# Breakdown of Self/Nonself Recognition in Cannibalistic Strains of the Predatory Slime Mold, *Dictyostelium caveatum*

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*Abstract.* *Dictyostelium caveatum* amebas feed upon both bacteria and the amebas of other cellular slime molds. The capacity to feed extensively upon other cellular slime molds is unique to *D. caveatum* amebas. They are able to phagocytose amebas larger than themselves by nibbling pieces of the cells until they are small enough to ingest. Here we report the isolation from previously cloned stock cultures of stable, cannibalistic strains of *D. caveatum* in which self/nonself recognition has broken down. Because of the extensive cannibalism, amebas of these strains do not complete multicellular development, and instead wander about for long periods while feeding upon each other. Although the cannibalistic behavior resembles that exhibited by the presumably diploid giant cells in the sexual cycle of other cellular slime molds, these strains are haploid and do not form macrocysts.

THE cellular slime mold, *Dictyostelium caveatum*, is a particularly good organism for the study of cell–cell recognition mechanisms. *D. caveatum* amebas, like those of other slime molds, can be grown upon bacteria in large quantities; however, they have the unique capacity to feed upon the amebas of other species (6, 8). To accomplish this efficiently, they must recognize the difference between amebas of other species and other *D. caveatum* amebas and this implies that a mechanism exists to distinguish self from nonself. The recognition event in this system is transduced into a signal which determines whether to ingest a potential prey cell, a process which can be observed directly and quantitated (9).

One of the principal advantages of studying recognition in cellular slime molds is the capacity to isolate and genetically characterize mutant strains. Here we report the isolation of cannibalistic strains of *D. caveatum*. After bacteria are exhausted, the cells of these strains feed upon each other and therefore can no longer distinguish self from nonself. We propose that *D. caveatum* amebas recognize self via a species-specific receptor that the cannibalistic strains lack.

Since we believe that cannibalism is caused by a single-step mutation, this raises the question of how multicellularity is maintained in this species. In cellular slime molds, starvation induces the chemotactic aggregation of previously autonomous amebas into mounds that later differentiate into fruiting bodies that consist of stalk and spore cells. A consequence of the cannibalism in these strains is that they cannot complete multicellular morphogenesis, presumably because they cannot starve. Consequently, they cannot form multicellular dispersal structures or spores.

## Materials and Methods

### Growth of Amebas

Cells were grown in suspension cultures in association with a rough strain of *Salmonella minnesota* (R595). The bacteria were grown in suspension on nutrient broth (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl, 1% glucose), harvested at late exponential phase, and washed five times with 17.5 mM phosphate buffer (pH 6.0). A bacterial suspension ($A_{420} = 10$) was inoculated with amebas. The doubling time under these conditions was 4–5 h at 27°C. The cells were harvested during exponential growth. Alternatively, amebas were inoculated at the edges of *Escherichia coli* bacterial lawns growing on lactose peptone agar (1.5% Bacto-Agar [Difco Laboratories Inc., Detroit, MI], 0.2% Bacteriological Peptone [Oxoid], 0.2% lactose, 2 mM $KH_2PO_4$, 2 mM $Na_2HPO_4$).

### Cell Density and Cell Volume Determination

Cell counts were carried out in duplicate. 1-ml aliquots were incubated on ice for 15 min and then vortexed until a single cell suspension was obtained. The samples were then counted using an electronic particle counter (Coulter Electronics Ltd., Harpenden, England). To determine cell volume, dissociated cells were counted at different thresholds on the particle counter. The threshold scale was calibrated with latex particles of known size.

### Phagocytosis Assay

The phagocytosis assay used here involves the removal of non-ingested amebae by phallolysin, a lytic protein isolated from *Amanitin* mushrooms (4, 9). A cannibalistic strain which carries a resistant mutation to the drug is used, the prey cells being phallolysin sensitive. This assay has been described in detail elsewhere (9). Briefly, aliquots were diluted 10-fold with distilled water and vortexed to dissociate the cells. The cell density was determined and phallolysin was then added. After 30 min, the cell density was re-determined to obtain the number of resistant cells. The number of wild-type cells was determined by subtraction from the cell count before lysis.

