Acanthamoeba Discriminates Internally between Digestible and Indigestible Particles

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ABSTRACT The capacity of *Acanthamoeba* to distinguish nutritive yeast particles from nonnutritive plastic beads during phagocytosis was investigated. When cells were allowed to phagocytose yeast to capacity, endocytosis stopped and subsequent presentation of particles (either yeast or beads) did not result in further uptake. By contrast, when cells were allowed to phagocytose plastic beads to capacity and a second dose of particles was presented (either yeast or beads), the cells exocytosed the internal particles and took up new ones. Yeast rendered indigestible by extensive chemical cross-linking were taken up at rates similar to those of untreated yeast, but, like beads, they were exocytosed when a second dose of particles was presented. The results show that an internal distinction is made between vacuoles containing yeast and vacuoles containing plastic beads, and they are consistent with the hypothesis that the presence within the vacuoles of material capable of being digested prevents exocytosis.

Phagocytic uptake of particles depends on a specific interaction at the cell surface which results in a localized stimulus for the cell to engulf the particle. In many cells this interaction can occur with a number of indigestible substances. For example, amebas will ingest silica, carbon particles, plastic beads, and other materials of no nutritive value (1, 2). Plastic beads, in fact, are valuable tools for studying the kinetics and mechanisms of endocytosis in a wide variety of cell types because they are indiscriminately engulfed by most cells. To understand better how cells control particle turnover rates and the related membrane recycling, we have studied how *Acanthamoeba* handles a nutritive particle, yeast, as compared with a nonnutritive particle, plastic bead.

Acanthamoeba contains a vacuolar compartment that serves as its digestive system. This compartment is maintained by the cell at virtually constant volume and surface area under standard culture conditions (3). Nevertheless, considerable membrane flow occurs through the compartment because of endocytosis (3, 4). Membrane flow, as inferred from the rate of membrane ingestion by endocytosis, is rapid during pinocytosis (~9 surface equivalents/h) and close to zero in cells that have taken up particles to the limit of their capacity (that is, are saturated). When the cell is saturated with a digestible particle such as yeast, space for particle uptake gradually becomes available as digestion progresses and undigested residues are incorporated into defecation vacuoles to be expelled from the cell. In contrast, indigestible particles like plastic beads are ingested readily by the amebas, but cannot be reduced in volume. In the experiments reported here, in addition to testing turnover rates of different types of particles, we examined whether *Acanthamoeba* discriminates between inert and nutritive particles and whether they do so externally or internally.

MATERIALS AND METHODS

Culture Methods: *Acanthamoeba castellanii* (Neff strain) was cultured axenically as follows: 25-ml stock cultures of amebas were maintained in aerated tubes in a slightly modified optimal growth medium as described by Neff et al. (5). Aliquots of stock solutions were transferred aseptically to 1 liter of growth medium in low-form culture flasks. The cultures were incubated at 30°C on a rotating platform shaker (A. H. Thomas Co., Philadelphia, PA) for 2–4 d until appropriate cell densities were attained. Cell doubling times were on the order of 12 h. Cells were used for experiments when the cultures attained cell densities of 0.5–1.1 × 10^6 cells/ml. Routine cell counts were performed on a cell counter (Particle Data, Inc., Elmhurst, IL).

Particles: *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, MO) were lipid-extracted in cold chloroform/methanol (2:1) for several hours, collected on a Buchner funnel, rinsed with diethyl ether, air-dried, and stored as stock for the experiments. Approximately 60 mg of dry yeast was suspended per ml of 0.1 M sodium phosphate buffer, pH 6.8, and rinsed twice by low-speed centrifugation to remove fragments and lysed yeast. Extensively cross-linked yeast were prepared by suspending the lipid-extracted cells in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8, for 6–10 d, followed by thorough washing in buffer and resuspension for 2–3 h in 0.1 M glycine in phosphate buffer to block any free aldehyde groups. The yeasts were rinsed again in phosphate buffer before use in the experiments.

