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Response of club cells in the skin of the carp *Cyprinus carpio* to exogenous stressors

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Abstract. The ultrastructure of club cells and neighbouring filament cells and leucocytes in the epidermis of carp, was studied under normal conditions and after exposure to several stressors: acid water, heavy metals, organic manure, brackish water and wounding. The effects of the stressors were remarkably similar. The club cells increased in size and contained more endoplasmic reticulum and Golgi areas. In both control and stressed fish, most mitotic figures of the filament cells were found adjacent to club cells, as was demonstrated after colchicine injection. Whereas in the controls apoptosis of filament cells was scarce and limited to the upper layer of the epithelium, in the stressed fish it was commonly seen in close proximity to the club cells but not in other mid-epidermal parts of the epithelium. This indicates that club cells influence the cellular kinetics of the filament cells. Under stress conditions leucocytes infiltrated the epidermis. Some were seen inside club cells. Apparently these leucocytes were taken up in phagosomes and subsequently they showed signs of necrotic degeneration. Leucocyte incorporation and degeneration in club cells were not observed in control fish. Control of the cellular turnover of filament cells and the elimination of leucocytes may represent new functions for club cells, which have mainly been associated with the production of pheromones.

Key words: Skin – Club cells – Apoptosis – Necrosis – Filament cells – Leucocytes – *Cyprinus carpio* (Teleostei)

Introduction

Club cells are characteristic components of the epidermis of several orders of teleost fish (Pfeiffer 1977). They are giant ovoid cells located in the mid-epidermal layers (Whitear and Zaccone 1984; Iger and Abraham 1990) with a peculiar cytoplasm containing many tiny coiled filaments (Whitear 1986). In eels, there is also a secretory

vacuole (Whitear and Zaccone 1984). The primary function of these cells has not been established (Ralphs and Benjamin 1992). Club cells in fish from the super-order Ostariophysi, to which the Cypriniformes belong, have been suggested to be the source of a pheromone-like substance that elicits a fright reaction (Pfeiffer 1977). In other orders, the presence of club cells is probably not associated with the production of fright-inducing substances (Whitear 1986). Zaccone et al. (1990) demonstrated the presence of serotonin in club cells of 2 species of teleosts and also suggested a pheromonal function. Others have ascribed to these cells the formation of toxic or anti-pathogenic agents, substances that deter predators, or agents accelerating wound healing (Smith 1982; Suzuki and Kaneko 1986; Al Hassan et al. 1987). Whitear and Mittal (1983) suggested that in ostariophysan fish the released content of the club cells (“gel secretion”) has protective effects on a damaged epidermal surface. A phagocytic function has been suggested by Lufty (1964). The present study examines the club cells of carp exposed to different types of stressors: water pollution (acidified water, and water containing organic manure or cadmium, copper or lead), brackish water, and skin damage caused by wounding. It was felt that an investigation of the impact of all these stressors might provide an indication of the functional role of these cells.

Materials and methods

Juvenile male and female carp, *Cyprinus carpio*, weighing 3–15 g, were used. After an acclimation period of at least 3 weeks, the fish were kept for periods up to 30 days either in normal conditions (i.e. tap water at 22°C), or experimental conditions. For all control and experimental groups, the water was continuously changed (about 30% per day), well aerated, and filtered. The experimental conditions were:

1. Acidified water

For 2 carp groups the pH of the water was gradually lowered to pH 5 or 6 (over a period of 2.5 h) by slowly adding small amounts

Table 1. Semi-quantitative evaluation of the changes in the carp epidermis in response to stressors. +, Moderate increase; ++, marked increase; = no change; - moderate reduction; - marked

		a	b	c	d	e	f	g
pH 5	24 h	++	+	=	+	+	=	+
	3 d	+	++	+	+	+	+	+
	14 d	+	+	++	+	+	+	+
pH 6	24 h	+	+	=	=	+	=	+
	3 d	+	+	=	+	+	+	+
	14 d	+	+	=	+	+	++	+
Manure	24 h	++	++	=	+	+	=	+
	4 d	+	++	-	++	++	+	+
Cd, low	24 h	+	+	=	+	+	=	=
	7 d	++	+	+	++	=	+	+
Cd, medium	24 h	+	+	=	+	+	=	+
	7 d	++	+	+	++	+	+	+
Cd, high	24 h	+	+	=	+	+	=	+
	7 d	++	++	-	++	++	+	++
Pb	24 h	+	+	=	+	+	=	+
	7 d	++	+	+	++	++	+	+
Seawater	24 h	+	=	=	+	+	=	+
	6 d	++	+	-	++	++	++	++
Wounding	24 h	+	+	=	+	+	=	=
	8 d	+	=	+	+	++	++	+

reduction; a-e, club cells; a, rER and Golgi membranes; b, cell size; c, cell number; d, upward migration; e, leucocyte incorporation; f-g, filament cells; f, mitosis; g, apoptosis; h, hours; d, days

of a diluted solution of H_2SO_4 . The pH of the water was automatically adjusted with pH-stat equipment (Consort, Belgium). For more details about the procedures, see Iger and Wendelaar Bonga (1993).

2. Manure exposure

Chicken manure (0.3–0.7% w/w) was added to the water. For details of fish husbandry, manure composition and bacterial content of the water, see Iger et al. (1988).

3. Heavy metal exposure

Cadmium nitrate at concentrations of 22, 65 and 500 $\mu g \cdot l^{-1}$ Cd, and lead acetate at concentrations of 0.5 $mg \cdot l^{-1}$ Pb, were added to the water. For details of the experimental set up, see Iger (1992).