### Electron Microscopy

Cells from suspension cultures were fixed by pipetting a 0.2-ml aliquot into 4

ml of 1% glutaraldehyde in 25 mM cacodylate buffer (pH 7.1). 15 s later, 2 ml of 2% OsO₄ was added and the cells were incubated at room temperature for an additional 15 min. Cells were washed twice with cold cacodylate buffer by pelleting at 200 $g$ for 5 min. The cells were pipetted onto a pre-soaked Millipore filter and coated with a thin layer of 2% agar produced by dipping a metal ring in molten agar that had cooled to 40°C, and the coated filter was dropped immediately into a scintillation vial containing cacodylate buffer on ice. The filters were dehydrated through a cold ethanol and propylene oxide series and embedded in Polybed 812 media (Polysciences, Inc., Warrington, PA). After sectioning the grids were stained with lead citrate and uranyl acetate.

## Chromosome Staining

Cells were grown in suspension on *Salmonella minnesota* at 26°C. The mitotic inhibitor (2) CIPC (Isopropyl-*N*-[3-chlorophenyl]-carbamate, Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (100 mg/ml) and added to exponentially growing cultures (doubling time of 4 h) to give a final concentration of 30 μg/ml. The cultures were incubated for 3 h with the drug and the cells harvested by differential centrifugation at 0°C. The cells were washed twice with 17.5 mM phosphate buffer (pH 6.0) supplemented with 50 μg/ml CIPC. The cells were fixed in methanol/acetic acid (3:1, vol/vol), washed
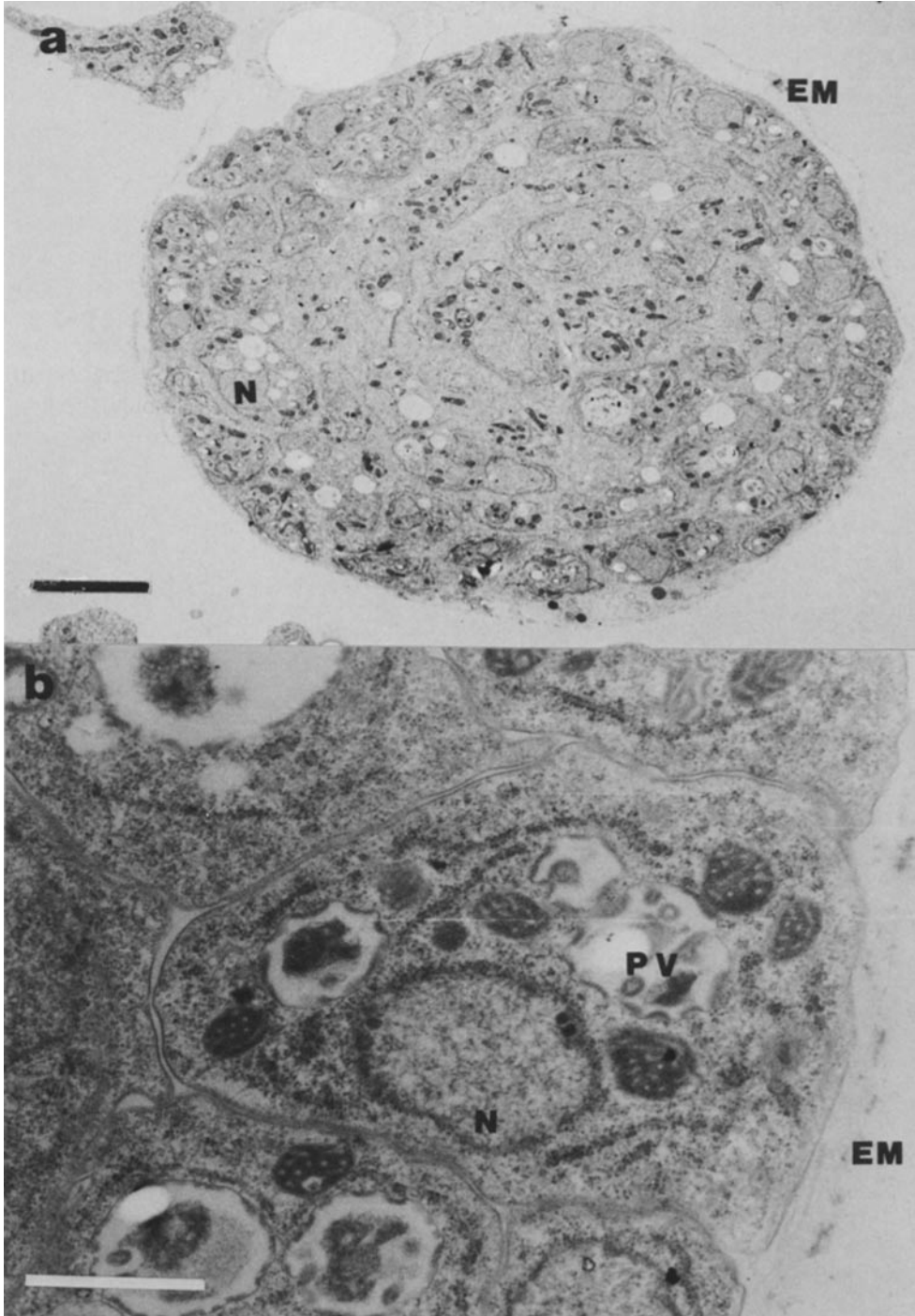