For fluorescently labeled yeast, yeast (100 mg/ml) were suspended in Lucifer

yellow VS (6) (1 mg/ml) in 0.5 M carbonate buffer, pH 9.0, for 2 h at room temperature. Fluorescent yeast were rinsed twice in carbonate buffer and twice in 0.1 M PO_4 buffer. The dye was covalently bound to the yeast and did not leach during the washes. Lucifer yellow VS was generously provided by W. W. Stewart (National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD).

Either 2.02-μm-diam polyvinyltoluene (Dow Chemical Co., Indianapolis, IN) or 1.8-μm-diam polystyrene, fluorescent, carboxylated (Polysciences, Inc., Warrington, PA) beads were used.

Experimental Protocol: All experiments followed the same general protocol. Amebas at a concentration of 1×10^6 cells/ml in 20 ml final volume were incubated at 30°C in Dubnoff reciprocating shaker in 50-ml Erlenmeyer flasks. Saturating loads of particles were added (3 mg yeast or 0.8 mg latex beads/10^6 amebas). Aliquots for monitoring uptake were taken at 15-min intervals and fixed in 1.5% glutaraldehyde for particle counts.

After 45 min, the amebas were washed in culture medium three times by low speed centrifugation to free them of unincorporated particles, allowed to reequilibrate for 15 min, and given a second load of particles. For controls, the particle content was also followed in: (*a*) amebas that were not washed, but were kept in incubation with the original particle load; (*b*) amebas that were washed but not given a second load of particles; and (*c*) amebas not given an initial load but carried through washes and given particles equivalent to a "second load." For quantification of uptake, a small drop of the fixed cells was flattened by allowing the slide to dry for 1–2 h at 60°C. The number of yeast or beads per cell was determined for each time point by counting 100 cells in the light microscope.

The average number of beads per cell (during initial loading) could also be determined by extracting the plastic in dioxane and measuring the uptake spectrophotometrically (7). When this value (from 2×10^6 cells) was compared with counts from relatively few cells in the light microscope, they were very similar. In one experiment with six time points, values calculated from extraction of cells were 67, 42, 26, 10, 6, and 7 beads/cell. Corresponding values from light microscopic counts of 75 cells were 66, 60, 32, 13, 7, and 4 beads/cell.

RESULTS

Ingestion of saturating loads of particles by *Acanthamoeba* is initially rapid but gradually slows and reaches a plateau at 45–60 min. To test turnover of particles at the plateau, cells were loaded with yeast, washed free of uningested yeast, and given latex beads. As shown in Fig. 1, very few beads were ingested by cells already loaded with yeast, whereas control cells taken through the same experimental manipulations, but not loaded with yeast, took up latex beads readily. The yeast-loaded cells retained all the yeast throughout the 3-h duration of the experiment. In the converse experiment (Fig. 2), amebas were given a saturating load of latex beads, washed, and reincubated with yeast. In this case, the amebas in the presence of yeast promptly cast out the latex beads and ingested yeast to the same extent as control cells. Bead-laden amebas that were not given a second stimulus to phagocytosis showed no loss of beads (Fig. 2). Taken together, these two experiments show that the amebas are able to distinguish yeast from latex beads, since they will exocytose beads and endocytose yeast, but not vice versa. Moreover, the data show that exocytosis of beads is induced by the presence of yeast.

Light micrographs were taken at two times in the experiment shown in Fig. 2. Images of amebas just after the addition of yeast to bead-loaded cells (Fig. 3 *a*) and 60 min later (Fig. 3 *b*) illustrate the virtually complete turnover in vacuolar content. The images also show that the cells can bind beads or yeast while loaded with either particle. Thus, failure to take up beads by yeast-loaded cells cannot be attributed to a failure of particle attachment.

