EXOCYTOSIS OF LATEX BEADS DURING
THE ENCYSTMENT OF ACANTHAMOEBA

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ABSTRACT

Cells of Acanthamoeba castellanii (Neff) are known to form mature cysts characterized by a
cellulose-containing cell wall when transferred to a nonnutrient medium. Amebas which
engulfed latex beads before encystment formed mature cysts essentially devoid of bead
material. The encystment of bead-containing cells appeared to be similar to that of control
cells since no important differences between the two were observed with respect to cellular
levels of glycogen or protein, cellulose synthetase activity, the amount of cyst wall polysaccharide formed, or the percentage of cysts formed. Actinomycin D and cycloheximide
inhibited encystment as well as bead expulsion. Ultrastructural analysis revealed that the
beads, which initially were contained in phagocytic vesicles, were released from the cell
by fusion of vesicular membranes with the plasma membrane. Exocytosis was observed in
cells after 3 hr of encystment, with most of the beads being lost before cyst wall formation.
Each bead-containing vesicle involved in expulsion was conspicuously demarcated by an
area of concentrated cytoplasm, which was more homogeneously granular than the sur-
rounding cytoplasm. Beads were not observed in the cytoplasm of mature cysts but were
occasionally found in the cyst wall.

MATERIALS AND METHODS

Materials

Monodispersed preparations of polyvinyltoluene latex beads (2.02 µ) were purchased from the Dow
Chemical Co. (Midland, Mich.), and the beads were
washed several times with distilled water and then dialyzed extensively against distilled water. Actino-
mycin D was obtained from the Nutritional Bio-
chemicals Corporation (Cleveland, Ohio) and cycloheximide from Calbiochem (Los Angeles,
Calif.). Spectral quality dioxane was used (Matheson Coleman & Bell, Norwood, Ohio).

Encystment

The maintenance and encystment of A. castellanii
(Neff) were carried out as previously described
(Weisman and Moore, 1969). 4-day old cells were
loaded with beads before encystment. Polyvinyl-
toluene latex beads at a final concentration of 0.3
mg/ml were added to amebas (10^6 cells/ml) in
growth medium (GM)\(^1\) for 1 hr at 30°C on a water
bath shaker. 10-ml vol were contained in 50-ml
erlenmeyer flasks (for electron microscopy) and 250-ml
vol in 1 liter flasks (for other purposes).

After this incubation, cells were washed twice
with encystment medium (EM) to remove free beads, and the cells were allowed to encyst as usual in 10-ml or
250-ml vol of EM. Portions of the original cultures
not incubated with beads were allowed to encyst in
parallel with the experimental cells as control sam-

Determination of Bead Content
during Encystment

The amount of polyvinyltoluene latex in the cells
at any time was determined by microscope exami-
nation or by dioxane extraction (Weisman and
Moore, 1969). In order to separate free beads in
EM from engulfed beads, 1 or 2 ml of cells were
layered over amebas (10^6 cells/ml) in growth medium
for 1 hr at 30°C on a water bath shaker. 10-ml vol were contained in 50-ml erlenmeyer flasks (for electron microscopy) and 250-ml vol in 1 liter flasks (for other purposes).

After this incubation, cells were washed twice
with encystment medium (EM) to remove free beads, and the cells were allowed to encyst as usual in 10-ml or
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not incubated with beads were allowed to encyst in
parallel with the experimental cells as control sam-

Analytical Methods

Changes in the cellular levels of glycogen were
determined as previously described by Weisman et al.
(1970), except that whole cells in EM were extracted
with alkali. Cellulose synthetase was extracted and
 assayed as described by Potter and Weisman (1971).
Protein determinations were performed by using the
procedure of Lowry et al. (1951) with bovine serum
albumin in Tris as the standard. The protein content
of cells was determined on extracts prepared by
passing cells in 0.05 M Tris-acetate (pH 7.4) through
the French press (18,000 lb/inch^2). Extracts were
stored frozen until the time of assay.

