

## CYTOPLASMIC FILAMENTS AND CELLULAR WOUND HEALING IN *AMOEBA PROTEUS*

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The flexibility and self-healing properties of animal cell surface membranes are well known. These properties have been best exploited in various micrurgical studies on living cells (2, 3), especially in amoebae (7, 20). During nuclear transplantation in amoebae, the hole in the membrane through which a nucleus passes can have a diameter of 20–30  $\mu\text{m}$ , and yet such holes are quickly sealed,

although some cytoplasm usually escapes during the transfer.

While enucleating amoebae in previous studies, we found that if a very small portion of a nucleus was pushed through the membrane and exposed to the external medium, the amoeba expelled such a nucleus on its own accord. When this happened, a new membrane appeared to form around the

embedded portion of the nucleus and no visible loss of cytoplasm occurred during nuclear extrusion.

In the present study, we examined amoebae that were at different stages of expelling partially exposed nuclei, to follow the sequence of events during the apparent new membrane formation. Unexpectedly, we found that a new membrane is not formed around the nucleus from inside but a hole is sealed primarily by a constriction of the existing membrane, and that cytoplasmic filaments are responsible for the prevention of the loss of cytoplasm.

## MATERIALS AND METHODS

### *Amoebae*

A strain of *Amoeba proteus* (D) cultured in a modified Chalkley's solution, with *Tetrahymena* as food organisms, was mainly used, but other amoebae including P and xD strains were also examined for comparison. The commonly used modified Chalkley's solution (6) was further modified to include more  $\text{Ca}^{2+}$ , and the solution consisted of 0.5 mM NaCl, 0.05 mM  $\text{NaHCO}_3$ , 0.05 mM KCl, 0.007 mM  $\text{Na}_2\text{HPO}_4$ , and 0.05 mM  $\text{CaCl}_2$ . The latter modification was made after it had been found that although amoebae grew well in the commonly used

medium, they were very sensitive to micrurgical manipulations apparently due to insufficient  $\text{Ca}^{2+}$ .

### *Micrurgy and Microscopy*

Amoebae were individually placed one at a time in small drops of culture medium on 0.6% agar (8), and their nuclei were partially exposed to the surrounding medium by means of a microprobe with a tapered tip. A drop of cold Karnovsky's (9) phosphate-buffered fixative was applied over each amoeba at different stages of nuclear expulsion. The amoeba was then quickly transferred into a dish with the same fixative, and was fixed for 1 h at 4°C. After washing overnight in 0.1 M phosphate buffer (pH 7.4) at 4°C, the amoebae were postfixated in 1% phosphate-buffered  $\text{OsO}_4$  for 1 h at room temperature, and were further processed for electron microscopy.

For transmission electron microscopy (TEM), the fixed amoebae were dehydrated in ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and were examined in a JEM 6C electron microscope. For scanning electron microscopy (SEM), the amoebae were dehydrated in acetone, dried in a Sorvall Critical Point Drier (Dupont Instruments, Sorvall Operations, Newtown, Conn.) with liquid  $\text{CO}_2$ , and placed on round cover glasses (1.2 mm in diameter) with the extruding nuclei facing upward. The specimens