To determine whether the recognition of particles occurred internally, or externally, in association with binding to the cell surface, we examined how presentation of particles of a particular type would affect retention of particles of that type. Cells were loaded with yeast, then washed and given a load of

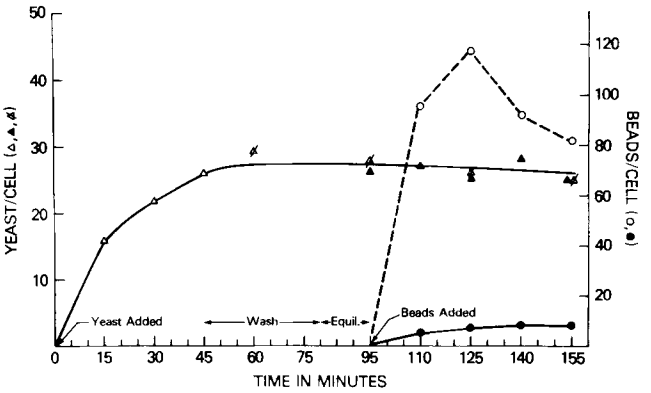

FIGURE 1 Bead uptake in cells saturated with yeast. Yeast were added to amebas and uptake was followed for 45 min (Δ), after which the cells were divided into two aliquots. One group was washed to remove free yeast. Both yeast content (▲) and bead uptake (●) were followed in those cells. Yeast-loaded cells (Ø) (not washed) were followed for the duration of the experiment. Cells not preloaded with yeast were incubated, washed, and equilibrated exactly as the yeast-loaded cells. They were given beads and the uptake was followed (O) as a control.

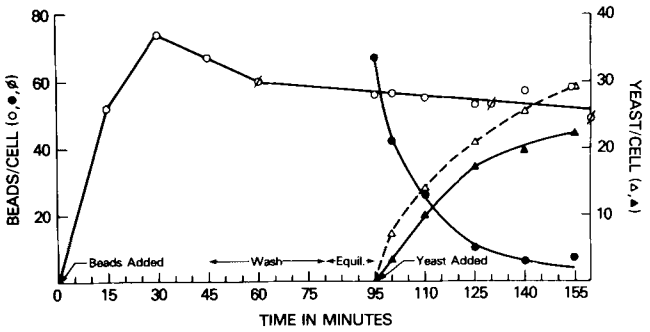

FIGURE 2 Yeast uptake in cells saturated with beads. Beads were added to amebas and uptake was followed for 45 min (O). The cells were divided into three aliquots. One group was washed to remove free beads. Both bead content (●) and yeast uptake (▲) were followed in these cells. The second group continued in incubation without being washed, and bead content was followed (Ø). The third group was washed, not given yeast, and bead content was followed (O). Cells not preloaded with beads (Δ) were incubated, washed, and equilibrated exactly as the bead-loaded cells. They were given yeast and the uptake was followed.

yeast covalently labeled with a fluorescent compound (Fig. 4). Control cells took up the fluorescently labeled yeast as rapidly as the nonfluorescent yeast, showing that the modified yeast were not treated differently by the cells, but the uptake of fluorescent yeast by amebas already loaded with yeast was less than one-third of the uptake of fluorescent yeast by control amebas.

Aliquots for photography were taken from the experiment shown in Fig. 4 just after giving yeast-loaded amebas (Fig. 5 *a*) or control amebas (Fig. 5 *c*) a load of fluorescent yeast and 60 min later (Fig. 5, *b* and *d*). In most experiments a few cells failed to take in any particles, and there was also variation in the degree to which individual cells appeared to be loaded. No uptake, as well as partial loading, may reflect a nonphagocytic period during a part of the cell cycle. If so, that period is relatively short since cells that failed to take in any particles amounted to only 1–2% of the population. The variability of fluorescent yeast uptake illustrated in Fig. 5 *b* presumably