*Figure 1.* Wild-type suspension culture. Cells were fixed 6 h after they were washed free of bacteria and suspended in buffer. (*a*) Tight agglutinate. Bar, 5 μm. (*b*) Higher magnification showing membranes separating cells and prespore vacuoles. *EM*, extracellular material; *PV*, prespore vacuole; *N*, nucleus. Bar, 1 μm.

once with the fixative, and dried onto clean microscope slides. The slides were treated for 90 s with 0.25% trypsin in 0.85% NaCl at room temperature, washed with distilled water, and stained with 10% Gurr's R66 improved Giemsa stain (BDH Chemicals Ltd., Poole, England) for 1 h at room temperature. After washing with distilled water, the slides were mounted with Euparal green.

## Results

The first cannibalistic strains of *D. caveatum* were obtained after extensive cultivation of the wild-type strain in suspension cultures. In wild-type cultures, the amebas grow exponentially until bacteria are consumed. As the amebas starve, they decrease in size and eventually form tight agglutinates consisting of several hundred cells which are difficult to dissociate

(Fig. 1 *a*). The clumps are surrounded by an extracellular material and the cells within the clumps show signs of differentiation such as the presence of prespore granules (Fig. 1 *b*). In contrast, the cultures which had become cannibalistic formed only loose clumps which could easily be dissociated to single cells by incubation for 15 min on ice. Many of the cells within these loose clumps were very large especially when compared to wild-type cells (note that the wild-type cell clump in Fig. 1 *a* is at the same magnification as the cannibalistic amebas in Fig. 2 *a*). The cannibalistic amebas are filled with phagosomes. Furthermore, the fresh phagosomes often contain within them other phagosomes, a consequence of extensive cannibalism (Fig. 2 *b*).
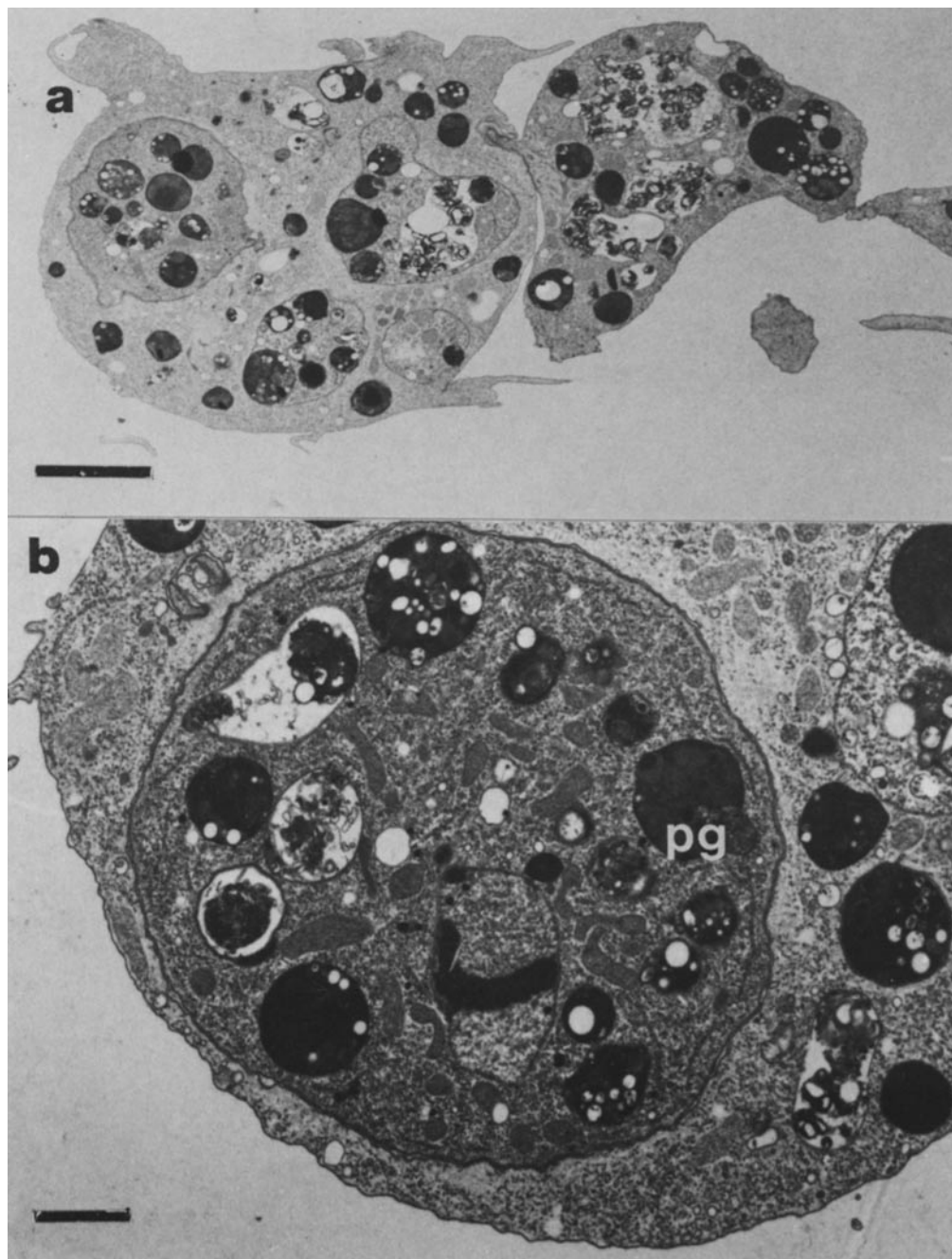