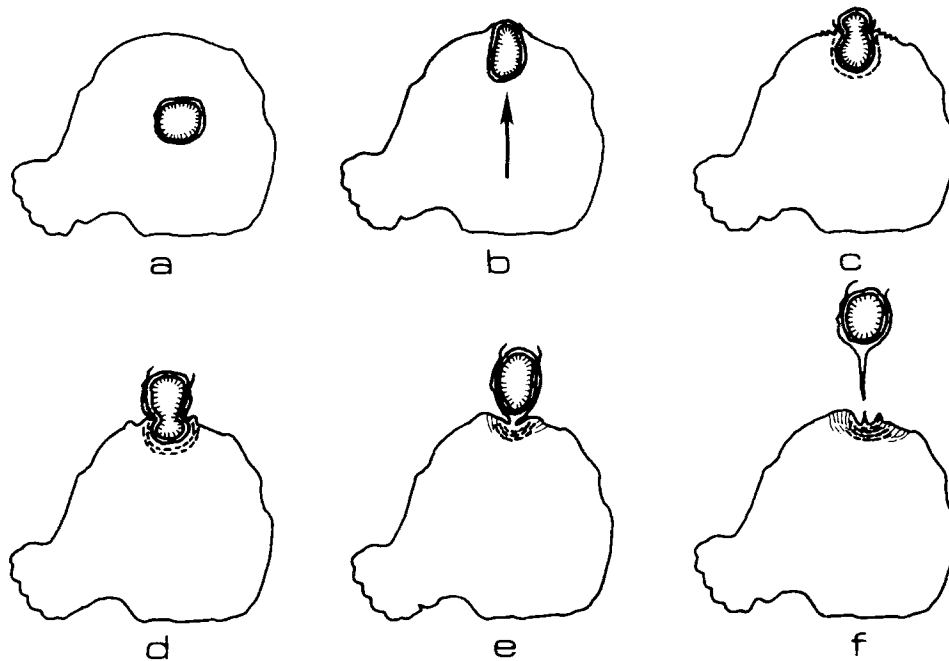


FIGURE 1 Schematic diagrams showing the sequence of events during the expulsion of a partially exposed nucleus. At stage *b*, a small portion of the nucleus is forced to come into contact with the external medium through the broken membrane. Subsequently, the amoeba expels the nucleus on its own accord (stages *c-f*).

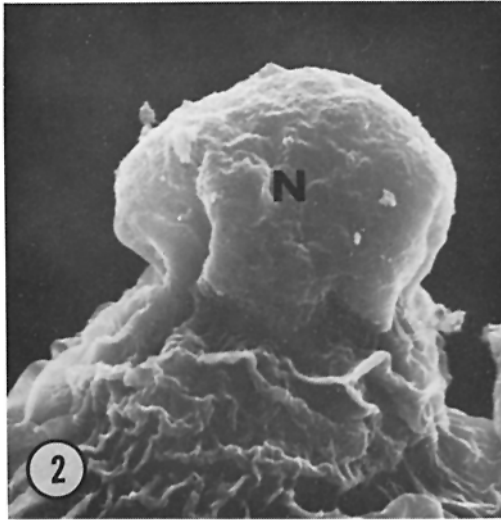


FIGURE 2 Scanning electron micrograph of a nucleus (N), about one-third of which has been expelled. Note the folded cell membrane below the constricted portion of the nucleus.  $\times 1,660$ .

were coated with palladium-gold and examined in an ETEC Autoscan (ETEC Corp., Hayward, Calif.).

## RESULTS

The sequence of events that occurred during the expulsion of an exposed nucleus and subsequent membrane healing is schematically shown in Fig. 1. These diagrams are based on more than 100 expulsions of nuclei observed under a light microscope, of which 10 were sectioned and examined by TEM, and another group of 8 by SEM. The expulsion of nuclei occurred only when a part of the nucleus was exposed to the external medium through a hole made in the cell membrane and the broken cell membrane was attached to the nucleus. If the exposed portion of the nucleus did not come into contact with the broken cell membrane, the nucleus was drawn into the cell even though a portion of it had been exposed to the environment. The process of autonomous expulsion and membrane healing usually took 10–15 s.

When a nucleus was being expelled into a medium containing  $\text{Ca}^{2+}$ , a constriction of the cell membrane developed around the nucleus at the level of the cell surface as though the hole resisted enlargement (Figs. 2 and 3). At the same time, an extensive folding of the cell surface took place around the constricted portion of the nucleus.

As soon as the expulsion started, sparsely distributed thick filaments (about 16 nm thick and

500 nm long) appeared around the portion of a nucleus still inside the cell (stage *c* in Fig. 1). At this time, these filaments were not associated with any visible thin filaments, and were not connected with each other. The thick filaments were never observed surrounding the nucleus of a control cell.

As the nucleus was further expelled, the number of thick filaments increased and they formed a barrier around the embedded portion of the extruding nucleus (stages *d* and *e* in Fig. 1, and Fig. 4). The filament barrier was separated from the nucleus by a layer of hyaloplasm.

By the time a nucleus was completely expelled (stage *f* in Fig. 1, and Fig. 5), the hyaline layer disappeared, and a considerable amount of cell membrane was detached from the cell. The extruding nucleus which was initially attached to the broken ends of the cell membrane became detached and lay by itself. The hole in the cell membrane was closed with no visible loss of cytoplasm, and immediately beneath the closed hole was a complex of thick and thin filaments (Fig. 6). The thin filaments were about 7 nm in diameter, and were associated with the cell membrane around the closed hole. A large amount of thin filaments not associated with any thick filaments were also observed to fill the hyaline cytoplasm surrounding the closed hole (cf. Fig. 5).