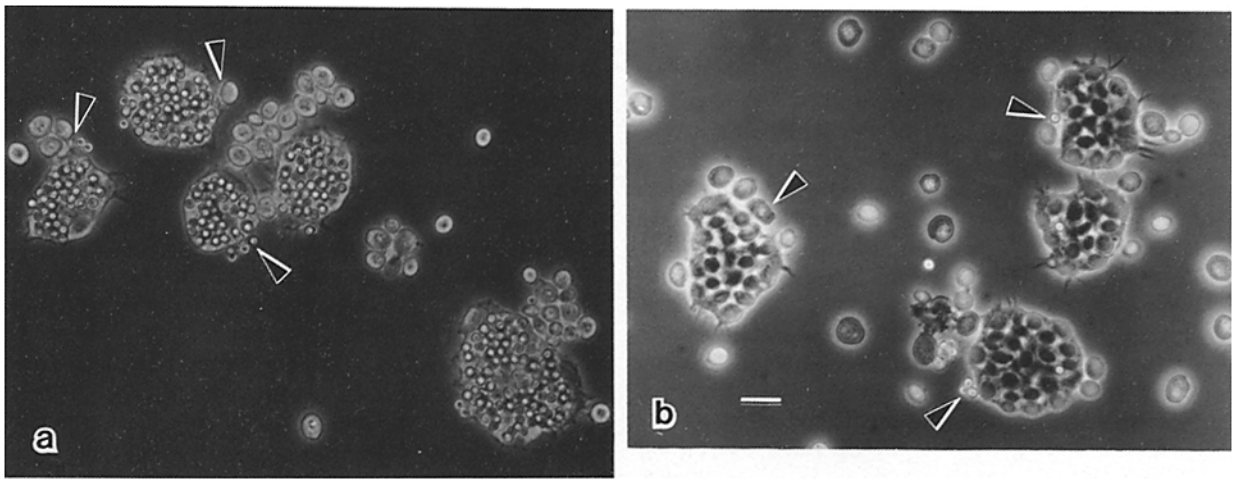

FIGURE 3 Light micrograph showing exchange of beads for yeast. Cells are from the experiment shown in Fig. 2. (*a*) Cells from 95-min time point (●); (*b*) cells from 155-min time point (●). Arrows indicate bound beads and yeast. Bar, 10 μM. × 510.

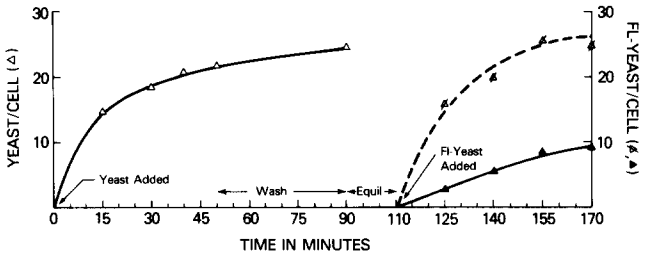

FIGURE 4 Yeast uptake by yeast-loaded cells. Cells were loaded with nonfluorescent yeast (△), washed, and equilibrated. Fluorescent yeast, added at 110 min, were poorly taken up by preloaded cells (▲). Control cells (taken through incubation and washes) took up fluorescent yeast (▲) at a rate comparable to the uptake of nonfluorescent yeast by cells not preincubated with yeast.

reflects incomplete loading in the initial yeast presentation. The results of the experiment shown in Figs. 4 and 5 indicate that a second stimulus to phagocytosis by yeast does not cause yeast to be exocytosed to the same degree as beads.

In experiments to show whether beads will displace beads readily, as they should if the external stimulus is nonspecific and the relevant property of the particles is recognized internally, we loaded cells with either polyvinyltoluene beads or fluorescent polystyrene beads and gave a second load of the other kind (Fig. 6). Only the second half of the experiment is plotted in this case. Neither the polyvinyltoluene nor the polystyrene beads were lost in the absence of a second phagocytic stimulus, but both showed a loss of 60% of the original load of beads an hour later, when the amebas were given a second load. It is possible that the apparent loss of beads levels out at 60% because the egested beads can be reingested, thus "diluting" the second load of particles. This is less likely to happen with yeast since they are normally digested before expulsion. These data support the hypothesis that retention of yeast is due to internal signals since neither beads nor yeast stimulate exocytosis of yeast, whereas both beads and yeast stimulate exocytosis of beads.

There are at least two obvious differences between the two types of particles used in these experiments which could operate in a signaling mechanism. One is the hydrophobic surface of the latex bead, which might be expected to interact with the ameba membrane differently than the largely carbohydrate surface of the yeast cell wall. Another difference is that yeast are digestible by the amebas and serve a nutritive function, whereas latex beads are clearly inert in this regard. We tested whether digestibility of yeast, rather than some surface property, was the relevant factor by rendering the yeast less digestible by extensive cross-linking of yeast proteins by glutaraldehyde.

