Initiation of Aggregation by *Dictyostelium discoideum* in Mutant Populations Lacking Pulsatile Signalling

By PETER M. GLAZER AND PETER C. NEWELL*

Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX1 3QU, U.K.

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The proportion of a wild-type population capable of initiating aggregation centres in starving fields of *Dictyostelium discoideum* was investigated using mixed populations of a few wild-type amoebae and a large excess of a mutant incapable of initiating aggregation signals but fully capable of responding to them. For this purpose an aggregation-deficient mutant (designated NP160) was isolated that showed high sensitivity to wild-type strains in synergy tests. This mutant formed normal cell-surface cyclic AMP receptors and phosphodiesterases and was fully capable of chemotaxis to cyclic AMP. Although it had an active adenylate cyclase and formed cyclic AMP, it was deficient in the pulsatile regulation of this enzyme and did not initiate or relay pulsatile cyclic AMP signals and did not form cell adhesive 'contact sites A' unless given artificial cyclic AMP pulses. When synergistic mixtures of this mutant and the wild-type were made, the mutant formed fruiting bodies at frequencies indicating that up to 1 in 5 of the wild-type cells could initiate aggregation centres when present at a ratio of between 1 in 500 and 1 in 10 000 of the mixed amoebal population.

INTRODUCTION

Whether the amoebae that initiate the aggregation phase of *Dictyostelium* form a genetically distinct subpopulation has been a matter of controversy for many years. Although it is now commonly stated that cells capable of initiation are not genetically different but arise at random in a starving population, no conclusive evidence for this notion has been reported.

The idea of a genetically distinct population of 'Initiator' (or 'I') cells was first proposed by Sussman & Noel (1952) and evidence for their existence was presented by Ennis & Sussman (1958*a*). I cells were reported to be morphologically distinct from other cells, being two to three times larger in diameter, and they were found at a fixed proportion of about 1 cell in 2000, even in the pre-aggregative growth phase. The I cells could be correlated with the sites at which aggregation centres formed and it was found that one aggregate was formed for approximately every 2200 cells of the wild-type in agreement with the observed frequency of I cells. Konijn & Raper (1961), however, questioned the existence of a special cell type occurring at a definite proportion that was exclusively capable of autonomy. They found that aggregates almost always developed in populations even as small as 100 cells, and they were unable to detect any morphologically distinct cells among these populations. They did observe some larger-than-average cells, but they could not correlate the presence of these cells with aggregation. They proposed the existence of a single population of cells with a continuous size distribution instead of the discontinuous distribution of initiator and responder cells described by Ennis & Sussman (1958*a*). Gerisch (1961) confirmed the capacity of small, isolated populations to aggregate by showing that aggregation occurred regularly in populations of 100 to 200 amoebae. Like Konijn & Raper (1961), he was unable to find a correlation between cell morphology and aggregation, and he concluded that if I cells exist,

they must occur at least twenty times as frequently as Sussman and his co-workers proposed.

Subsequently, the question of the nature of the autonomous cell was taken up again by Raman *et al.* (1976) who sought to quantify the intrinsic probability that a given cell will express autonomy as a function of the time of development, the size of total cell population, and the cell density. By observing small populations of cells on agar carefully controlled for these parameters, and by measuring the absence rather than the occurrence of aggregation (where aggregation occurs it is impossible to say how many autonomous cells were present), they found that the probability of autonomy saturates by 24 h after starvation and is significantly non-zero only after about 8 to 10 h. They also showed that the saturation value for the probability of autonomy was inversely proportional to the total cell population for a fixed cell density. The highest value found for this probability was 10^{-2} , in agreement with the data of Konijn & Raper (1961), but they were also able to reproduce the numerical results of Ennis & Sussman (1958*a, b*) at the appropriate cell density and population. Raman (1976) argued that such evidence concerning aggregation in small populations of cells cannot resolve whether or not there exists a special subset of cells predetermined to be exclusively capable of autonomy.