4. Brackish water exposure

Commercial sea salt was added to the water at a concentration of 5 $gr \cdot l^{-1}$ (Iger 1992).

5. Wounding

With a razor blade a small incision was made in a scaleless area of the dorsal body wall. Details of the healing process have been reported by Iger and Abraham 1990.

The duration of exposure to the stressors is listed in Table 1. For the detection of mitotic figures, 6 fish from each group were intramuscularly injected with colchicine (Sigma; 2 mg/100 g body weight) dissolved in saline (0.6 g NaCl per 100 ml). Controls were injected with saline only. These fish were sampled 20–22 h after injection.

Six fish per group were examined. For electron microscopy pieces of skin (3×3 mm) were taken from the dorsal part of the head of fish anaesthetized with Hypnocalmer (Jungle, Texas) and prefixed in 3% glutaraldehyde buffered in 0.09 M sodium cacodylate, pH 7.4. Fixation was carried out in 1% osmium tetroxide in the same buffer. The tissues were dehydrated in ethanol and embedded either in LX-112 or in Spurr's resin. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined in a Jeol 100 CX electron microscope.

Fig. 1. Control. A typical club cell with a polymorphic nucleus (*n*) surrounded by central cytoplasm (*arrow*), and an electron transparent periphery (*p*). *nf* Nucleus of a filament cell. $\times 3400$

Fig. 2. Acid water (pH 5; 14 days). A mitotic filament cell (*f*) adjacent to club cell (*c*). $\times 8200$

Fig. 3. Acid water (pH 5; 3 days). An early stage of apoptosis of filament cell (*arrow*) adjacent to club cell (*c*). The nucleus of the filament cell (*n*) is still visible. $\times 4000$