Extensively cross-linked cells were readily ingested by the amebas (Fig. 7). The initial uptake of the fixed yeast was only slightly less than uptake of unfixed yeast. When both groups of amebas were given a second load of fluorescent unfixed yeast, however, the rate of loss of fixed yeast was significantly higher than the rate of loss of unfixed yeast. As seen in the previous experiments, the fixed yeast were not exocytosed in the absence of a second load of yeast.

Electron micrographs of cells 200 min (Fig. 8 *a*) after a load of heavily cross-linked yeast show that the yeast are morphologically similar to unfixed yeast, but do not show signs of digestion during the interval monitored. For comparison, Fig. 8 *b* shows partial digestion of unfixed yeast had already taken place in a sample fixed after 165 min. The results with fixed yeast suggest that the property of digestibility of the yeast may play a role in their retention within the cell upon a second phagocytic stimulus.

DISCUSSION

Acanthamoeba, like other cells (1, 8, 9), shows phagocytic discrimination between materials presented externally. For example, we have observed that it ingests *Saccharomyces* and *Aerobacter* readily, but *Schizophyllum* and *E. coli* very poorly. Studies of *Acanthamoeba* by Rabinovitch and DeStefano (10) show that surface properties of a particle are important in determining ingestion rates, but the specifics of the surface interactions with the ameba are not known. Certainly failure of particles to bind to the ameba will result in little or no uptake, but it is not clear from the available data whether some types of particles that are poorly taken up bind but fail to be ingested, as has been observed in macrophages (9).

In the present study we have investigated the capacity of *Acanthamoeba* to make an internal distinction between two types of particles which are readily bound and avidly ingested. Plastic beads appear to be taken up somewhat better than

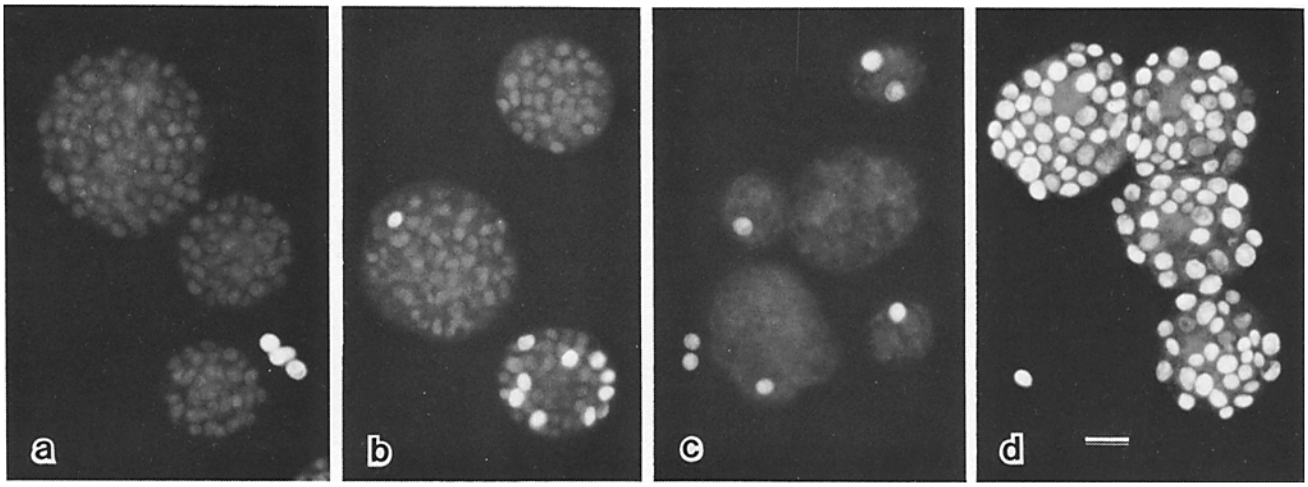

FIGURE 5 Light micrographs from experiment shown in Fig. 4. Fixed cells were flattened for photography. (*a*) Cells (loaded with nonfluorescent yeast) immediately after the addition of fluorescent yeast (110-min time point, Fig. 4). (*b*) Same cells as in *a* at 170 min. Cells have retained nonfluorescent yeast and have taken up fluorescent yeast only where they were not saturated by previous load. (*c*) Control cells at 110 min time point. Only one to two yeast have been ingested per cell. (*d*) Same cells as in *b* at 170 min. Cells are loaded with fluorescent yeast, showing that no deficiency in uptake can be attributed to fluorescent labeling of yeast. Bar, 10 μm. × 540.