One way to resolve this question would be to look at smaller and smaller populations of cells. But aggregation among wild-type cells is infrequent in populations below about 100 cells, and aggregation of a single cell is an absurd notion. Therefore, the question cannot be answered by looking only at homogeneous cell populations. However, as Raman (1976) suggested, it 'can perhaps be resolved only through a detailed study of mixed populations of wild-type amoebae and mutants that do not show autonomy', for in such mixtures the capacity of individual wild-type cells to initiate aggregation can theoretically be measured. It was this experimental approach that was adopted for the work described in this paper.

For such an investigation it was necessary to have an aggregation-deficient mutant which could not generate autonomously signalling cells but which could show synergism with signalling-competent wild-type cells and which possessed sufficient sensitivity to aggregate when initiated by very small numbers of the wild-type. Early work by Sussman (1952) and Ennis & Sussman (1958*b*) with synergistic mixtures of wild-type and aggregation-defective mutants used mutant strains that lacked this sensitivity. To be sensitive to isolated wild-type cells the mutant would have to be normal in forming cell-surface cyclic AMP receptors and phosphodiesterases and be chemotactically competent. This combination of characteristics is not commonly found in non-aggregating strains and does not, for example, fit any of the categories of aggregation mutants of Darmon *et al.* (1977) or Juliani & Klein (1978). After initial screening, strain NP160 was selected for further study as it showed an unusual ability to synergize not just with wild-type strains but also with many mutant strains that could achieve the loose cell aggregate stage in development. Furthermore, spores of the fruiting bodies produced by synergism of NP160 with the wild-type yielded amoebal populations reflecting the original proportion of strains mixed, whereas for other mutants tested the wild-type was normally favoured by a factor of 10^2 to 10^4 . Mutant NP160 seemed, therefore, to be a good prospect for the desired mutant, as was confirmed in the biochemical and genetic work outlined below.

METHODS

Media and chemicals. The composition and methods of preparation of nutrient (SM) and selective media, and the chemicals used were as described previously (Mosses *et al.*, 1975; Ratner & Newell, 1978).

Strains. All *Dictyostelium discoideum* strains used are derivatives of NC4 (Raper, 1935). Strain M28, originally obtained from Dr Eugene Katz, S.U.N.Y., Stonybrook, N.Y., U.S.A., is a developmentally competent strain that bears the genetic markers *bwnA* (production of brown pigment), *tsgE* (temperature sensitive for growth) and *sprA* (round spores) (Katz & Sussman, 1972). Strain NP160 was isolated as a spontaneous mutant of strain M28 that was unable to aggregate.

Maintenance of stocks. All strains of *D. discoideum* were grown at 22 °C in the dark on SM agar in association with a cobalt-resistant mutant of *Klebsiella aerogenes* designated OXF1 (Williams & Newell, 1976). Working

stocks were maintained by weekly clonal passage in association with bacteria. The primary stock of strain NP160 was kept as spores produced in synergism with strain M28. The mixture of NP160 spores and M28 spores was maintained desiccated in silica gel at 4 °C. A stock of M28 spores alone was also kept in silica gel, and a secondary stock of NP160 amoebae, derived from a single clone from the above mixture of spores, was stored frozen in horse serum containing 5% (v/v) dimethyl sulphoxide in plastic straws immersed in liquid nitrogen.

Parasexual genetic manipulations. These were carried out using previously described methods (Williams *et al.*, 1974; Ratner & Newell, 1978; Ross & Newell, 1979).

Cyclic AMP binding assay. The assay used was that described by Green & Newell (1975), except the Nuclepore polycarbonate filters were used instead of Millipore membrane filters (Mullens & Newell, 1978). For the time course of cyclic AMP binding, cells were harvested, washed free of bacteria, resuspended at 1×10^7 amoebae ml^{-1} in 17 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.1, and then shaken at 22 °C in a rotary shaker. At intervals, samples of the cell suspensions were withdrawn and washed twice with 50 mM ice-cold $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.2, and readjusted to 10^7 cells ml^{-1} for the assay, which was performed in duplicate.