*Figure 2.* Cannibalistic amebas in suspension culture. Cells were fixed 16 h after removal of bacteria. (*a*) A pair of amebas feeding upon each other. (*b*) Higher magnification of a fresh phagosome containing older phagosomes. *pg*, phagosome. Bars, (*a*) 5 μm and (*b*) 1 μm.

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After exhaustion or removal of bacteria in suspension cultures of cannibalistic strains, the cell number decreased steadily for long periods (Fig. 3). Washed cells exhibited a constant rate of decrease with a half-life of 6.9 h during the first 24 h. During this period, the cell density decreased more than 10-fold. This indicates that the cell density did not strongly affect the rate at which cells fed upon each other. This was probably due to the fact that the cells fed upon each other in clumps that formed rapidly after the cells were inoculated into the flasks. The cannibalistic strains appeared to exhibit little or no stationary phase but began to feed upon each other as soon as bacteria were exhausted. In some experiments using washed amebas, a lag period was observed before cell numbers began to decrease. However, since *D. caveatum* amebas begin by nibbling pieces of cells, this probably reflected the time necessary for the first cells to be completely ingested. This also occurs when wild-type cells are fed amebas of other species (9).

The size distribution of cannibalistic amebas feeding upon each other was very broad (Fig. 4). The smallest cells probably represented those being fed upon by other cells. Since *D. caveatum* amebas can feed by nibbling, smaller cells can feed upon larger cells. However, larger cells can take larger bites and hence would be favored. Despite the presence of many small cells, the mean cell volume of amebas feeding upon each other for 24 h ($1.02 \times 10^{-6}$ $\mu$l) was greater than that of bacterially grown amebas ($0.667 \times 10^{-6}$ $\mu$l). This size increase was probably due to the amebal diet since wild-type amebas also increase in size when feeding upon other amebas as opposed to bacteria (9). However, the mean cell volume of bacterially grown cannibalistic amebas was also greater than that of bacterially grown wild-type amebas ($4.62 \times 10^{-6}$ $\mu$l), so this may not completely explain the size difference.