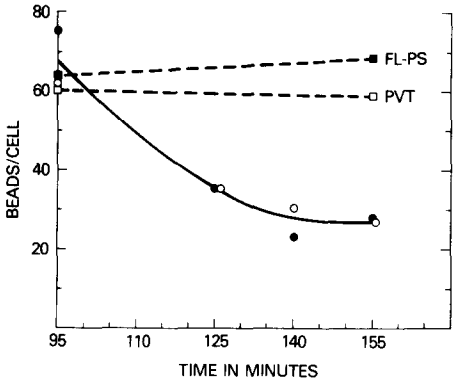

FIGURE 6 Bead turnover induced by beads. Two groups of cells were preloaded with 1.8-μm fluorescent polystyrene beads (*FL-PS*) or 2.02-μm polyvinyltoluene beads (*PVT*). The amebas were washed free of beads, divided into two aliquots, and equilibrated (similar to experiment shown in Fig. 2). Only bead loss was followed in these experiments. Loss of beads without a second load is shown by dotted lines (□, *PVT*; ■, *FL-PS*). Loss of beads in presence of second load of other type is shown by solid line (○, *PVT*; ●, *FL-PS*).

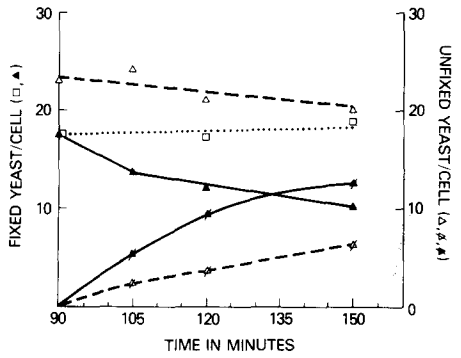

FIGURE 7 Enhanced turnover of fixed yeast. Only the second part of the experiment is plotted. Protocol was identical to Fig. 1. Data from three groups of cells are plotted. Control cells (□) were loaded with fixed yeast, washed, and reincubated with no further yeast load. Cells loaded with fixed yeast (▲) were given unfixed fluorescent yeast and uptake was followed (▲). In 1 h, approximately half the yeast had turned over. Cells loaded with unfixed yeast (△) were also given unfixed fluorescent yeast (▲). Both the loss and uptake of additional yeast were smaller in these cells.

yeast, since the initial slope of the uptake curve is greater and the saturation of the amebas is achieved in ~30 min vs. 45 min for yeast (Figs. 1 and 2). However, plastic beads and fixed yeast, but not unfixed yeast, are rapidly exocytosed when an engorged ameba is presented with a second load of either type of particle.

It is possible that differences in the surface interactions of the particles with the ameba membranes may in some way affect the subsequent handling of the ingested particle by the ameba. Plastic beads are thought to attach through nonspecific, hydrophobic interactions (11). The binding of yeast, on the other hand, appears to be mediated through specific interactions with the carbohydrate polymers of the yeast wall (11–13). Glutaraldehyde fixation of the yeast would not be expected to greatly modify the outer surface of the yeast since aldehydes react poorly, if at all, with carbohydrates (14). Despite the considerable differences in surface properties of the particles, both yeast and bead phagosomes rapidly acquire lysosomal enzymes (1, 15–18), and beads taken in individually can be consolidated into a single vacuole (15, 19) so that physiological fusions occur with both types of phagosomes. Once inside the cell the particles often lose the close association with the membrane that is characteristic of the ingestion phase. Fusions of lysosomes, pinosomes (20), and fusions between individual phagosomes lead to both addition and deletion of membrane from the vacuoles. Such events would likely dilute and disrupt any specific associations of the particles with membrane components. We cannot rule out particle/membrane interactions as playing some role in the mechanism of internal discrimination, but the available evidence does not suggest a decisive role.