Electron Microscopy

Cells (2 ml) for electron microscopy were removed
from EM by centrifuging in B.E.E.M. capsules
(Better Equipment for Electron Microscopy, Bronx,
N.Y.) and fixed in a 1:1 (v/v) mixture of 6% glutaral-
dehyde in 0.05 M cacodylate (pH 6.8) and OsO4
(1% aqueous solution). Cells were agitated with the
glutardehyde-OsO4 fixative and, after 5-6 min,
centrifuged. This fixative was removed, and fresh,
cold fixative was added to the pellet; samples were
kept on ice for 30 min. After one rinse in H$_2$O, the
samples were fixed for 30 min in uranyl acetate (0.5%
aqueous solution) on ice. The samples were dehy-
drated for 10 min in cold absolute alcohol on ice.
After the alcohol was removed, the pellets were
mixed with a small amount of epoxy resin (Spurr,
1969) and placed in a vacuum overnight at 30°C
to allow for evaporation of the remaining alcohol.
This epoxy resin was replaced with fresh plastic
before polymerization for 1 day at 45°C and 1 day
at 65°C. As pointed out previously (Korn and
Weisman, 1967), epoxy resins are solvents for poly-
vinyltoluene, and hence the beads are often partially
dissolved during the embedding procedure.

Thin sections were cut on a Sorvall Porter-Blum
MT-2 microtome (Ivan Sorvall, Inc., Norwalk,
Conn.) with a Dehmer diamond knife, picked up
on 300-mesh unsupported copper grids, and stained
with lead citrate (Reynolds, 1963) and uranyl
acetate (saturated in 50% ethanol). Grids were
examined with an RCA-EMU 3G electron micro-
scope at 50 or 100 kv.

RESULTS

Loss of Beads during Encystment

The kinetics of bead loss during encystment
were similar whether determined by a count of beads
cells per cell (Fig. 1) or by the dioxane extrac-
tion of bead material from cells (Fig. 2). Although
the exact kinetics of bead expulsion during en-

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1 Abbreviations: EM, encystment medium; GM, growth medium.
cystment varied slightly with different cell batches, the loss of beads began soon after transfer to EM for most cells (see also Fine Structure of the Bead Expulsion Process) and in all cases mature cysts contained little if any latex material. Less than 10% of the original bead material remained by the time the cell population contained 60 mature cysts.

Comparison of Encystment in Bead-Containing and Control Cells

The encystment of cells containing beads appeared to be essentially the same as that of control Acanthamoeba cells as judged by a comparison of several changes occurring during encystment (Table I). The parameters selected for this comparison were: (a) glycogen levels, which decrease to about one-third of the level in trophozoites during the first 8 hr of encystment (Weisman et al., 1970); (b) total cellular protein levels, which fall during encystment but not as rapidly as glycogen levels (Bowers and Korn, 1969; Neff and Neff, 1969); (c) cellulose synthetase activity, which is not detectable until the time of cell wall synthesis (Potter and Weisman, 1971); and (d) the amount of cyst wall polysaccharide (Griffiths and Hughes, 1969). In each case, no important difference was observed between bead-containing and control cells after 8 and also 28 hr of encystment. In addition, morphological examination by both phase contrast microscopy and electron microscopy (see below) supported this conclusion.

Bead Expulsion in the Presence of Inhibitors

Actinomycin D (100 µg/ml) and cycloheximide (20 µg/ml) each inhibited the encystment process as judged by microscope examination. At the same time, bead expulsion was prevented (Table II). On a weight basis, 70-90% of the bead material originally present remained within the cells after 30 hr of incubation in EM containing one of the inhibitors.

Fine Structure of the Bead Expulsion Process

Cyst formation and bead expulsion kinetics for the culture from which samples were taken for electron microscopy were similar to those shown in Fig. 1. The electron micrograph of a representative cell at the beginning of encystment indicated that the majority of the beads are contained within individual phagocytic vesicles (Korn and Weisman, 1967) (Fig. 3). Phagocytic vesicles were occasionally found fused with other vesicles (Fig. 3, arrow) or with food vacuoles (Fig. 7). The membranes usually surrounded the beads very closely, but occasionally appeared more distantly

Figure 1. The loss of beads from amebas during encystment as determined by particle count. Cells were loaded with beads and allowed to encyst as described in Materials and Methods. The percentage of cells in the total population containing more than 15 beads per cell (○) and the percentage of mature cysts in the population (●) were determined microscopically.
removed in those vesicles in which the beads or portions of beads had been extracted or fused by the embedding plastic.