A corresponding phenotype was observed when the cannibalistic strains were inoculated at the edges of bacterial lawns (Fig. 5, a–c). At the feeding front, the wild-type and cannibalistic strains exhibited a similar phenotype. Behind the feeding front, the amebas of both strains aggregated as they began to starve. In the wild-type strain, these aggregates were stable and developed into fruiting bodies consisting of stalks and spores. On the other hand, the cannibalistic strains
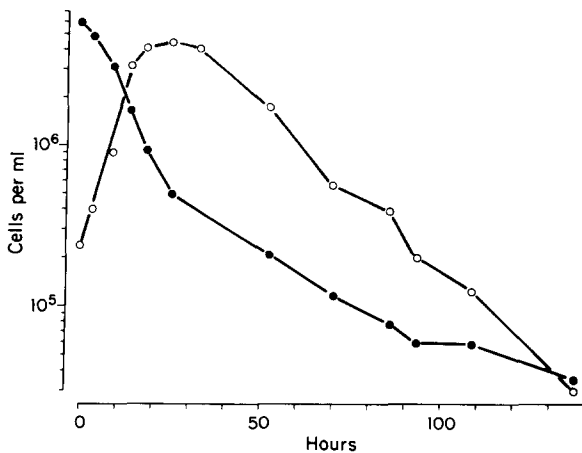


Figure 3. Cell growth kinetics of a cannibalistic strain. The growth kinetics were followed in a culture with bacteria (O) and in a culture in which the cells were washed free of bacteria and resuspended to a cell density of 5 × 10⁶ per ml (●).
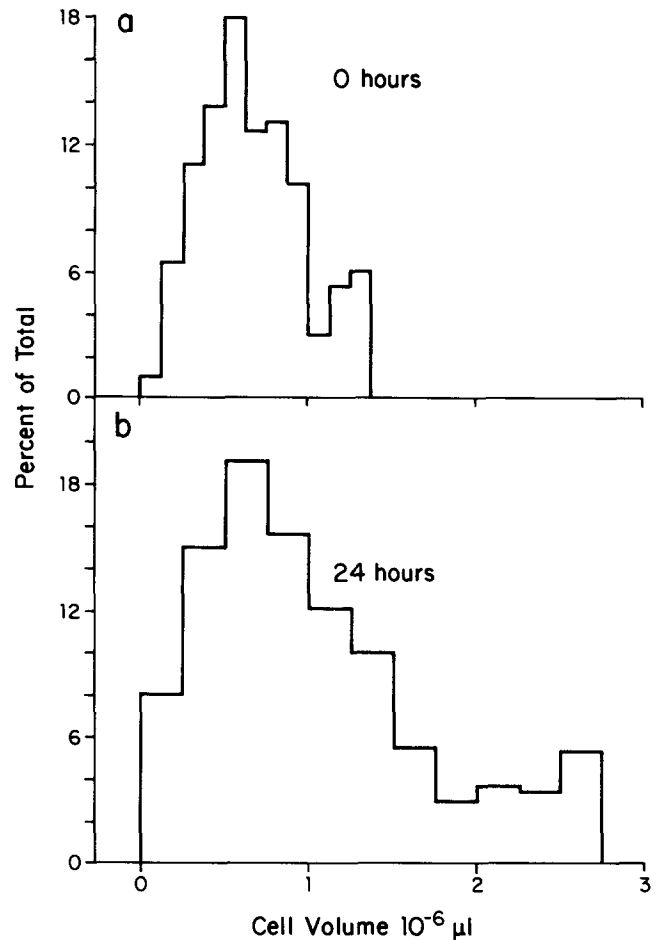


Figure 4. Cell volume distributions of amebas of a cannibalistic strain immediately after washing free of bacteria (a) and 24 h later (b).

formed only loose aggregates which later dispersed. Further behind the feeding front, the cell density had decreased dramatically and only a few large amebas which leave tracks on the agar as they migrated could be seen (Fig. 5c). These large wandering amebas have been observed on the plates 4 wk after bacteria are consumed. After recognizing this phenotype as cannibalistic, we have isolated several cannibalistic strains based simply on their appearance in agar culture. We currently have 14 independent isolates.

We have tried to obtain morphogenesis of cannibalistic strains by imposing conditions which are known to favor fruiting body formation. Cannibalistic strains do not progress beyond the aggregate stage when cultured with charcoal, in small populations, or when mixed with other cellular slime molds. Fruiting in wild-type strains was particularly vigorous when mixed in low ratios (1:10,000) with *Dictyostelium discoideum* and inoculated on non-nutrient agar. Under these conditions, *D. discoideum* slugs were formed which initially contained only a few *D. caveatum* amebas. The slugs migrated normally before stopping and being consumed by the *D. caveatum* amebas. Numerous tips arose from the carcasses of each slug and formed bouquets of *D. caveatum* fruiting bodies by 3 d. When cannibalistic strains were used in these mixtures no fruiting bodies were formed by 3 d (Fig. 6b) and the remaining cell masses decreased in size so that by 4 d only slime material and a few cells could be seen (Fig. 6c).