Assay of membrane-bound and extracellular phosphodiesterase. Phosphodiesterase activity was assayed using the method of Ross & Newell (1981) which is a modified form of the chromatographic assay of Malchow *et al.* (1972).

Test for chemotaxis. Agar plates containing 2% (w/v) Oxoid agar and 25 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.0 were made up with and without cyclic AMP. For each strain, the series of plates included a control plate with no cyclic AMP and plates containing cyclic AMP at concentrations of 10 nM, 100 nM, 1 μM , 10 μM and 100 μM . Cells were harvested, washed free of *K. aerogenes* and resuspended in 17 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.1 at 2×10^7 cells ml^{-1} . Fifteen drops of about 20 μl each from the appropriate cell suspensions were put down in a grid on to each of the plates. The plates were kept at 22 °C for 7 h and then examined for evidence of chemotaxis. Chemotaxis was indicated by increased outward movement of the cells from their original small drops, as compared with the cells on the control plates lacking attractant. Cells in the drops on the unsupplemented agar tended to stay within the area of the small drop, but in the plates containing cyclic AMP, spatial gradients of cyclic AMP were created around the small drops by the action of the extracellular phosphodiesterase produced by the cells. If the cells were chemotactically competent, they responded to these gradients by moving outward from the confines of their small drop. Hence, a positive result in this test indicated that the cells were capable of both chemotaxis and of extracellular phosphodiesterase production. A negative result meant that the cells lacked one or both of these capacities.

Light scattering in cell suspensions. Cells were harvested, washed free of bacteria and resuspended in 17 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.1 at 2×10^7 cells ml^{-1} . The cell suspension was shaken at 22 °C, and at various times during development 30 ml samples were withdrawn. Cells were washed twice with phosphate buffer and finally resuspended in 3 ml of buffer, at about 2×10^8 cells ml^{-1} . This suspension was placed in a 3 ml spectrophotometer cuvette with a wax slope in the bottom (Gerisch & Hess, 1974). Oxygen was bubbled through the suspension in the cuvette through two syringe needles. Cyclic AMP was added to the cuvette in 100 μl quantities from solutions containing approximately 30 times the final concentration of cyclic AMP desired. Changes in the absorbance at 405 nm of the cell suspension were monitored using a recording spectrophotometer.

Cyclic AMP assays. The nucleotide was assayed using the cyclic AMP assay kit from Amersham. Cells were harvested, washed free of bacteria, resuspended at 2×10^7 cells ml^{-1} in 17 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.1, and shaken at 22 °C. At intervals, 10 ml samples were withdrawn and the cells were separated from the supernatant by centrifugation for 3 min. The cell sediment was resuspended to a final volume of 300 μl in Tris/EDTA buffer. From this suspension, four 35 μl samples were taken at 90 s intervals and added to a microfuge tube already containing 20 μl of 4 M-perchloric acid and 5 μl of universal indicator; the pH was adjusted to 7.5 with 4 M-KOH. The samples were collected in four parts at 90 s intervals in order to average fluctuations in cyclic AMP pool levels, and they were mixed with perchloric acid so that cellular reactions, especially phosphodiesterase activity and continued cyclic AMP production, would be uniformly halted. After neutralization with KOH, each sample was centrifuged for 2 min. The supernatant, which contained the cyclic AMP, was assayed in duplicate: 50 μl supernatant was placed in each of two separate microfuge tubes in an ice bath and to each tube was added 50 μl of the cyclic [^3H]AMP solution (containing 0.9 pmol) and 100 μl of binding protein solution, both from the assay kit. After mixing, the tubes were left at 4 °C for 2 h, and then 100 μl of activated charcoal suspension was added to each tube. Each tube was shaken thoroughly, left at 4 °C for exactly 2 min and then centrifuged for 2 min to separate the charcoal fraction. A 200 μl portion of the supernatant was mixed in a scintillation vial with 3 ml of toluene/triton/2,5-diphenyloxazole scintillant, and the radioactivity in each vial was recorded. Serial dilutions of a standard cyclic AMP solution were treated in the same way as the unknowns.