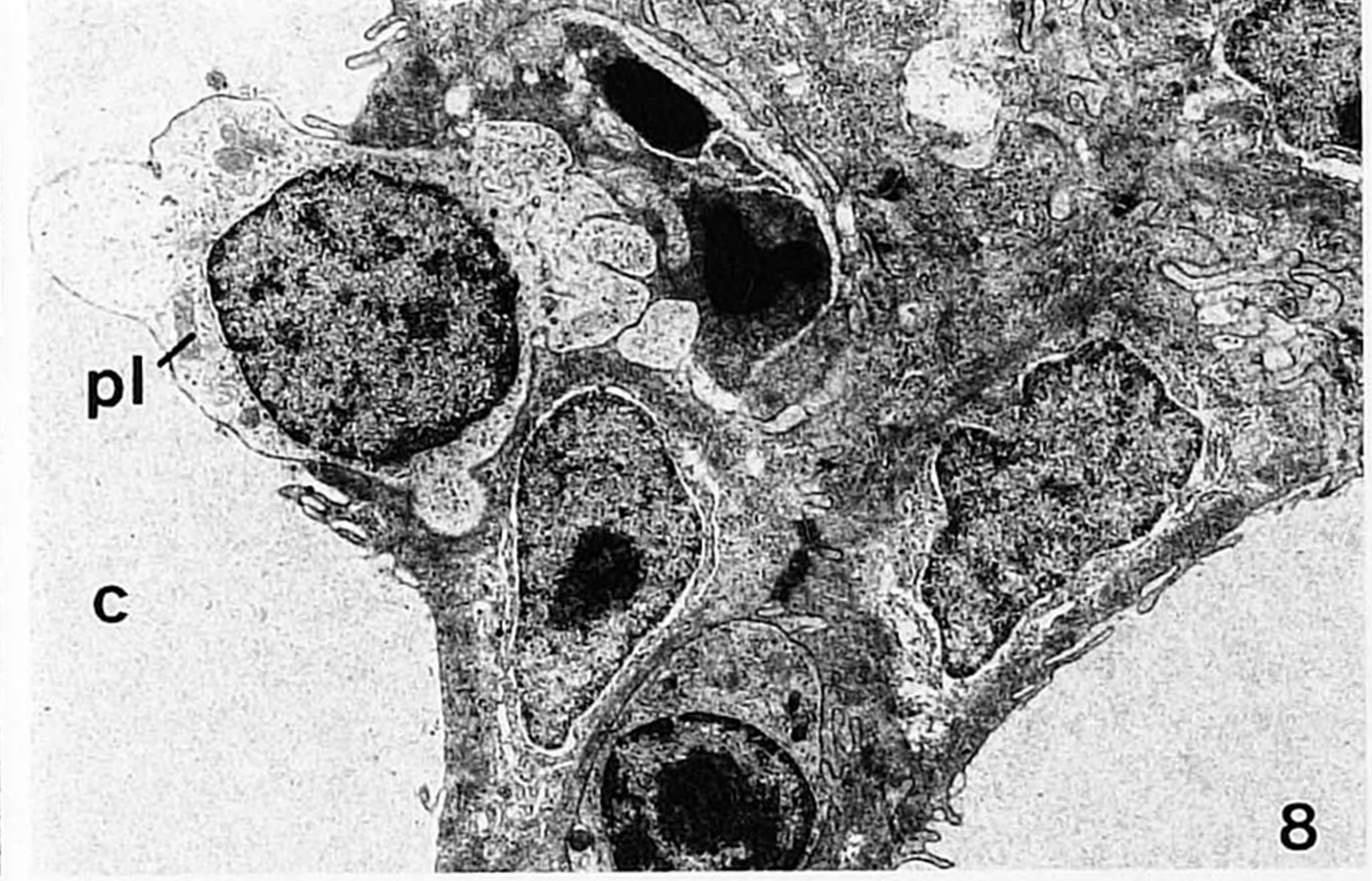
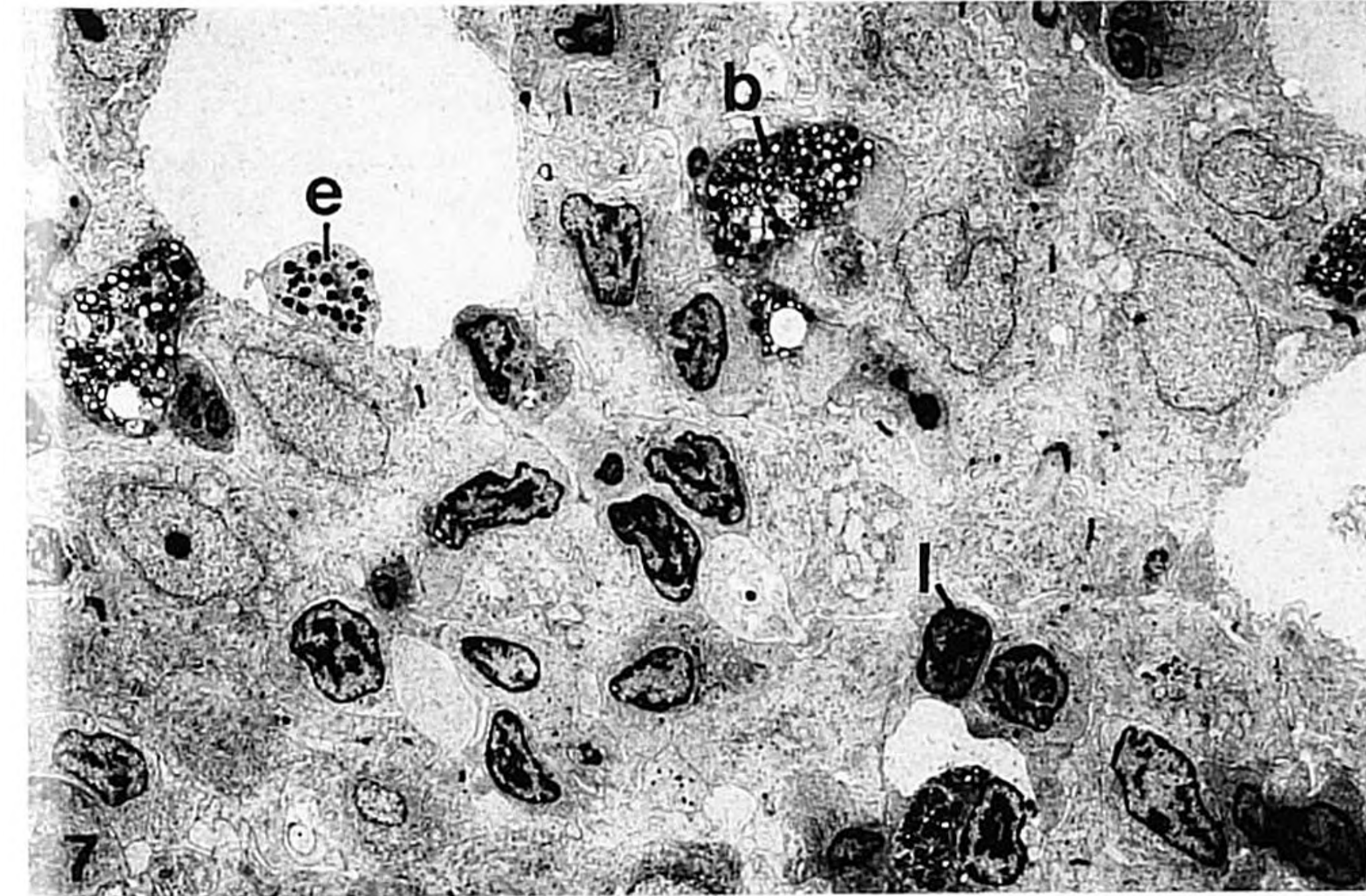
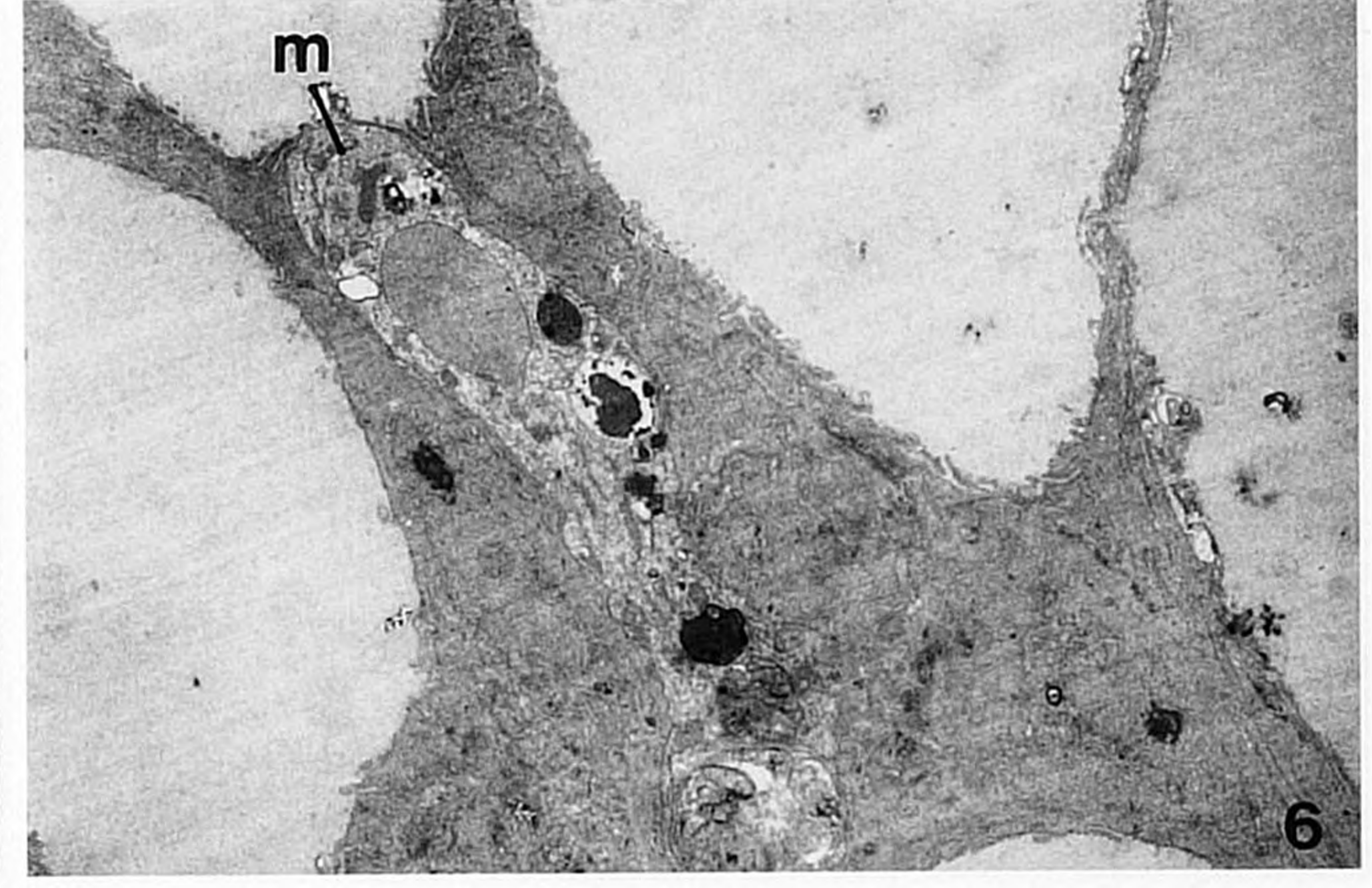