*Figure 5.* Wild-type and cannibalistic strains feeding on bacteria. (*a*) Wild-type (BW1). (*b*) Cannibalistic strain (BW36). *FE*, feeding edge; *AG*, aggregates; *FB*, fruiting bodies; *CA*, cannibalistic amebas. Bar, 1 mm. (*c*) Tracks left by cannibalistic amebas. Bar, 0.5 mm.

When cannibalistic strains were mixed with wild-type strains, the wild-type amebas were rapidly devoured. In 50:50 mixtures of wild-type and cannibalistic strains, the wild-type amebas were consumed after ~8 h (Fig. 7). Although wild-type amebas appear to be preferentially lost, the cannibalistic amebas are probably not distinguishing between the two cell types. As they starve, the wild-type cells become smaller (9), and being fed upon by the cannibalistic amebas must accelerate this process. Their smaller size probably makes them more susceptible to phagocytosis.

In the sexual phase of the life cycle of other species of cellular slime molds, the fusion of haploid cells of opposite mating types leads to the formation of presumably diploid giant cells (3, 7). The giant cells attract and feed upon a limited number of neighboring amebas during a morphogenetic process which leads to the formation of a resistant structure called a macrocyst. Since cannibalistic amebas resemble in many ways giant cells, we postulated that the event which causes a strain to become cannibalistic might involve diploid formation. Therefore, we determined the karyotype of both a cannibalistic and wild-type strains by using the Giemsa staining procedure (1, 2). Both the wild-type and cannibalistic strains exhibited a haploid set of six chromo-

somes (Fig. 8). Therefore, we believe that the cannibalistic behavior is due to a specific site mutation. It is also reassuring that the karyotypes of wild-type and cannibalistic strains were identical since this essentially ruled out the possibility that the cannibalistic strains are contaminants of another amebal species.

When cultured by serial transfer on bacterial carpets, the cannibalistic strains were very stable. When the amebas of a previously cloned cannibalistic strain were recloned after growth on bacteria, out of 13,858 clones only a single clone was obtained which could form fruiting bodies. This suggests that a mutation or a stable genetic switch is responsible for cannibalistic behavior. Recently, using parasexual genetic methods (5), we have selected diploids between independently derived cannibalistic strains and between these strains and wild-type strains. These diploids all form normal fruiting bodies, thus indicating that a recessive mutation is responsible for the cannibalistic phenotype, and that there are at least two complementation groups involved.