Assay of contact sites A. Cells were harvested, washed free of bacteria and resuspended in 17 M- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer pH 6.1 at 1×10^7 cells ml^{-1} . For each strain the cell suspension was divided into two parts: the first was left stirring at 22 °C, while the second 50 ml portion was stirred at 22 °C and treated with cyclic AMP pulses at 5 min intervals starting 1.5 h after starvation. Pulses were delivered in the form of 27 μl drops of a 185 μM -cyclic AMP solution, adding 5 nmol cyclic AMP per pulse to a final concentration of approximately

100 nM. The drops were generated by carefully controlling the flow of a cyclic AMP solution from a large reservoir in a separatory funnel through clamped rubber tubing and a drawn out Pasteur pipette. At each time point, two 0.1 ml samples of each suspension were taken to assay for the formation of EDTA-resistant cell contacts. To one of these samples, 0.5 ml of a 0.01 M-EDTA solution was added; the other received 0.5 ml of buffer alone. Both mixtures were shaken vigorously for 15 s in order to dissociate possible existing agglutinates. The mixtures were left for 2 min and then examined by microscope for the presence of cell clumps.

Synergism of strains M28 and NP160. Square agar chips of area 2.25 cm² were cut from 2% agar plates containing 25 mM-K₂HPO₄/KH₂PO₄ buffer pH 6.2. Freshly harvested cells of M28 and NP160 were carefully spread on to the agar chips to the appropriate density. Volumes of between 1 and 50 µl of appropriately diluted cell suspensions were spread; all agar chips received a total of about 45 000 cells, made up of different proportions of M28 and NP160 cells. They were incubated at 22 °C under controlled humidity and monitored for 5 d for the development of fruiting bodies.

RESULTS

Genetic locus of strain NP160

Mutant NP160 arose spontaneously in the developmentally proficient strain M28. The mutant produced clones which grew at the normal growth rate on agar plates inoculated with lawns of *K. aerogenes* but failed completely to aggregate and consequently had a flat morphology inside the clonal growth ring. The mutation (*agg-87*), which was stably inherited, was located on linkage group IV by its segregation pattern from diploids DP1028 and DP1029 constructed from NP160 and the tester strains XP95 and XP92, respectively (Table 1). The spontaneous occurrence and simple pattern of segregation indicated that the mutation was probably at a single locus.

Complementation studies in diploids constructed with other aggregation-deficient strains showed that the mutation was in a different complementation group from previously published mutations and the locus was, therefore, designated *aggL*.

Formation of the chemosensory apparatus

Cyclic AMP receptors. The ability of strain NP160 to form cell-surface cyclic AMP receptors was tested by the binding of cyclic [³H]AMP to amoebae and separating amoebae from the unbound radioactivity using the membrane filter technique (Green & Newell, 1975; Mullens & Newell, 1978). The degree of binding (Fig. 1) found for NP160 was similar to that for strain M28 and clearly showed an adequate degree of binding in the mutant for efficient cyclic AMP detection. Analysis using Scatchard plots showed normal formation of both high and low affinity forms of the receptor.

Phosphodiesterases. When phosphodiesterase activity was measured both in the extracellular supernatant (Fig. 2) and associated with the cell-surface membranes (Fig. 3) the mutant was again found to resemble the parental strain M28 over the first 8 to 10 h of development. The amount of phosphodiesterases produced by NP160 is sufficient for signal destruction (needed as part of the signal detection system) and indeed is slightly higher than for some other parental types that are aggregation competent.