The expulsion of beads from the amebas was found to occur most commonly in cells after 3 hr of encystment. Each bead-containing vesicle involved in expulsion was conspicuously demarcated by an area of concentrated cytoplasm, more homogeneously granular than the surrounding cytoplasm (Figs. 4, 5, 6 a, 9) and frequently accompanied by bundles of fibrils (Figs. 5, 6 b). There commonly appeared dark "blebs" which appear to be tangential sections of invaginated membrane (Fig. 6 b; Fig. 7, arrow). These granular, concentrated, cytoplasmic areas are assumed to be directly involved in the expulsion of beads and will be referred to as "expulsion cups". The most representative micrographs of expulsion cups are shown in Figs. 4–6 a, in which cases beads were expelled singly, and in Fig. 7, in which case they were expelled in a group, and sometimes along with cell debris. Less representative cups were observed at the end of cytoplasmic extensions (Fig. 6 b) and inside of very immature cyst walls (Fig. 9).

Fragments of vesicular membranes were frequently seen adhering to expelled beads (Figs. 5, 6 b, 8, 9), but membrane-bounded beads were never found outside of cells (Figs. 4, 7–10). Electron microscope examination of thin sections of free beads expelled from encysting cells (17 hr from initiation of encystment) and isolated by layering over sucrose (see Materials and Methods) further supported the conclusion that beads are not membrane-bounded when expelled. Occasionally a nonmembranous, electron-opaque layer was observed surrounding engulfed as well as expelled beads (Figs. 5, 8). Similar amorphous material was described in earlier work with latex beads and is thought to represent either a contaminant of the bead preparation or cell material picked up by the beads during phagocytosis (see Korn and Weisman, 1967). Bundles of fibrils comparable to those previously reported in the hyaline cytoplasm of *Acanthamoeba* (Bowers and Korn, 1968) are visible in expulsion cups (Figs. 5, 6 b) and are presumably involved in the movement of beads, although it is not known whether these fibrils are contractile. Typical microtubules measuring 200–260 A in diameter were found in the cytoplasm (Figs. 5, 6 b) but were not observed in expulsion cups.

Very young immature cysts in samples taken after 24 hr of encystment were occasionally ob-
At 0, 8, and 28 hr of encystment the bead content, cellular protein content, cellular glycogen content, and cellulose synthetase activity of cells were determined as described in the text. The cyst wall fraction was isolated at 28 hr. Cells were washed once with EM, suspended in 0.05 M Tris-acetate (pH 7.4), and passed through the French press at 18,000 lb/inch². Centrifugation of the extract at 1000 g for 10 min resulted in the pellet used as the cyst wall fraction. Assay was carried out by the anthrone procedure (Dische 1962), with glucose as the standard. The mature cyst population was determined by phase contrast microscopy.

* Percentage of the population containing more than 15 beads per cell.
‡ Values given are mg/10⁸ cells.
§ Values given are ug/10⁶ cells.
¶ Values given are nmoles/min per mg protein and represent the glucose incorporated from UDP-[¹⁴C]glucose into alkali-soluble plus alkali-insoluble material.
¶¶ Values given are µg/10⁶ cells of anthrone-reacting material.
** The percentage of mature cysts in the population.

| Time of encystment | Bead content* | Protein content | Cellular glycogen | Cellular cellulose synthetase activity || Content of cyst wall fraction¶ | Mature cysts** |
|-------------------|---------------|----------------|-----------------|------------------------------------|-----------------------------|---------------|
| hr                | C  B          | C  B           | C  B            | C  B                               | C  B                        | C  B          |
| 0                 | 0  0          | 0.20 0.20      | 80 80           | 0 0                                | —  —                       | 1  2          |
| 8                 | 0  0          | 0.13 0.17      | 20 26           | 0 0                                | —  —                       | 2  3          |
| 28                | 0  0          | 0.08 0.09      | 8 12            | 1.1 1.1                            | 5.5 6.5                    | 48 46         |

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|-------------------|---------------|----------------|-----------------|------------------------------------|-----------------------------|---------------|
| hr                | C  B          | C  B           | C  B            | C  B                               | C  B                        | C  B          |
| 0                 | 0  0          | 0.20 0.20      | 80 80           | 0 0                                | —  —                       | 1  2          |
| 8                 | 0  0          | 0.13 0.17      | 20 26           | 0 0                                | —  —                       | 2  3          |
| 28                | 0  0          | 0.08 0.09      | 8 12            | 1.1 1.1                            | 5.5 6.5                    | 48 46         |

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¶¶ Values given are µg/10⁶ cells of anthrone-reacting material.
** The percentage of mature cysts in the population.