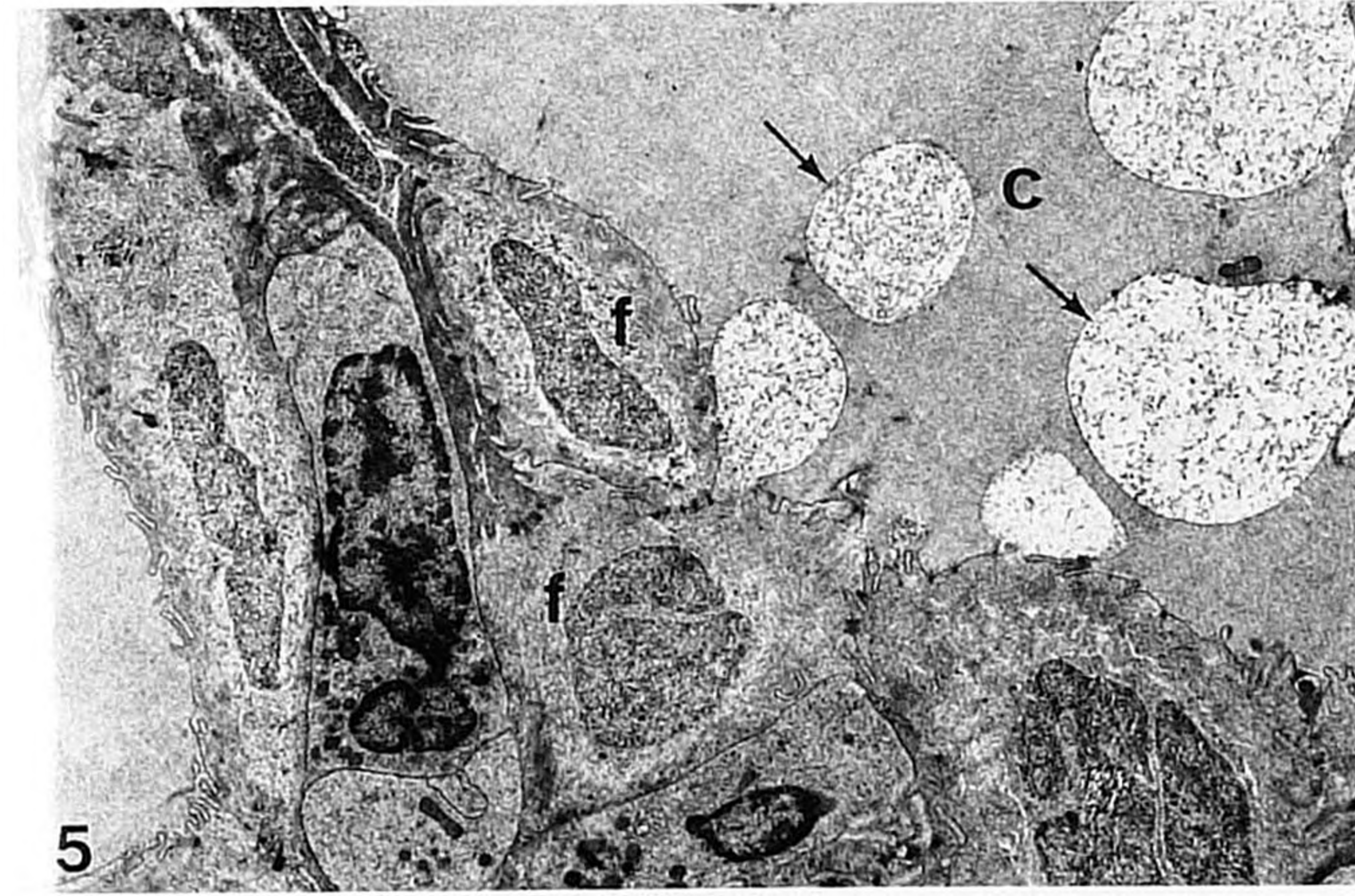
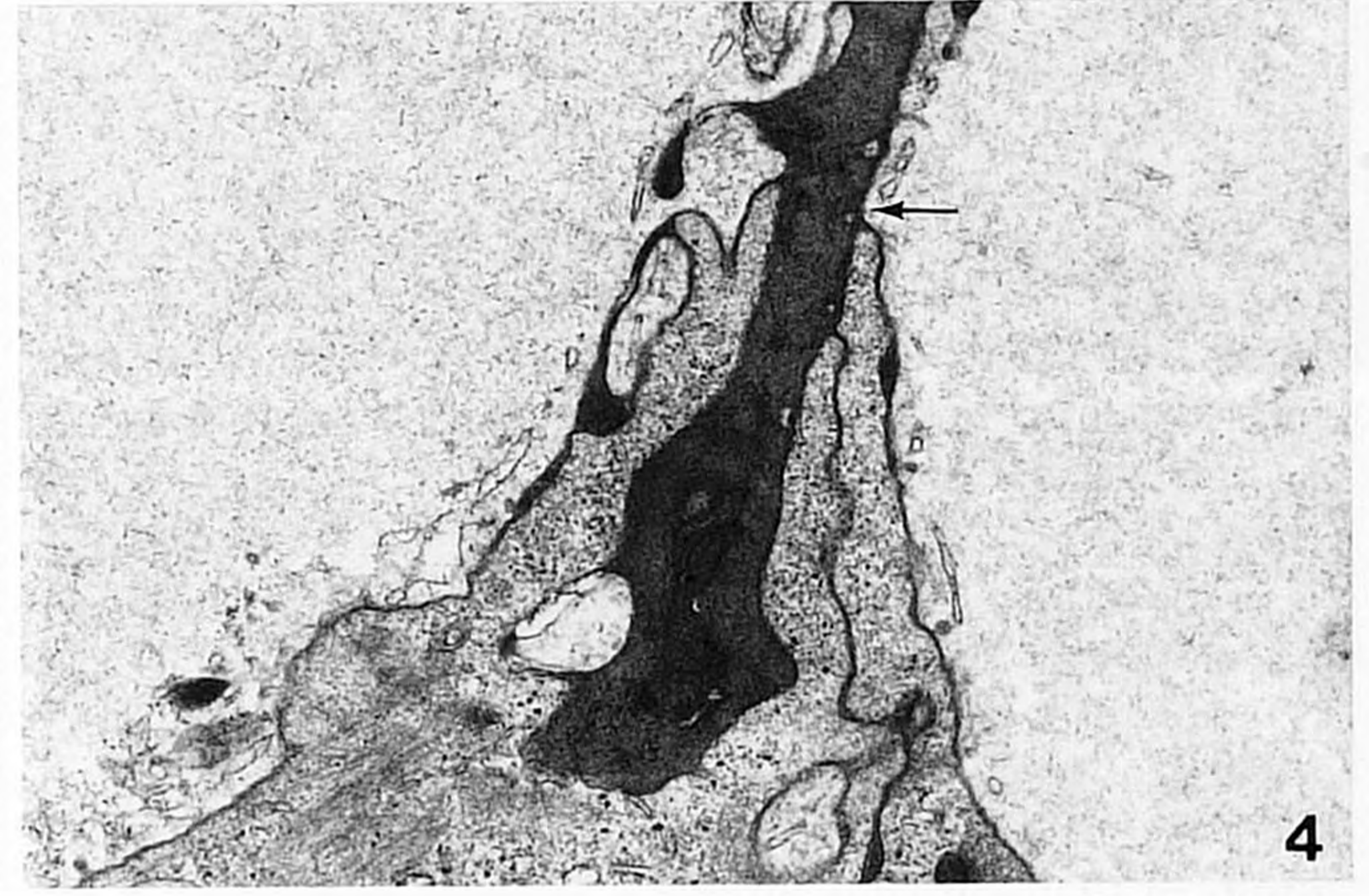