In order to check the role of  $\text{Ca}^{2+}$  in wound healing, we observed the nuclear expulsion and subsequent membrane healing in the absence of  $\text{Ca}^{2+}$ . Amoebae were washed several times in a culture solution lacking  $\text{Ca}^{2+}$  and were enucleated on agar prepared with Ca-free medium. Under these conditions, the expulsion of a nucleus took less than a few seconds, and the hole in the membrane was closed slowly over a period of 10 s or more, resulting in a loss of much cytoplasm. Sometimes, a hole was not closed and the enucleated cell cytolysed. Since the expulsion took such a short time, it was not possible to process the amoebae to examine the ultrastructural aspects of nuclear expulsion taking place under these conditions. The results were essentially the same in other strains of amoebae studied for comparison.

## DISCUSSION

What appeared in the beginning to be a new membrane forming around a nucleus being expelled has turned out to be a complex of cytoplasmic filaments. The possible involvement of contractile filaments in cellular wound healing was

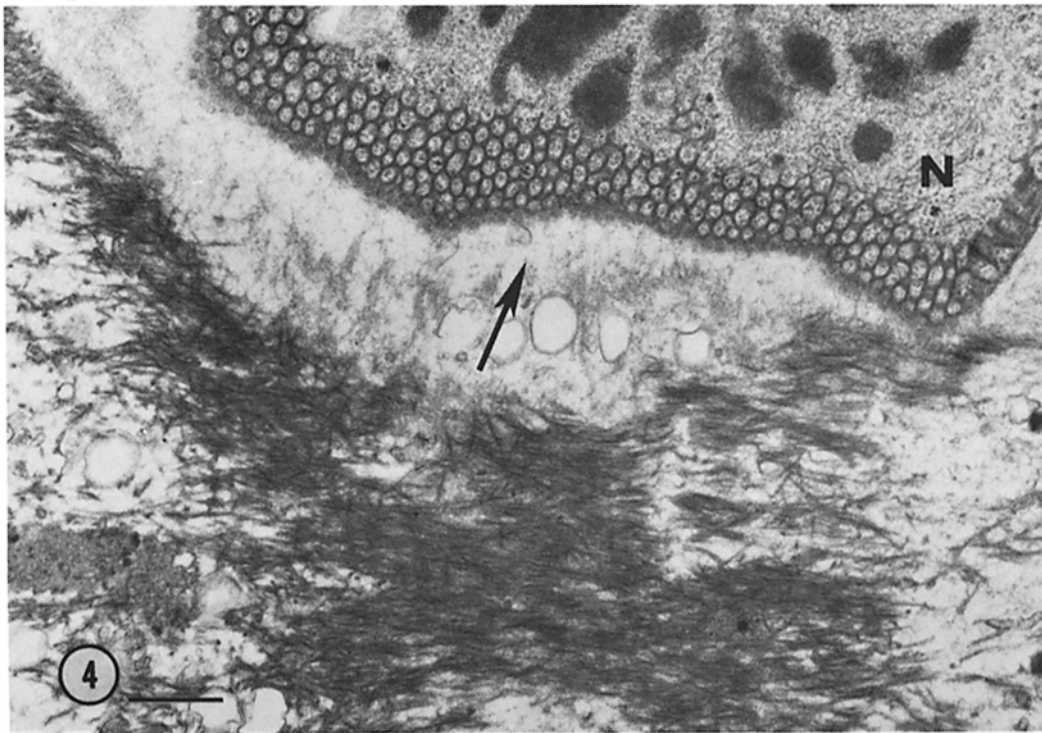


FIGURE 3 Photomicrograph of a 0.3- $\mu\text{m}$  resin section of an amoeba in the process of expelling its nucleus. Note the constriction around the upper portion of the nucleus, and the folded cell surface. Stained with azure II (5).  $\times 1,230$ .

FIGURE 4 Electron micrograph showing a portion of the filament complex surrounding the embedded part of a nucleus (N) being expelled. The arrow indicates the direction of expulsion. The scale in this and other figures represents 1  $\mu\text{m}$ .  $\times 12,200$ .

first suggested by Gingell (4) in *Xenopus* eggs, and the presence of microfilaments in contracting cortex has been confirmed in *Xenopus* and *Ambystoma* eggs (1, 11). However, in the amphibian eggs, only actin-like thin filaments have been found with no accompanying thick filaments. It is not clear how the thin filaments alone could contract to bring about membrane folding and subsequent wound healing.