Chemotaxis. The chemotactic ability of NP160 was assayed in two ways. Firstly, the movement of amoebae was observed in a drop of cells on buffered non-nutrient agar containing cyclic AMP at a concentration of 10 nM to 100 µM. Over a period of 7 h both M28 and NP160 were seen to move outward as a ring. This test not only demonstrated the chemotactic ability of the mutant but also confirmed the existence of a functional phosphodiesterase, as the movement is a response to a cyclic AMP gradient created by the phosphodiesterase of the cells in the drop (Bonner *et al.*, 1969). The second chemotactic test made use of the changes in optical properties of starving amoebae in suspension in response to pulses of cyclic AMP (Gerisch & Hess, 1974). During the response the absorbance of the cell suspension decreases transiently in a characteristic manner that is correlated with chemotactic movement (Ross & Newell, 1981). The sensitivity to pulses of cyclic AMP

Table 1. *Phenotype pattern of haploid segregants selected from heterozygous diploids formed from NP160 and XP92 (DP1029) and from NP160 and XP95 (DP1028)*

By convention, the recombinant segregants lie on the south west-north east diagonal in each set of four figures, so zeros in this diagonal indicate linkage of the two loci involved. The zeros in parentheses indicate that the given class of haploid segregant was necessarily excluded by the haploid selection technique used; from DP1029 no *Cyc*⁺ segregants were possible, and from DP1028 no *Acr*⁺ segregants were possible, because the segregants from these diploids were selected on plates containing cycloheximide and methanol, respectively. The tester strain phenotype is represented in the north west corner; the NP160 mutant phenotype is in the south east corner.

Heterozygous diploid	Parental haploids	Aggregation phenotype	Linkage group						
			I	II	III	IV	V	VI	VII
DP1029	{ NP160 XP92 }	Agg ⁺ Agg	+ <i>tsgE sprA</i> <i>cycA</i> + +	—	+ +	<i>bwnA</i> +	—	+ <i>manA</i>	+ <i>cobA</i>
			<i>Cyc</i> <i>Cyc</i> ⁺	—	<i>tsgA axeB</i>	<i>Bwn</i> ⁺ <i>Bwn</i>	—	<i>Man</i> <i>Man</i> ⁺	<i>Cob</i> <i>Cob</i> ⁺
			12 (0)	—	<i>Tsg</i> <i>Tsg</i> ⁺	12 0	—	11 1	8 4
			38 (0)	—	26 12	0 38	—	19 19	27 11
DP1028	{ NP160 XP95 }	Agg ⁺ Agg	+ <i>tsgE sprA</i> <i>Spr</i> ⁺ +	+ +	+ +	<i>bwnA</i> <i>bwnA</i>	—	+ <i>manA</i>	+ <i>cobA</i>
			<i>Spr</i> ⁺ <i>Spr</i>	<i>whiA acra axeA</i>	<i>tsgA axeB</i>	<i>Bwn</i> ⁺ <i>Bwn</i> ⁺	—	<i>Man</i> <i>Man</i> ⁺	<i>Cob</i> <i>Cob</i> ⁺
			51 17	<i>Acr</i> <i>Acr</i> ⁺	<i>Tsg</i> <i>Tsg</i> ⁺	68	—	38 30	25 43
			† †	68 (0) 122 (0)	29* 28 98* 24	122	—	87 35	48 74

* The *Tsg* class may be over-represented in this linkage group because the segregants from DP1028 may contain not only the *tsgA* marker from XP95 but also the *tsgE* marker from NP160. This problem does not arise with segregants from DP1029 because they were selected on cycloheximide plates which exclude the *tsgE* marker from NP160.

† Since the aggregation-deficient segregants produce no spores, they have no *Spr* shape phenotype.

‡ As both NP160 and XP95 carry the *bwnA* marker, no *Bwn*⁺ segregants were expected, and none were found.

Fig. 1. Time course of cyclic AMP binding to whole cells of parental strain M28 (O) and mutant NP160 (●).

Fig. 2

Fig. 3

Fig. 2. Activity of extracellular cyclic AMP phosphodiesterase during the first 14 h of development of parental strain M28 (O) and mutant NP160 (●) in starving cell suspensions.