A significant factor in the mechanism of discrimination appears to be the quality of digestibility of the particle. That yeast rendered undigestible after prolonged fixation in a glutaraldehyde are expelled from the cell at a significantly faster rate than unfixed yeast provides evidence that digestibility is

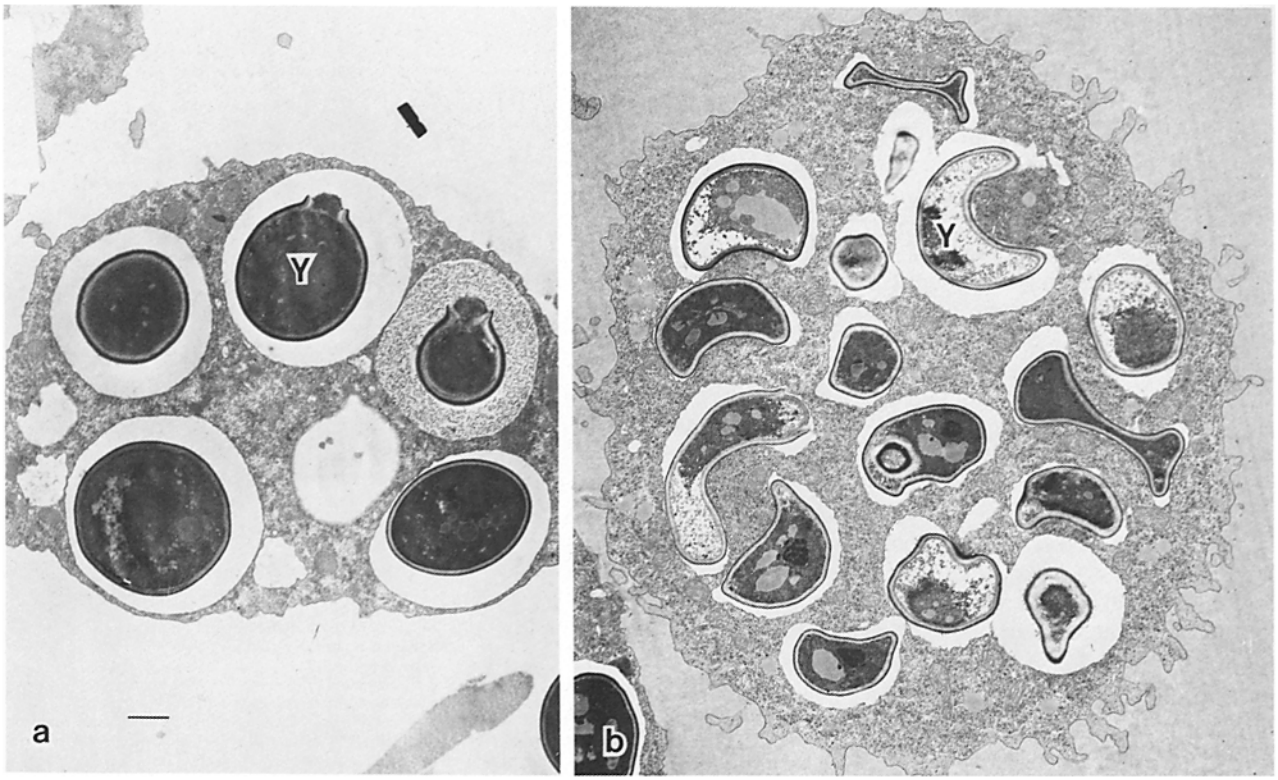

FIGURE 8 Electron micrographs of cells from Fig. 7. (*a*) Thin section of *Acanthamoeba* showing condition of fixed yeast 3.3 h after ingestion. Yeast show no signs of digestion. (*b*) Thin section of *Acanthamoeba* showing condition of unfixed yeast 2.75 h after ingestion. Loss of content and collapsed walls are indications of partial digestion of yeast. Y, yeast. Bar, 1 μm. × 5,100.

an important component. Cells that depend on endocytosis for nutrition normally have efficient mechanisms for defecation of residues left after digestion. This capacity presumably involves intracellular selection of vacuoles either for retention or for exocytosis, since all stages in the ingestion cycle are present simultaneously. It seems likely that the results we observe with the rapid defecation of bead-containing vacuoles and retention of digestible yeast reflect these same physiological mechanisms.