## Discussion

Cannibalism in cellular slime molds has been reported to occur during macrocyst formation (3, 7) and other phases of
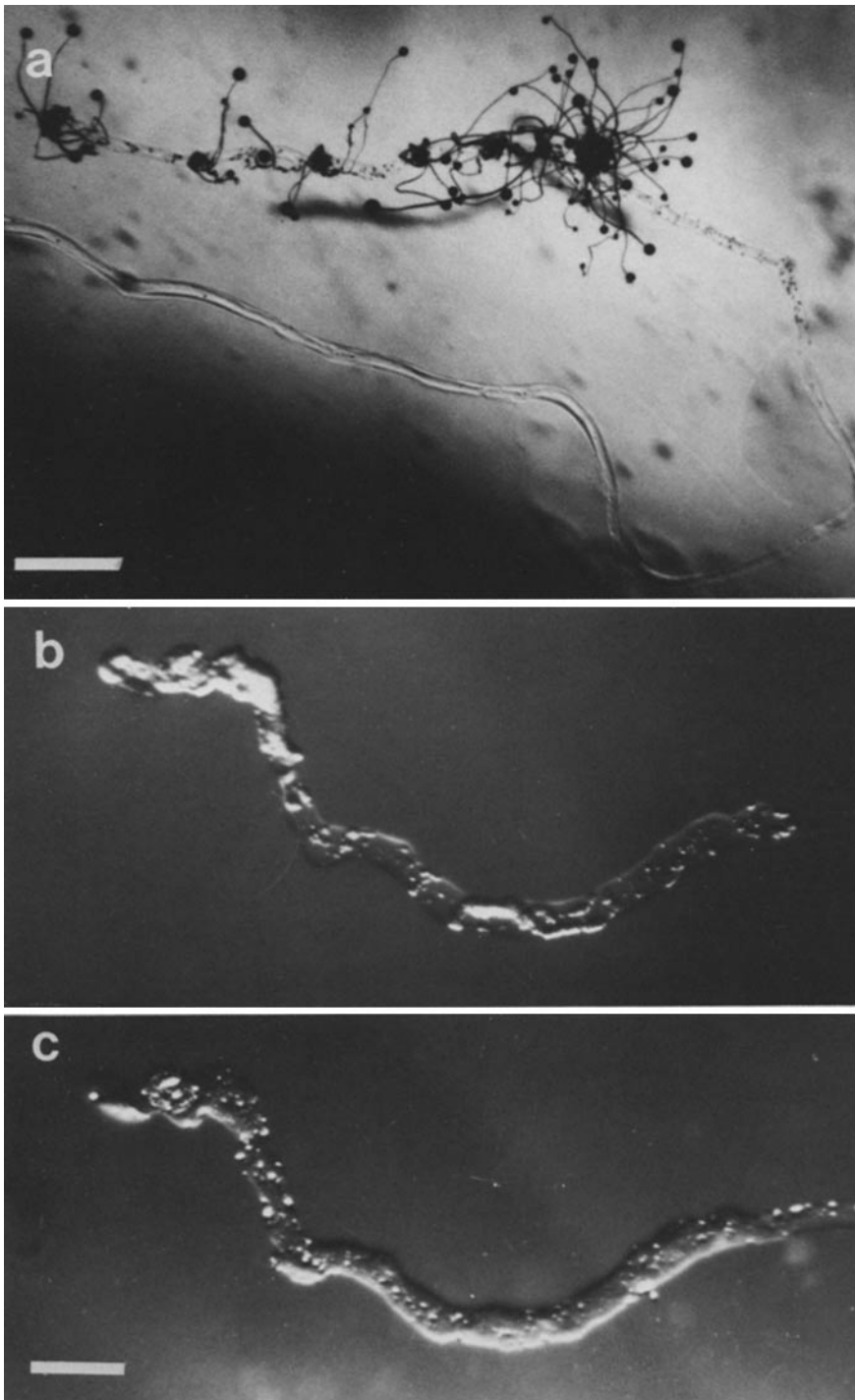
*Figure 6.* Wild-type and cannibalistic strains feeding on *D. discoideum* slugs. (*a*) *D. caveatum* fruiting bodies arising from the remains of a *D. discoideum* slug infected with wild-type amebas (BW1) 3 d after being mixed at low ratios (1:10,000) with *D. discoideum* (Dd61). Bar, 1 mm. (*b*) Remains of a *D. discoideum* slug infected with amebas of a cannibalistic strain (BW36) 3 d after mixing in the same ratio with *D. discoideum*. (*c*) The same slug in *b* 1 d later. Bar, 0.5 mm.

the life cycle. However, the cannibalism reported here differs in several important respects from these. During macrocyst formation, a special population of giant cells which are formed by fusion of amebas of opposite mating types are responsible for the cannibalism. Furthermore, the cannibalistic behavior is restricted to a defined period of a developmental program that ends in the formation of a resistant cyst. Since this occurs

under starvation conditions, the cannibalized cells probably increase the probability that the cyst will be able to survive until conditions improve. In this sense the contribution of the cannibalized cells is analogous to that of dead stalk cells in fruiting bodies. This developmental program is also initiated by starvation and the stalk cells themselves do not contribute to the next generation of amebas but probably increase the
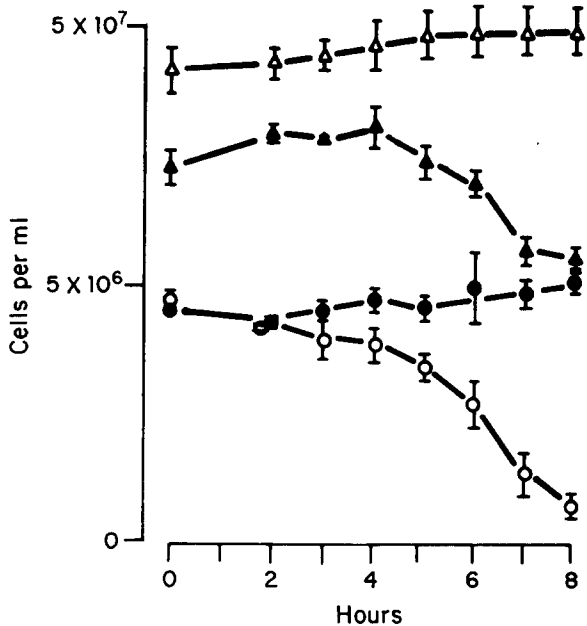
*Figure 7.* Phagocytosis of wild-type amebas. Both wild-type and cannibalistic amebas were grown in suspension in association with bacteria. Each strain was washed free of bacteria and resuspended in phosphate buffer. Alone: wild-type (△), cannibal (▲); together: wild-type (○), cannibal (●).

chances of dispersal or survival of the spores.