Fig. 3. Activity of cell-associated cyclic AMP phosphodiesterase during the first 14 h of development of parental strain M28 (O) and mutant NP160 (●) in starving cell suspensions.

between 10 μ M and 100 pM was determined for NP160 and was found to be almost identical to that for the parental strain M28 (Fig. 4). The refractory period for the chemotactic response was also investigated by using two pulses of cyclic AMP given at intervals of 2 min or a few seconds apart. As shown in Fig. 5, the response for a second pulse

Fig. 4. Light scattering responses of mutant NP160 and parental strain M28 produced by pulses of cyclic AMP. Oxygenated suspensions of starving amoebae ($2 \times 10^8 \text{ ml}^{-1}$) were monitored for absorbance at 405 nm after 9 h of development at 22 °C. The effect of pulses of cyclic AMP from 10 μM to 100 pM (final concentration) was recorded.

Fig. 5. Light scattering responses of mutant NP160 and parental strain M28 to two 100 nM pulses of cyclic AMP (final concentration) delivered with a short interval between them. Conditions were as described in Fig. 4. The cyclic AMP pulses were delivered with 2 min, 1 min and 30 s intervals at times indicated by the arrows.

could be seen in both strains even if this was given only 30 s after the first. Clearly this facet of the chemosensory apparatus is not deficient.

Cyclic AMP formation. The basal cellular concentration of cyclic AMP was measured using a binding assay to determine whether or not the mutant possessed active adenylate cyclase. The results (Fig. 6) indicate that the pattern of cyclic AMP concentration was similar in NP160 and M28 at early times of starvation but after 6 to 7 h the parental strain showed a considerably higher concentration. Although the assay could not detect the individual fluctuations of cyclic AMP that would be expected in a signalling-competent strain, these fluctuations would tend to increase the average concentration observed by this method, thereby producing the increase in the cyclic AMP concentration observed in M28. Direct assay of adenylate cyclase (kindly performed by Dr M. B. Coukell, using the methods of Coukell & Chan, 1980) confirmed that the mutant possessed the basal activity of this enzyme and that this increased during the first 6 h of development in NP160 to approximately 50% of

Fig. 6. Cellular cyclic AMP concentrations in parental strain M28 (○) and mutant NP160 (●) during the first 12 h of development in cell suspension. These curves represent the average of two separate experiments.

the activity of the parental strain. [NP160 activity rose from 0.3 units (mg protein)⁻¹ at the end of the growth phase to 9 units (mg protein)⁻¹ after 6 h of development compared with the parental strain increasing from 0.8 to 22 units (mg protein)⁻¹ in the same time period. One unit is the activity producing 1 pmol cyclic AMP min⁻¹.] It is deduced that the primary lesion of NP160 does not lie in the basal activity of adenylate cyclase but the data are not inconsistent with a fault in the periodic activation of the enzyme needing for signalling.

Signal relay. Evidence that mutant NP160 is deficient in signal relay and autonomous signal generation came from visual observations of starving amoebae placed as lawns at high density (8×10^5 cm⁻²) on buffered agar. Under dark-field illumination (Gross *et al.*, 1976) relay may be visualized in the wild-type as bright and dark bands moving outward from the aggregation centres. These bands correspond to cells moving towards the centres (light bands) and stationary cells awaiting the next signal (dark bands) (Alcântara & Monk, 1974). Under such conditions NP160 produced no bands at all, the cells merely remaining as a randomly moving lawn. When agar blocks bearing amoebae showing moving light and dark bands were cut out of control plates and carefully placed in direct contact alongside blocks bearing NP160, the relayed waves were not continued by the mutant but failed abruptly at the junction of the two blocks. There was some evidence, however, of local chemotactic movement of the NP160 on to the agar with the relaying cells, indicating that the mutant could perceive the signals.

