that contained aggr 50–4 (Table 3 and Gerisch et al. 1975b). The aggregation-stimulating effect extended about 1 cm distant from the filter. This indicates that aggregation in aggr 50–4 is stimulated by a diffusible factor, ASF, which is released from a variety of non-aggregating mutants. No aggregation was obtained when aggr 50–4 was separated from donor strains by a dialysis membrane, suggesting that ASF is a macromolecule. The chemical nature requires further examination.

Biochemical phenotype of donor mutants

In all donor mutants tested, an extracellular inhibitor of cyclic-AMP phosphodiesterase was found (Table 1). In the wild type, the inhibitor is an early marker for cell differentiation from the growth-phase stage to aggregation-competence. This result indicates that the donor mutants are blocked at a later stage of development than aggr 50–4. In these studies, inhibitor formation has been determined in suspension cultures (Riedel et al. 1973). To ensure that under agar plate conditions development stops at the same stage as in suspension cultures, phosphodiesterase and the inhibitor were measured in the fluid extracted from agar plates on which Wag-11 or aggr 50–2 clones had been grown. Table 2 shows that Wag-11 forms inhibitor, whereas the two 50–2 clones showed not more than background levels.

It should be emphasized that the phosphodiesterase inhibitor is most likely
not identical with ASF, although both are released from the cells at an early stage of differentiation. No aggregation was observed after adding 480 units of partially purified inhibitor per plate along a line in front of the growth zone of aggr 50–4. Several inhibitor-producing mutants did not act as ASF-donors (Table 1). Aggr 75 is a donor (Table 3) although in suspension cultures, it overproduces extracellular phosphodiesterase (Riedel et al. 1973). On agar plates, the enzyme activity was not so high. Nevertheless, an excess of extracellular phosphodiesterase between 20 and 68 units per ml was found during the whole period between 3 h before and 9 h after the end of growth. (For definition of units see ‘Methods’.) The excellent donor mutant Wag-11 also produces an excess of extracellular phosphodiesterase in agar plate cultures (Table 2).

Extracellular phosphodiesterase is certainly not identical with ASF. This is demonstrated by the action of ga 86 and 88 as donors (Table 3). In suspension cultures, both mutants produce an excess of the inhibitor of extracellular phosphodiesterase (Riedel & Gerisch, 1971). Similarly, ga 88 shows on agar plates much inhibitor and almost no activity of the enzyme (Fig. 2).

**Figure 1.**

Cell aggregation and sexual differentiation in aggr 50|Wag-11 pairs. (A): Aggregates at the boundary between aggr 50–2–8 (left) and Wag-11–26 colonies (right) on agar. (B), (C): A motile giant cell at the boundary of Wag-11–20 and aggr 50–2–5 colonies, at an interval of a few minutes. Normal-sized amoebae are also present. (D), (E): Rounded giant cell (D) and macrocyst cluster (E) at the zone of contact between aggr 50–2–5 and Wag-11–28.
Table 1. *Induction of aggregation in mutant combinations*

<table>
<thead>
<tr>
<th>Mutant no. ...</th>
<th>aggr 20-2 (i+)</th>
<th>aggr 39 i+</th>
<th>aggr 52-2 i-</th>
<th>aggr 66-1 i-</th>
<th>aggr 70-1 (i+)</th>
<th>aggr 85 i+</th>
<th>aggr 50-4 i-</th>
<th>Wag-2-1 (i+)</th>
<th>Wag-3 (i-)</th>
<th>Wag-4 i+</th>
<th>Wag-6 i+</th>
<th>Wag-8 (i+)</th>
<th>Wag-11 i+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 20-2</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 39</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 52-2</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 66-1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 70-1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 85</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggr 50-4</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-2-1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-3</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-4</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-6</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-8</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wag-11</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutants i+ or i- were classified on the basis of explicit determinations of cyclic-AMP phosphodiesterase inhibitor in heated culture fluids as described by Riedel et al. (1973). In the case of aggr 50-4, the absence of inhibitor was tested in its descendant, aggr 50-2. In (i+) labelled mutants only inactivation of phosphodiesterase at 5 or 9 h after the end of growth was checked. Except in aggr 20-2 and aggr 70-1, where the PD activity was reduced by 56% and 17%, respectively, the inhibition was > 90%.