Cannibalism has also been observed in cells treated with a conditioned medium (I. Tatischeff and C. De Chastellier, personal communication). However, the cannibalism was a rare event occuring in at most 1% of the cell population and was not a stable property of a strain.

The existence of cannibalistic strains in *D. caveatum* is probably related to their unusual capacity to prey upon other species. Under certain conditions it may be adaptive to maintain a population of actively migrating vegetative cells which could prey upon other cellular slime molds for long periods. In this light, the cannibalism of cells of their own genotype could be viewed as a method to allow longer searching times. However, it is also possible that cannibalism is an inherent problem for strains which specialize in phagocytosing other amebas and may explain why these strains are so uncommon.

Since we believe that the spontaneous mutations that gave rise to cannibalism in our cultures also arise in natural populations, this raises a paradox: Since cannibalism would always seem to be favored in mixtures of wild-type and cannibals, how can a multicellular cycle be maintained in the presence of such behavior? Cannibalism seems to require the sacrifice of the capacity to form both a dispersal structure and resistant spores. It is possible that under certain conditions cannibalistic strains can be induced to undergo multicellular development. However, we have imposed conditions which are known to favor fruiting body formation without success. Perhaps a genetic switch exists that regulates switching between the two phenotypes under appropriate environmental signals. A deeper understanding of the molecular nature of the mutations which lead to cannibalism may help us resolve this paradox.

We are currently using *D. caveatum* as a system to understand the mechanism of self/nonself recognition. Because this
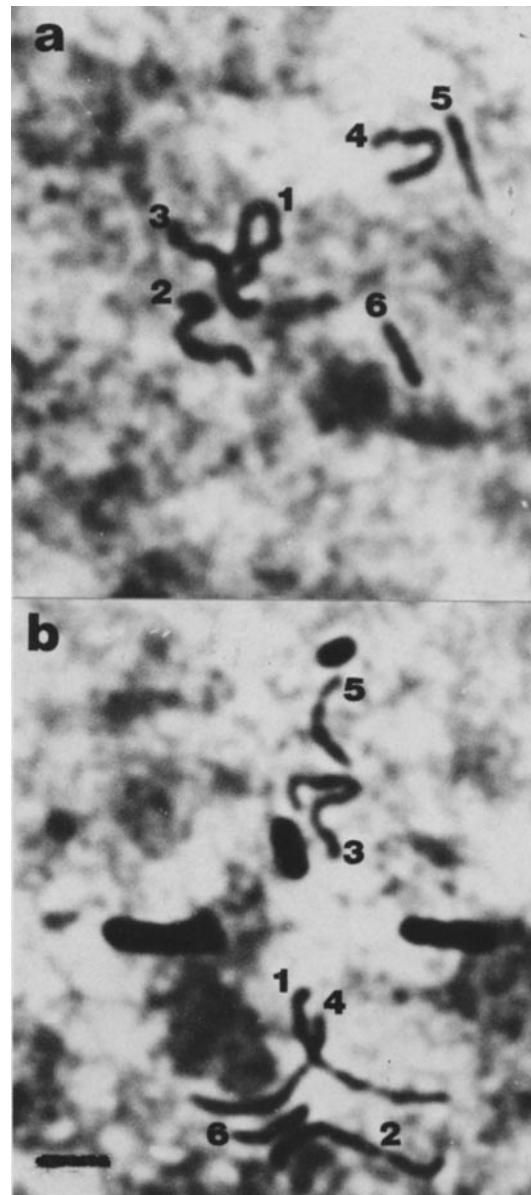


*Figure 8.* Representative chromosomal sets of wild-type and a cannibal. (a) Wild-type (BW1). (b) Cannibal (BW36). Bar, 2 μm.

recognition is disrupted in the cannibalistic strains, they should be useful in aiding us in this endeavor. Our current hypothesis is that a cell surface molecule present on both cannibalistic and wild-type cells marks a cell as a *D. caveatum* cell. When pseudopods of a wild-type *D. caveatum* cell encounter a surface containing these molecules they are inhibited in some way so that engulfment or nibbling does not occur. This hypothesis suggests that cannibalistic cells could lack a receptor for this molecule or that the receptor is not able to transduce its signal and inhibit phagocytosis.

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