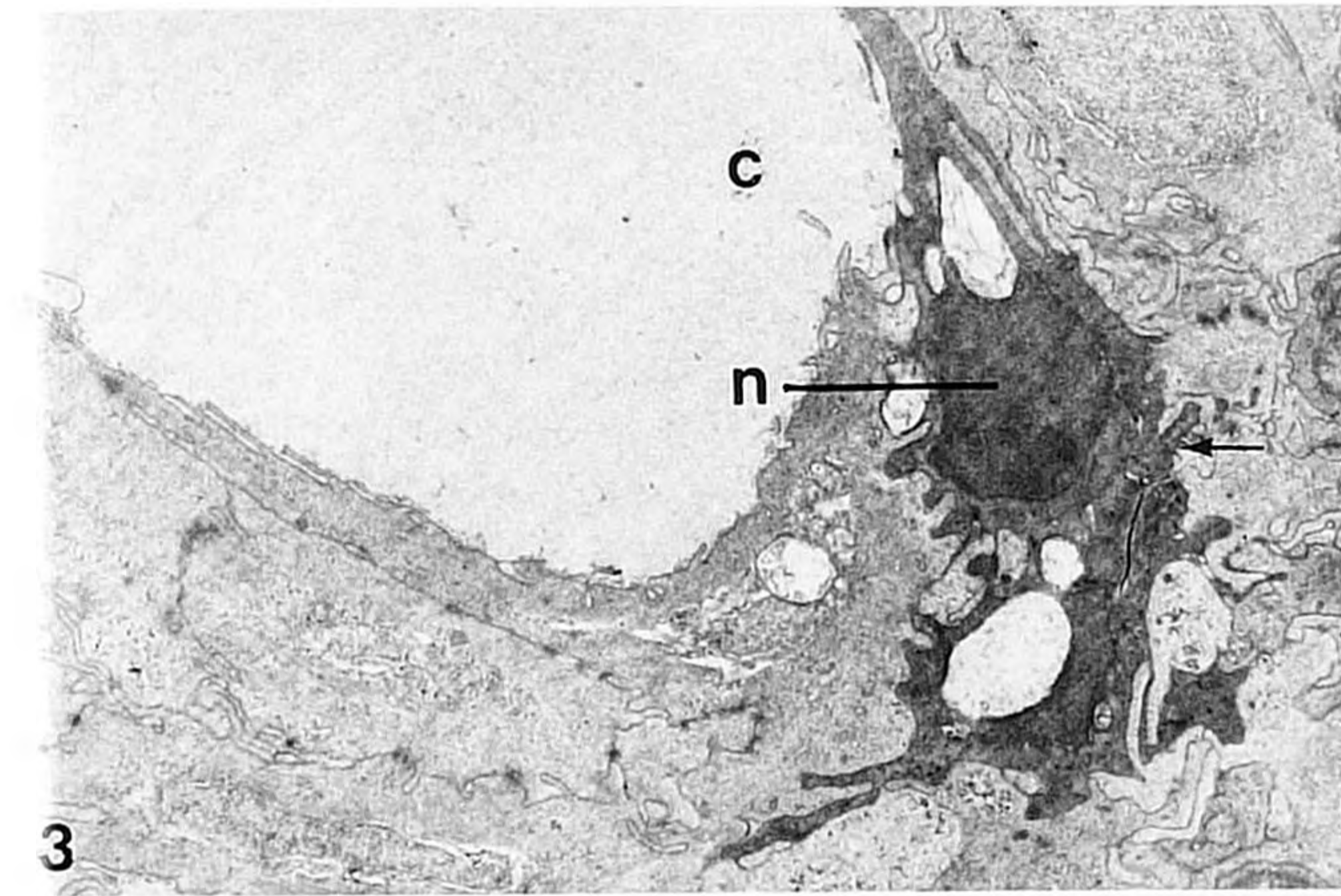
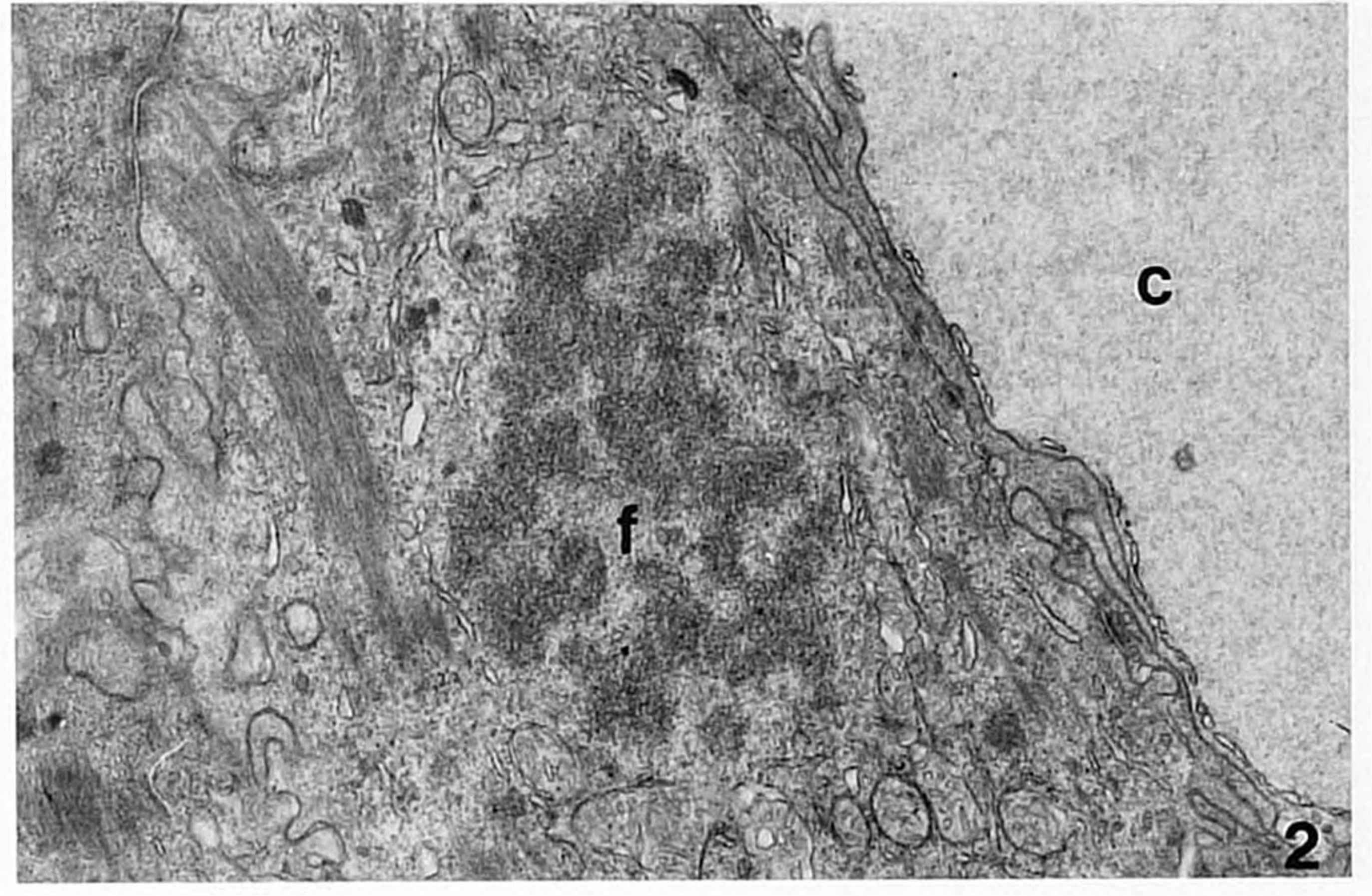
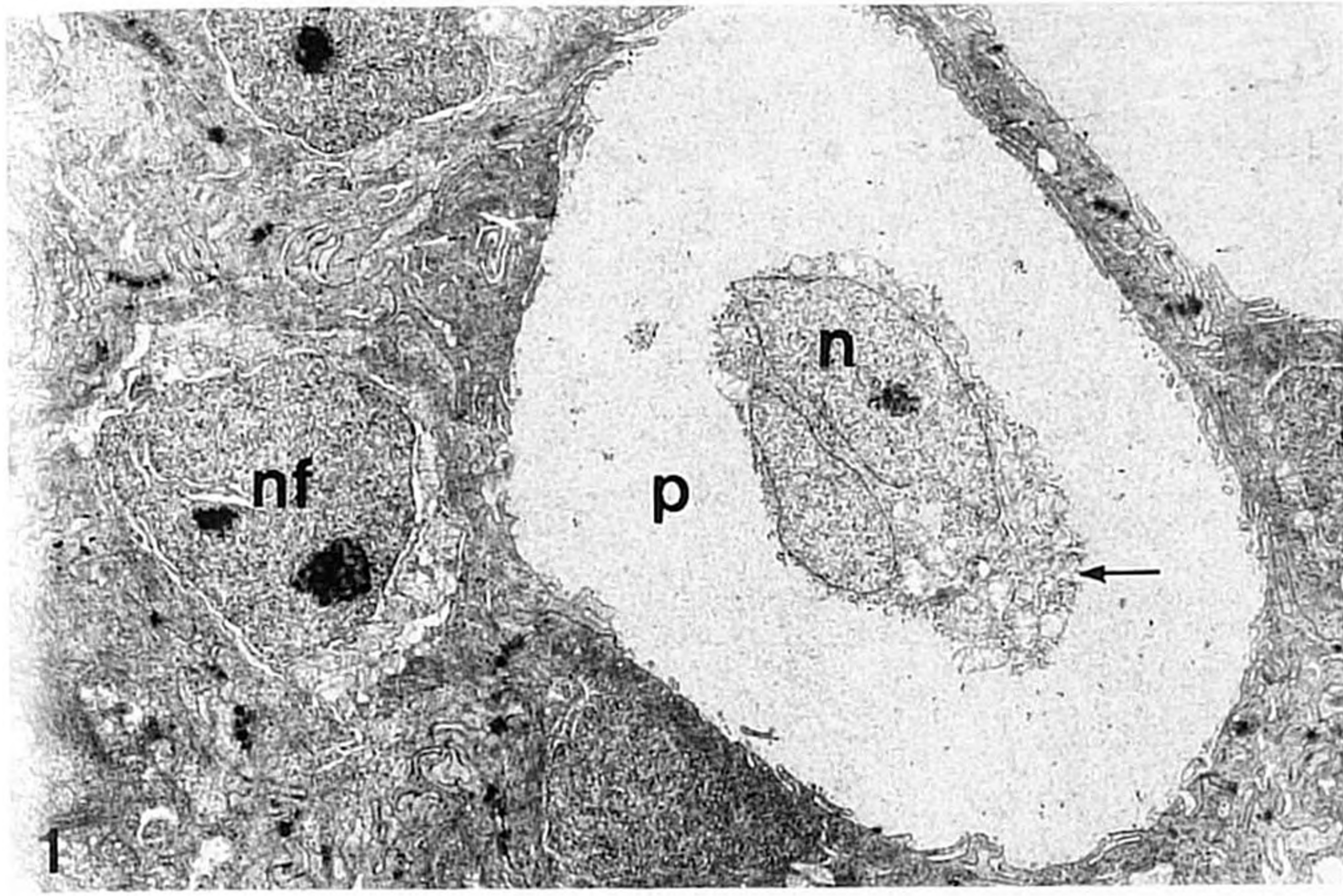
Fig. 4. Cadmium contaminated water (22 $\mu g \cdot l^{-1}$; 7 days). Part of an apoptotic filament cell. The apoptotic material (*arrow*) fills the intercellular spaces between club cells. $\times 14500$