The thick and thin filaments found near the wounded areas in amoebae are similar in dimensions to those shown to be involved in cytoplasmic contraction in amoebae and other motile cells (10, 12–18), and probably represent myosin and actin, respectively. There appear to be two separate forces involved in the nuclear expulsion and membrane healing; viz., one to expel the nucleus and another to pull the membrane together to mend the broken area. It is easy to speculate how the thick and thin filaments aggregated as shown in Figs. 5 and 6 might contract in the usual manner (18) to generate forces to pull the edges of membrane around the open hole. However, the same filaments cannot generate force for the expulsion of nuclei, at least during the initial stage of expulsion, since, during the early stage, only thick filaments are found around the nucleus and they are not connected with each other. In addition, the expulsion of a nucleus occurs as usual in the absence of extracellular  $Ca^{2+}$ , whereas the wounds are healed very slowly or not at all without  $Ca^{2+}$  both in amoebae and in amphibian eggs (4). Therefore, the initial motive force for the expulsion of a nucleus must be derived from another source, which does not require extracellular  $Ca^{2+}$ .

With the expulsion of a nucleus, a considerable amount of cell membrane attached to the nucleus is lost (cf. Fig. 5), and the loss would have to be replaced. However, since the amoeba seems to be able to build up some reserve membrane, e.g., by folding as shown in Figs. 2 and 3, an immediate replacement of the lost membrane may not be needed. The new membrane formation reported by Szubinska (19) was not observed in our study.

The membrane healing in amoebae appears to be unique in that it takes only a few seconds, whereas the same process takes several minutes in amphibian eggs (4). It is not known whether the thick and thin filaments that appear around the nucleus and wounded area are formed *de novo* from their precursors or whether existing filaments are simply rearranged. Although the filaments are often found in normal amoeba cytoplasm (10),

they are not so abundant as in wounded amoebae and their locations have to be sought (12). Therefore, it is more likely that the filaments are assembled *in situ* in response to wounding. It is also not clear how the signal initiated by the partial exposure of a nucleus and its contact with the cell membrane is transmitted to the interior of the cell to stimulate the aggregation of thick filaments. The fact that a nucleus has to come into contact with the broken cell membrane for expulsion suggests that the nuclear membrane might act as a transmitter of the signal. In *Xenopus* eggs, an influx of  $Ca^{2+}$  caused by a change in membrane permeability has been suggested as the stimulus for the cytoplasmic contraction (4).

Further work is in progress to find answers to these questions. Meanwhile, it is interesting to note that the involvement of cytoplasmic filaments appears to be a universal phenomenon in cellular wound healing.