### Table I

**Comparison of Bead-Containing (B) and Control (C) Cells during Encystment**

| Time of encystment | Bead content* | Protein content | Cellular glycogen | Cellular cellulose synthetase activity || Content of cyst wall fraction¶ | Mature cysts** |
|-------------------|---------------|----------------|-----------------|------------------------------------|-----------------------------|---------------|
| hr                | C  B          | C  B           | C  B            | C  B                               | C  B                        | C  B          |
| 0                 | 0  0          | 0.20 0.20      | 80 80           | 0 0                                | —  —                       | 1  2          |
| 8                 | 0  0          | 0.13 0.17      | 20 26           | 0 0                                | —  —                       | 2  3          |
| 28                | 0  0          | 0.08 0.09      | 8 12            | 1.1 1.1                            | 5.5 6.5                    | 48 46         |

### Table II

**The Effect of Actinomycin D and Cycloheximide on Bead Loss during Encystment**

| Addition to encystment medium | Value for cells after 30 hr of encystment |
|------------------------------|------------------------------------------|
|                              | Bead content*  | Mature cysts  |
| µg/10⁶ cells                 | %             |
| None                         | 6  50          |
| Actinomycin D                | 23  3          |
| Cycloheximide                | 30  10         |

Bead-containing and control cells were allowed to encyst in the presence of actinomycin D (100 µg/ml) and cycloheximide (20 µg/ml) as indicated. Bead content and the percentage of mature cysts in the population were determined as described in the text. After 30 hr of encystment, the number of cells per milliliter in each of the three types of cultures was approximately the same.

* Bead content at zero time was 33 µg/10⁶ cells.

Bead-containing and control cells were allowed to encyst in the presence of actinomycin D (100 µg/ml) and cycloheximide (20 µg/ml) as indicated. Bead content and the percentage of mature cysts in the population were determined as described in the text. After 30 hr of encystment, the number of cells per milliliter in each of the three types of cultures was approximately the same.

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* Bead content at zero time was 33 µg/10⁶ cells.
FIGURE 3  An electron micrograph of a representative cell at the beginning of encystment (0 hr) showing that each bead (B) is contained within an individual phagocytic vesicle. One vesicle contains more than one bead (arrow). Contractile vacuoles (CV), food vacuoles (FV), lipid droplets (L), and a nucleus (N) are also shown. Scale marker, 1 μ. X 3300.
differentiation that occurred differed from the described pattern not in nature or sequence of the phenomena observed but merely in the time of the occurrence of these phenomena (Fig. 6 b, 30 hr trophozoite).

DISCUSSION

A few hours after transfer to EM, Acanthamoeba cells become rounded and a thin layer of wall is formed at the cell surface (Bowers and Korn, 1969). Eventually a mature cyst wall is formed. Amebas which have engulfed latex beads into phagocytic vacuoles discharge these beads as encystment proceeds, thus removing particulate material from their cytoplasm while maintaining an intact plasma membrane and carrying out the differentiation process of encystment. The encystment of bead-containing cells appears indistinguishable from that of control cells. No important differences between the two were observed, as judged by several criteria involving chemical and enzymatic changes during encystment (Table 1). The results suggest that the loss of particulate material may not be fundamentally different from the discharging of cellular debris during encystment in the absence of beads. This conclusion is further supported by the similarities in the fine structure of bead-containing and control cells during encystment, by the fact that both beads and cell debris can be observed in the cyst wall, and by the ob-
FIGURE 5 Expulsion cups (EC) in the process of expelling beads (B) from a cell after 7 hr in EM. Note the membrane fragments (F), nonmembranous material (N), and bundles of fibrils (BF). A microtubule (MT) is also shown. × 21,000.
FIGURE 6  (a) An expulsion cup (EC) in a cell, after 12 hr in EM, in process of expelling a bead (B). The area indicated by arrows suggests that the membranes have not rejoined (see Fig. 12 c). Mitochondrion (M). $\times 29,500$. (b) A bead (B) in an expulsion cup (EC) contained in a cytoplasmic extension of cell after 30 hr in EM. The fragments (F) may arise by rejoining of vesicular membrane and plasma membrane (see Fig. 12 d). Invaginations of membrane into the expulsion cup appear as blebs (BL). Lipid droplets (L), microtubules (MT) and bundles of fibrils (BF) are also shown. $\times 29,500$. 
FIGURE 7  Phagocytic vesicles depositing their beads (B) to the outside of a cell after 3 hr in EM. Invaginations of membrane into the expulsion cup (arrow) often appear as biebs (BL) when cut in tangential section. Lipid droplets (L) and a food vacuole (FV) containing cellular debris (D) and a bead are also seen. × 8400.