Kitching (1), noting an observation of Metalnikov in ciliates (reported in 1916) that food vacuoles containing digestible material circulate much longer than those containing inert material, speculated that it is the outward passage of dissolved food substances from the vacuole that in some way inhibits the defecation process. An interesting, similar observation has been reported recently by Hohman et al. (21). They found that translocation of photosynthate from symbiotic algae resident in the gut cells of hydra is a factor in the protection of symbionts from intracellular digestion; that is, lysosomes were prevented from fusing with phagosomes containing photosynthesizing algae. Although we do not yet have direct evidence, it is our working hypothesis that the flow of digestive end-products into the cytoplasm prevents premature exocytosis of vacuoles containing digestible particles.

Our observations that additional beads or yeast in the medium accelerate expulsion of indigestible particles by *Acanthamoeba* has a counterpart in observations of Chapman-Andersen on the giant ameba *Chaos* (22). She found that amebas fed Alcian blue accumulated dye-containing granules which were lost from the cell on refeeding, whereas they persisted in starved cells. Similar experiments using ferritin (23) showed that the iron was also defecated more rapidly with a subsequent feeding. Although the time scale in these experiments was days, rather than hours, and the influence of starvation per se is an issue, the phenomenon appears to be similar to that which we observe in *Acanthamoeba.*

Stiemerling and Stockem (8) have made the interesting observation in *Amoeba proteus* and *Physarum* that mixed aggregates of digestible and indigestible materials are "sorted" by the ameba as the initial phagosome is fragmented into smaller vacuoles. The end result is a separation of digestible and indigestible material into different vacuoles, thus allowing rapid excretion of indigestible material without loss of foodstuff.

The requirement for a second phagocytic load to obtain rapid exocytosis of indigestible particles implies that exocytosis is in some way stimulated by endocytosis. It is unlikely that defecation is the exact reverse of endocytosis. Particle binding to the vacuole membrane does not appear to be a factor in exocytosis, as it is in endocytosis, since detritus is not usually closely associated with the vacuole membrane. Moreover, exocytosis would seem to require that the vacuole move close enough to the cell surface to permit membrane fusions before the assembly of filaments at the membrane that may be required for expulsion. Such assemblies have been observed consistently during endocytosis of particles in all cell types, and presumably are stimulated by particle binding. Stockem, in reviewing the sparse available evidence (8), concludes that exocytosis probably does not result in the addition of vacuole membrane to the surface. In the model he proposes, the vacuole approaches the plasma membrane and fuses, releasing the content of the vacuole, but the membrane is retrieved intracellularly.

The "stimulus" to exocytosis may be a specific effect on

the mechanism of movement of certain types of vacuoles to the surface or their fusion. Alternatively it may be a nonspecific effect generated by a limited vacuolar space. In *Acanthamoeba* the vacuolar system has a defined capacity. This is shown by reproducible saturation curves in particle uptake studies (24) and by morphometric measurements that show similar total vacuolar volumes whether the vacuoles are filled with soluble medium, particles, or a combination of both (3). It should be noted here that the phenomena we report in this study are readily demonstrated only because of this cellular property. In a saturated ameba there is no available space for additional uptake without some defecation. If vacuoles containing nondigestible particles are not inhibited from exocytosis, then the overload of one or two newly ingested particles could promote contact of some vacuoles with the surface and expulsion of their contents. On the other hand, if vacuoles containing digestible material are prevented from exocytosis, then no more endosomes can be formed until the necessary space becomes available. This explanation implies that there need be no specific signal generated by the digestible particle that prevents endocytosis; rather, only a condition that prevents exocytosis need occur. The failure to endocytose may be a volume-related phenomenon due to a filled vacuolar system. In any event, the consequences of the behavior we observe is that the turnover rate and the attendant membrane flow depend on the type of particle.

We thank F. Fantoli for excellent photographic assistance and E. D. Korn, T. C. Hohman, and B. J. Clarke for a critical reading of the manuscript.

Received for publication 15 November 1982, and in revised form 28 March 1983.

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