Contact site A. No contact sites A were formed during starvation in liquid suspension culture. Contact sites A are glycoproteins that are formed during starvation and mediate intercellular binding (Beug *et al.*, 1973; Huesgen & Gerisch, 1975). They are regulated by periodic cyclic AMP signalling during aggregation (Gerisch *et al.*, 1975) and are not formed in the absence of pulsatile signalling. To see if contact sites A could be induced in NP160 by artificial cyclic AMP pulses, cyclic AMP was added at 5 min intervals to suspensions of the mutant (to a concentration of 100 nM) over a period of 8 h. Visual inspection clearly demonstrated the presence of cell clusters and chains which were not disrupted by 5 mM-EDTA, indicative of contact sites A. Control suspensions of NP160 without cyclic AMP pulses showed only single cells. It is deduced that NP160 is only able to form contact sites with the aid of artificial pulses and we infer that such cyclic AMP pulses are normally lacking in the mutant.

Mutant NP160 is, therefore, an excellent mutant for use in experiments designed to test the capacity of the wild-type strain to initiate aggregation. It is unable to initiate or relay pulses of cyclic AMP itself but can respond to signals from the wild-type with a normal chemotactic response and form fruiting bodies. It is able to receive and process cyclic AMP signals with a

Fig. 7

Fig. 8

Fig. 7. Number of fruiting bodies formed in mixtures of wild-type strain M28 and mutant NP160 on non-nutrient agar chips. The total cell density was kept at $2 \times 10^4 \text{ cm}^{-2}$ but the relative density of the two strains was varied from one extreme to the other.

Fig. 8. Number of fruiting bodies formed by homogeneous populations of wild-type strain M28 on non-nutrient agar chips at 22°C . Note that at a cell population density above $4 \times 10^4 \text{ cm}^{-2}$ the number of fruiting bodies formed sharply declined. This point represents the critical density at which cells are close enough for signal relay to operate and thereby cause an increase in the size of the aggregation territory.

Fig. 9. Number of fruiting bodies formed in synergistic mixtures of wild-type strain M28 and mutant NP160 on non-nutrient agar chips in the absence (●) and presence (▲) of 10 mM-L-histidine. The data represent the mean of six experiments. The number of NP160 cells was kept at $2 \times 10^4 \text{ cm}^{-2}$ and the number of M28 cells was varied from 0 to 50 cm^{-2} . The number of M28 cells plated was determined by careful serial dilution of suspensions dense enough to be counted in a haemocytometer and then checked by plating samples of the diluted suspension on to lawns of *K. aerogenes* to determine viable counts.

sensitivity that would enable it to respond to weak signals from low numbers of wild-type amoebae.

Ability of the wild-type to form aggregation centres in mixtures with mutant NP160

The ability of strain M28 to initiate aggregation centres was tested using starving mixed populations of M28 and NP160 amoebae on non-nutrient agar. The number of fruiting bodies formed by such mixtures was assessed under the strictly standardized conditions outlined in Methods. The total population per agar chip was kept at 4.5×10^4 (i.e. $2 \times 10^4 \text{ cm}^{-2}$) and the

ratio of the strains was varied from one extreme to the other. The results (Fig. 7) indicate that the number of fruiting bodies formed was dependent on the number of M28 amoebae present. It was also noticeable that with increasing numbers there was substantial competition among M28 amoebae in the formation of these centres. This competition, however, was not due to signal relay as the highest concentration of M28 cells used ($2 \times 10^4 \text{ cm}^{-2}$) was below the critical threshold needed for signal relay (Fig. 8) as determined by the method of Gingle & Robertson (1976). This competition decreased the apparent efficiency of centre formation by the M28 cells to approximately 1 centre for every 1000 M28 cells plated. To overcome this competitive effect, further experiments were carried out to look at the number of fruiting bodies formed in mixed populations containing very few M28 cells (between 2 and 50 cells cm^{-2}) (Fig. 9). With so few wild-type cells, the number of aggregates varied linearly with the number of M28 cells plated, presumably because the M28 cells were too sparsely spread for competitive interactions to occur. Under such conditions it may be calculated from the slope of the graph that 1 fruiting body was formed for every 10 M28 cells present.