FIGURE 8  A bead (B) expelled from a cell after 5 hr in EM. Note the membrane fragments (F) and nonmembranous, electron-opaque material (N) adhering to the bead. × 29,500.
Figure 9  A portion of an immature cyst (24 hr in EM) in which the expulsion cup (EC) and expelled bead (B) are separated by a thin layer of cyst wall (W). A Golgi apparatus (G) and membranous fragments (F) are also shown. X 21,000.

The observation that Actinomycin D and cycloheximide, both of which prevent encystment, may also prevent bead expulsion. These findings do not eliminate the possibility that bead expulsion is not part of the normal encystment mechanism for removing cytoplasmic material, but each of the observations would be expected if this was in fact the case.

Fine structural analysis of bead expulsion during *Acanthamoeba* cyst formation indicates that this process of expulsion can properly be called exocytosis. From the second or third hour after transfer of amebas to EM, the bead-containing vesicles are observed to fuse with the plasma membrane so that expulsion of the beads occurs. In this sense, the morphological events in bead expulsion are like those in exocytosis in other biological systems, e.g., the secretion of zymogen from pancreatic exocrine cells into the lumen of the pancreatic duct (Caro and Palade, 1964; Jamieson and Palade, 1967). From the kinetics of bead expulsion compared to the kinetics of cyst wall formation and from ultrastructural analysis, it is apparent that most of the beads are lost before the formation of the wall. However, since the cytoplasm of some sections of immature cysts with a thin wall layer contained beads whereas the cytoplasm of mature cysts never did, the pos-
FIGURE 10  A portion of a mature cyst after 30 hr in EM showing beads (B) trapped in the wall (W) and one bead in the ostiole (O). Numerous autolysosomes (A) are visible. $\times$ 10,500.

FIGURE 11  A portion of a mature cyst after 30 hr in EM showing cellular debris (D) in an ostiole (O) of the cyst wall (W). $\times$ 21,000.
Diagrammatic representation of proposed mechanism for bead expulsion. As a bead is pushed toward the cell surface (a), portions of plasma membrane and vesicular membrane break (b), allowing the free edges of membrane to rejoin (c) and the naked bead to be released (d). Electron micrographs in Figs. 6a, b illustrate the fine structure encountered at intervals c and d, respectively. PM, plasma membrane; VM, vesicular membrane; B, bead.

The present report provides information about exocytosis in a protozoan under rather specialized conditions. The mechanism by which the cytoplasmic vacuole fuses with the plasma membrane and expels its content remains unclear. The scheme proposed for the sequence of events in bead expulsion (Fig. 12) is based on morphological evidence (Figs. 6a, b) particularly the finding that numerous membranous fragments appear to be associated with the expelled beads (Figs. 5, 8, 9). In this view, when a bead is pushed toward the cell surface both the plasma membrane and the vesicular membrane are eventually broken. This allows the free ends of the membranes to rejoin and the naked bead to be released. The proposed mechanism shown in Fig. 12 is highly theoretical and diagrammatic, and one must appreciate that there is no direct evidence other than the morphological observations to support it. Furthermore, at the present time one cannot exclude the possibility that the vesicular membrane and the plasma membrane fuse without fragmentation (Bennett, 1969) and that the membrane fragments found in the area of bead expulsion represent degraded cell membranes released in digestion vacuoles in a process that is independent of and separate from the process of bead expulsion.

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