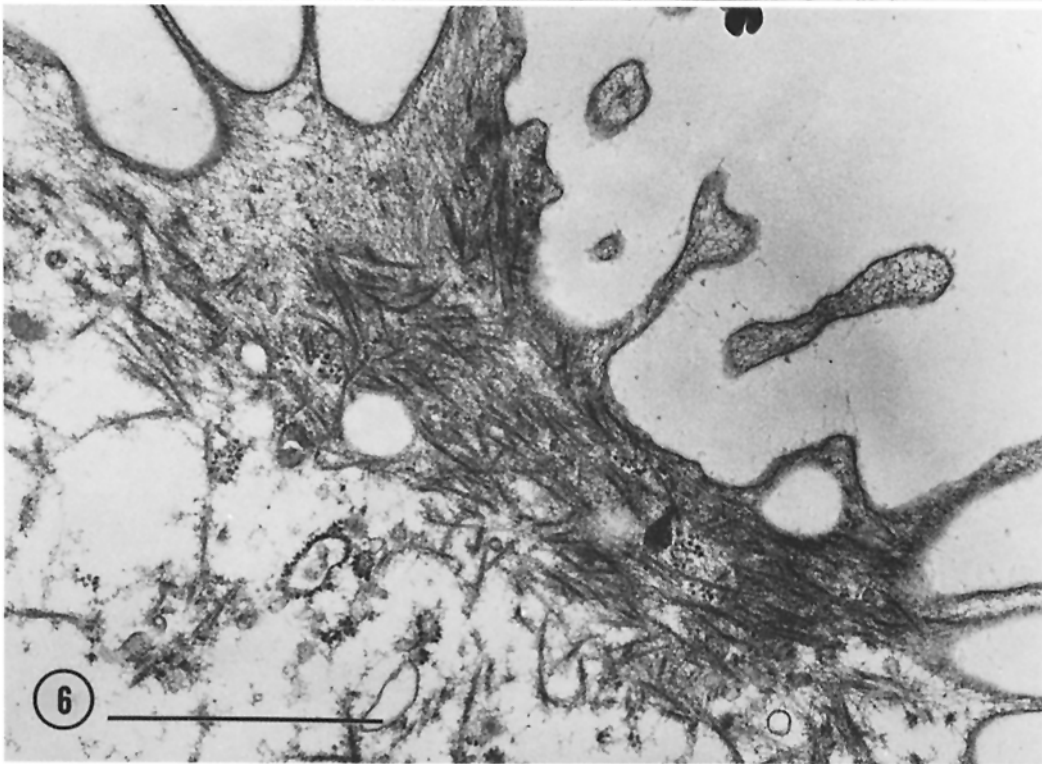
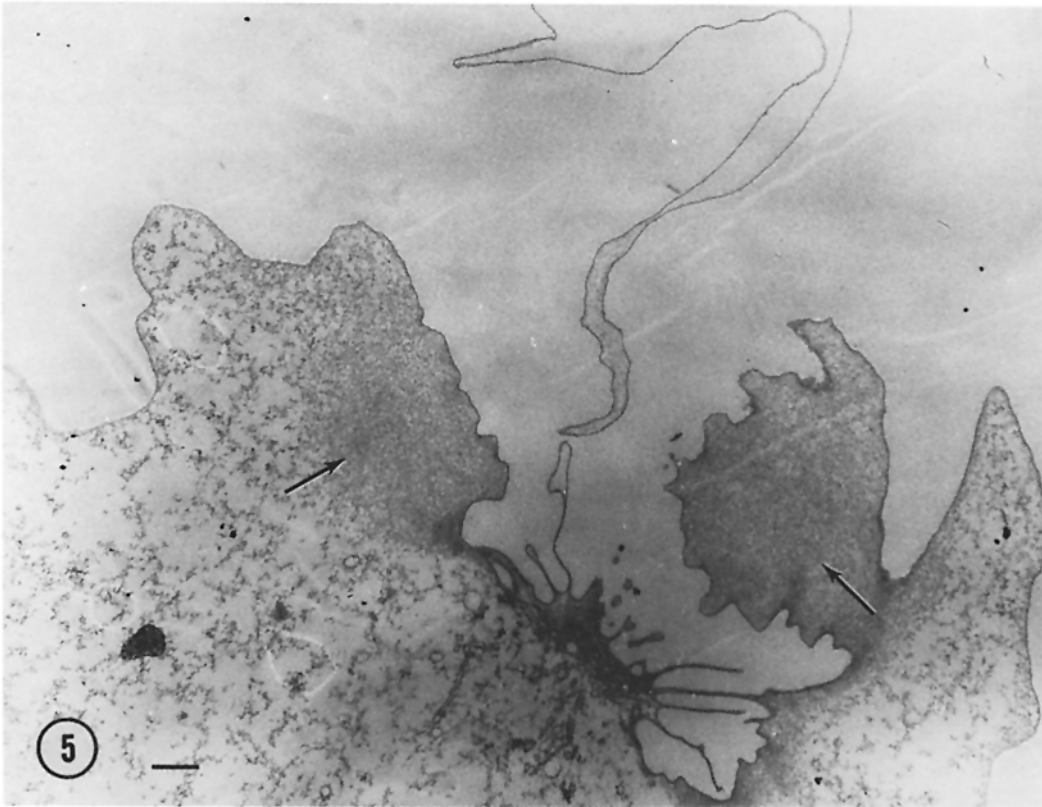
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FIGURE 5 Electron micrograph of an amoeba which has just completed the expulsion of its nucleus. Note the membrane piece which is attached to the expelled nucleus (out of sight) and its base. Immediately beneath the closed hole is a complex of thick and thin filaments (cf. Fig. 6), and the cytoplasm around the closed hole is filled with actin-like thin filaments (arrows).  $\times 5,800$ .

FIGURE 6 A higher magnification of a portion of the amoeba shown in Fig. 5. Note the association of thick and thin filaments, and the apparent attachment of the thin filaments to the inside of the cell membrane.  $\times 36,500$ .