Whether or not an M28 cell triggers chemotaxis and aggregation by nearby NP160 cells must depend on the excitability of the M28 cell and on the sensitivity of the responding NP160 cells. In an attempt to increase this sensitivity, a number of agents were added to the agar. Over a wide range of concentrations Ca^{2+} had no apparent effect, while phosphodiesterase (from beef heart) caused only a marginal increase in the number of fruiting bodies produced by the synergistic mixtures. However, when L-histidine, which was shown by Bradley *et al.* (1956) to stimulate aggregation, was added to the agar, a marked increase in the number of fruiting bodies produced by the synergistic mixtures was observed (Fig. 9), the slope of the graph indicating that under these conditions 1 in 5 of the M28 cells was initiating fruiting body formation.

DISCUSSION

Previous attempts at finding the number of cells in an amoebal population that could initiate aggregation either used homogeneous populations containing several thousand cells and found initiators at a frequency of roughly 1 in 2000, or used small drop populations of 100 to 200 cells and found initiators at a frequency of roughly 1 in 100. It is clear from the present study, however, that substantial competition for centre formation arises with populations containing more than 100 cells cm^{-2} and as a consequence only minimal estimates for centre-forming ability may be made in such studies. On the other hand, populations of much less than 100 wild-type cells on agar do not consistently initiate aggregates. The present study obviated this dilemma by using very small numbers of wild-type cells mixed with a large population of a mutant that was itself unable to initiate centres.

Most aggregation mutants would have been unsuitable for such a study as they either do not interact to produce fruiting bodies at high frequency in mixtures with the wild-type, or they fail to produce components of the chemosensory apparatus (such as phosphodiesterases) that ensure high sensitivity to signals produced by wild-type cells present in population ratios of only 1 in 10000. Mutant NP160 was found to show unusually high synergism with the wild-type and detailed studies of its genetics and biochemical abilities revealed that it possessed a mutation on linkage group IV that prevented it from initiating or relaying cyclic AMP signals in the aggregation phase but that in other important respects it appeared normal. It grew with the vigour of the wild-type and for the first 8 h of aggregation it produced cell-surface cyclic AMP receptors, cyclic AMP and extracellular and membrane-bound phosphodiesterases in amounts similar to the parental strain. It showed normal chemotactic ability in artificial cyclic AMP gradients and, as would be expected for such a mutant lacking signal initiation, contact sites A were not formed in the absence of artificial cyclic AMP pulses. Its properties, therefore, made it highly suitable as a synergistic partner

for testing the initiative ability of the wild-type present at low frequency, and in a wider context it should prove valuable for other studies requiring a mutant with these unusual characteristics.

Synergism tests of strains NP160 and M28 showed that when as few as 2 cells cm^{-2} (5 cells per 2.25 cm^2 agar chip) were present, M28 could initiate aggregation and fruiting body formation in NP160. The ratio of initiation of M28 (found over the range 2 to 50 cells cm^{-2}) was 1 cell in 10 on standard buffered agar and 1 cell in 5 when the sensitivity of the responding amoebae was raised by adding 10 mM-L-histidine to the buffered agar. Although this does not show that *every* M28 is capable of initiation, the finding that 1 in 5 of the population have that capability makes it extremely unlikely that a special subset of initiator cells is responsible for initiating aggregation. If such a subset existed it would comprise 20% of the population even during the growth phase, with the probability that, under most conditions, only 1 cell in 200 of the subset would be needed and used for initiation. Since such a large subpopulation does not have genetic or phenotypic characteristics that have been reported, its existence seems highly unlikely, and we suggest that the data presented is strong evidence that initiator cells are not a pre-existing cell type but arise from the starving population of amoebae during the phase of aggregation.

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