+, Aggregation streams and pseudoplasmodia, i.e. slug-like structures formed.

++, In addition, rudimentary fruiting bodies formed.
Aggregation deficient mutants of Dictyostelium

Using membrane filters for separation, aggregating strains including wild types could be tested as ASF-donors (Table 3). Among three wild-type strains, ASF activity was observed only in the clone v-12/M1. This strain is known for its slow development to aggregation-competence as tested in suspension-cultures (Gerisch, 1962). One possible reason for the low efficiency of wild-type strains as donors could be the formation of ASF within a short transient period

Table 2. Cyclic AMP phosphodiesterase and its inhibitor in Wag-11 and aggr 50–2 clones

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phosphodiesterase (u./ml)</th>
<th>Inhibitor (u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wag-11</td>
<td>233</td>
<td>87</td>
</tr>
<tr>
<td>aggr 50–2–5</td>
<td>186</td>
<td>5</td>
</tr>
<tr>
<td>aggr 50–2–8</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>E. coli B/2 (control)</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

The inhibitor was determined after heating of the extracellular fluid 10 min at 80 °C. This procedure inactivates the enzyme and liberates the inhibitor in an active state (Riedel et al. 1973). The extracellular fluid was taken not earlier than 12 h after the exhaustion of food bacteria.

Table 3. Development of aggr 50–4 induced by donor cells acting through a membrane filter

<table>
<thead>
<tr>
<th>No.</th>
<th>Phenotype</th>
<th>Aggregation streams</th>
<th>Pseudo-plasmodia</th>
<th>Rudimentary fruiting bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-12</td>
<td>M1 Wild type</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>v-12</td>
<td>M2 Wild type</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ax-2</td>
<td>Axenic wild type</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Wag-2–1</td>
<td>No aggregation</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Wag-3</td>
<td>No aggregation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wag-11</td>
<td>No aggregation, except of travelling cell bands (Gerisch et al. 1975b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>aggr 39</td>
<td>No aggregation</td>
<td>not observed</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>aggr 70–1</td>
<td>No aggregation</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>aggr 75</td>
<td>Fruiting bodies, no streams formed during aggregation</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>ga 86 and ga 88</td>
<td>Fruiting bodies, large aggregation territories</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

+ and ++ indicate arbitrary classifications into weak and strong responses.
between growth phase and the acquisition of aggregation competence. One
would expect then efficient donor mutants to exist which are arrested at the
early stage of differentiation during which ASF is formed. This possibility was
tested in the efficient donor, *Wag-11*, by the determination of contact sites A.
These constituents of the cell membrane appear simultaneously with the
ability of aggregating cells to associate into streams (Beug, Katz & Gerisch,
1973a). Fig. 3 shows that *Wag-11* is defective in the expression of contact

![Fig. 3. Contact sites A in two ASF donor mutants. Similar to aggregation-competent wild-type *Ax-2* cells (○), *aggr 39* (■) absorbed *F*_ab specific for contact-sites A (Beug et al. 1973). The same immunoassay of contact sites A showed that *Wag-11* (▲) equals wild-type *Ax-2* cells of the growth-phase stage (○) where these sites are almost absent. (The weak absorption found can be attributed to contact sites B, which are already present in growth phase cells and not fully blocked by 10 mM EDTA (Beug et al. 1973).) Mutant cells were harvested for *F*_ab absorption at a time when their wild-types would have reached full aggregation-competence: *aggr 39* at 5 h, *Wag-11* at 9 h after the end of growth.

sites A. This result indicates that certain donor mutants are blocked after in-
hbitor formation and before the appearance of contact sites A. However,
in other donors like *aggr 39* contact sites A can be expressed (Fig. 3). De-
velopmental arrest at a stage preceding contact-site A formation is therefore
consistent with, but not indispensable for the action of a mutant as an efficient
donor.

**Macrocyst formation.**

Macrocyst formation in wild-type cultures is preceded by the development of
giant cells growing-up by phagocytosis of normal cells (Filosa & Dengler, 1972).
These cells are most probably zygotes (MacInnes & Francis, 1974). Certain
clones of *aggr 50–2* formed macrocysts in combination with *Wag-11* (Fig. 1E).
In addition to mature macrocysts, actively motile giant cells were found (Fig.
1B, C), as well as rounded giant cells containing multiple contractile vacuoles
(Fig. 1D). In the *aggr 50–2–5/Wag-11* combinations shown in Fig. 1B–E, only
Aggregation deficient mutants of Dictyostelium

sporadically aggregation streams were observed although these combinations belong to the best macrocyst forming ones. These mutants indicate, therefore, that all factors necessary for the mating reaction are expressed in the absence of full aggregation-competence. Giant cells can exist in these mutant combinations in a free motile state obviously because of the lack of tightly adhering cells around them. In a wild-type strain it is difficult to isolate the giant cell from peripheral cells (Filosa, Kent & Gillette, 1975).

**DISCUSSION**

Synergism between aggregation-deficient mutants has been repeatedly observed (Sussman & Lee, 1955; Ennis & Sussman, 1958; Weber & Raper, 1971; Yamada, Yanagisawa, Ono & Yanagisawa, 1973) since its first investigation by Sussman (1954). In most of these cases immediate contact between the mutant cells is required for synergism. The present case represents the other extreme: Aggregation is stimulated by a diffusible factor (ASF) which extends the influence of the donor mutant over a longer distance. Since the responder strain retains the phenotype of a non-aggregating mutant when grown on washed *E. coli* B/r, and does so also when washed after the end of growth (Huesgen, 1973), the synergism is not of the type reported by Weber and Raper (1971). In this case the responding mutants are able to develop normally in the absence of a helper mutant when freed from bacterial metabolites.

ASF-donor strains exhibit a great variety of phenotypes (Table 3), so that the ASF-donor activity cannot be associated with a specific morphogenetic character. The obvious reason is that the ASF-donor activity is expressed at an early stage of cell differentiation, prior to the expression of any specific morphogenetic function. ASF-donors are found among mutants exhibiting opposite defects in the regulation of cyclic-AMP phosphodiesterase and its inhibitor (Fig. 2 and Table 2). This indicates that neither the enzyme nor the inhibitor activity is required for ASF action. The inhibitor, however, appears to be a fairly reliable marker for the developmental stage which a mutant has either to reach or to pass in order to produce ASF. This stage is reached before the acquisition of full aggregation-competence, as suggested by the ASF-donor activity of a mutant which does not express contact sites A (Fig. 3).

The absence of any differentiation marker in the ASF-requiring mutant has lead to the conclusion that cell differentiation from growth phase to aggregation-competence is blocked at an initial step in this mutant and, as far as aggregation is concerned, ASF is able to bridge this deficiency (Gerisch et al. 1975b). These results are summarized in Fig. 4. The effects of ASF and of cyclic-AMP pulses (Gerisch et al. 1975a; Gerisch, Fromm, Huesgen & Wick, 1975c; Darmon, Brachet & Pereira da Silva, 1975) show that in *D. discoideum* populations, cell differentiation up to the aggregation stage is largely synchronized by chemical cell communication. A third factor, folic acid, identified by Pan, Hall & Bonner,
(1972) as a chemotactic agent, controls cell differentiation in a similar way as cyclic AMP does (Wurster, 1976). It remains to be shown if folic acid pulses are produced by developing *D. discoideum* cells.

The wild-type *NC-4* releases a gamone that induces a sexual response in the wild-type strain *v-12* (O’Day & Lewis, 1975; MacHac and Bonner, 1975). The induction of macrocysts by combination of two aggregation-deficient mutants, a *NC-4* descendant and a *v-12* derived one, indicates that *Wag-11* produces the gamone and that *aggr 50–2–5* is able to respond to it. Identity of the gamone with ASF is unlikely since ASF is produced in mutants of both wild-type strains *NC-4* and *v-12* (Table 1). Consequently, ASF is not limited to one specific mating type. The highly motile giant cells shown in Fig. 1B and C pose the question of how the contractile system of *Dictyostelium* is constituted in order to allow polar cell movement in a 10 μm cell as well as in a cell of more than 100 μm in length.

We thank Professor J. M. Ashworth, Colchester, for the Wag mutants, Miss E. Müller and Miss S. Wicki for careful assistance. This work was supported by the ‘Schweizerischer Nationalfonds’ and the ‘Deutsche Forschungsgemeinschaft’.

**REFERENCES**


Aggregation deficient mutants of Dictyostelium


(Received 15 June 1976)