Fig. 5. Brackish water (sea salt, 5 $gr \cdot l^{-1}$; 24 h). Necrotic processes (*arrows*) probably originating from filament cells (*f*) are seen in club cell cytoplasm (*c*). $\times 3400$

Fig. 6. Cadmium contaminated water (22 $\mu g \cdot l^{-1}$; 24 h). A macrophage (*m*) containing phagosomes and lysosomes between club cells. $\times 2750$

Fig. 7. Lead contaminated water (0.5 $mg \cdot l^{-1}$; 24 h). The epidermis is highly infiltrated by leucocytes. An eosinophilic granulocyte (*e*), indented in or inside a club cell. *b* Basophilic granulocyte; *l* lymphocyte. $\times 1200$

Fig. 8. Brackish water (sea salt, 5 $gr \cdot l^{-1}$, 6 days). A partially necrotic leucocyte, probably a plasma cell (*pl*) adjacent to a club cell (*c*). $\times 4200$



Results

Club cells in control fish

Club cells are common in the middle layers of the epidermis (Fig. 1). About 20–40 cells are present per 1 mm of length of cross-sectioned epidermis. Differentiating and mature club cells are found. The nucleus of the former is round, and surrounded by cytoplasm containing well developed endoplasmic reticulum, Golgi areas, mitochondria, polyribosomes and small vesicles of 100–130 nm. The periphery of the cell, a narrow belt of 1–2 μm width, contains fine coiled filaments. Occasionally, polyribosomes, intermediate filaments of 8–9 nm width, and desmosomes connecting the club cells with adjacent filament cells, are present.

Mature club cells are ovoid. The nucleus is polymorphic and surrounded by a thin rim of moderate electron-dense cytoplasm containing mainly mitochondria, Golgi elements and endoplasmic reticulum. Most of the cytoplasm is electron lucent, and occupied by fine coiled filaments. The cell periphery shows very few desmosomes and associated intermediate filaments. The cells share interdigitations, 15–25 per cell in cross section, with the surrounding filament cells. Small peripheral vesicles (Whitaker et al. 1991 a) were not observed.

After colchicine injection of control fish, the incidence of mitotic figures, seldom observed in sham injected fish, increases markedly in the epidermis, in particular in the mid-epidermal region. Most of the mitotic figures are found in filament cells adjacent to club cells (Fig. 2). In areas without club cells, or around immature club cells, mitotic filament cells are very scarce.

Club cells in fish under experimental conditions

The observations reported below were made under almost all experimental conditions, although to a degree depending on the length of exposure to the stressor or the type of stressor. A semiquantitative evaluation of the results is presented in Table 1. Only differences with the controls are reported. Already after a few days, the club cells of these fish, both mature and differentiating cells, are located closer to the skin surface than in the controls. During the whole period of exposure the mature club cells are enlarged, from about $40 \times 60 \mu\text{m}$ in the controls to about $50 \times 80 \mu\text{m}$ in the stressor-exposed fish. Mitosis of filament cells, as observed after colchicine injection, is more frequent and mainly restricted to the cells adjacent to mature club cells. It is hardly found in filament cells not in direct contact with the club cells. Another phenomenon observed around the mature club cells is the presence of apoptotic filament cells (Figs 3,4). These cells lose their desmosomes and show condensation and enhanced electron-density of their nuclei and cytoplasmic organelles. Remnants of apoptotic filament cells are found as apoptotic bodies inside macrophages. Neighbouring filament cells are active in endocytosis of vesicles of high electron-density originating from the apoptotic filament cells. Club cells adjacent to mitotic or apoptotic

filament cells contain well developed endoplasmic reticulum, extensive Golgi areas and many polyribosomes. Occasionally necrotic extensions of filament cells are found within club cells (Fig. 5). In controls apoptotic filament cells are only rarely found and restricted to the top layer of filament cells, the pavement cells. No consistent changes are observed in the numbers of club cells (Table 1).

During all experimental treatments the epidermis becomes heavily infiltrated by leucocytes (Fig. 7); lymphocytes as well as granulocytes and macrophages. Leucocytes are often seen adjacent to club cells (Figs. 6, 8), and some leucocytes are seen inside club cells (Figs. 7, 9–11). This phenomenon is commonly observed in fish of all experimental groups. Up to 3 leucocytes per club cell may occur (Fig. 10). At higher magnification, some of these leucocytes are surrounded by 2 membranes, indicating that they are located in phagosomes (Fig. 12). Other leucocytes are surrounded by 1 membrane. These often exhibit signs of necrosis (Fig. 8, 11) i.e., swelling of the cell and its mitochondria and granules, and disruption of membranes. This indicates that the membrane of the leucocytes disappears when lysis of these cells advances. Organelles located in the perinuclear cytoplasm of the club cells - Golgi systems, granular endoplasmic reticulum, polyribosomes, and mitochondria - are oriented in the direction of the incorporated cells (Fig. 11). Some of these club cells are in a state of degeneration; their nuclear membrane is disrupted and the contorted filaments aggregated, while the cytoplasm shows patches of high electron density (Fig. 14). In other club cells, as well as in the filament cells located apically from leucocyte-containing club cells, small vesicles appear (Fig. 13). The intercellular space between these filament cells becomes widened, and cytoplasmic extensions of club cells penetrate in these

Fig. 9. Cadmium contaminated water ($22 \mu\text{g}\cdot\text{l}^{-1}$, 7 days). A lymphocyte (*l*) inside a club cell. $\times 4200$

Fig. 10. Manured water (4 days). Leucocytes (*le*) and necrotic processes of filament cells (*f*) inside club a cell. $\times 4200$

Fig. 11. Lead contaminated water ($0.5 \text{ mg}\cdot\text{l}^{-1}$, 7 days). A slightly necrotic lymphocyte (*l*) inside a club cell. $\times 6400$

Fig. 12. Cadmium contaminated water ($22 \mu\text{g}\cdot\text{l}^{-1}$, 24 h). Detail of a necrotic lymphocyte (*l*) inside a club cell (*c*). The 2 membranes (*arrows*) indicate that the lymphocyte is located in a phagosome of the club cell. $\times 58000$

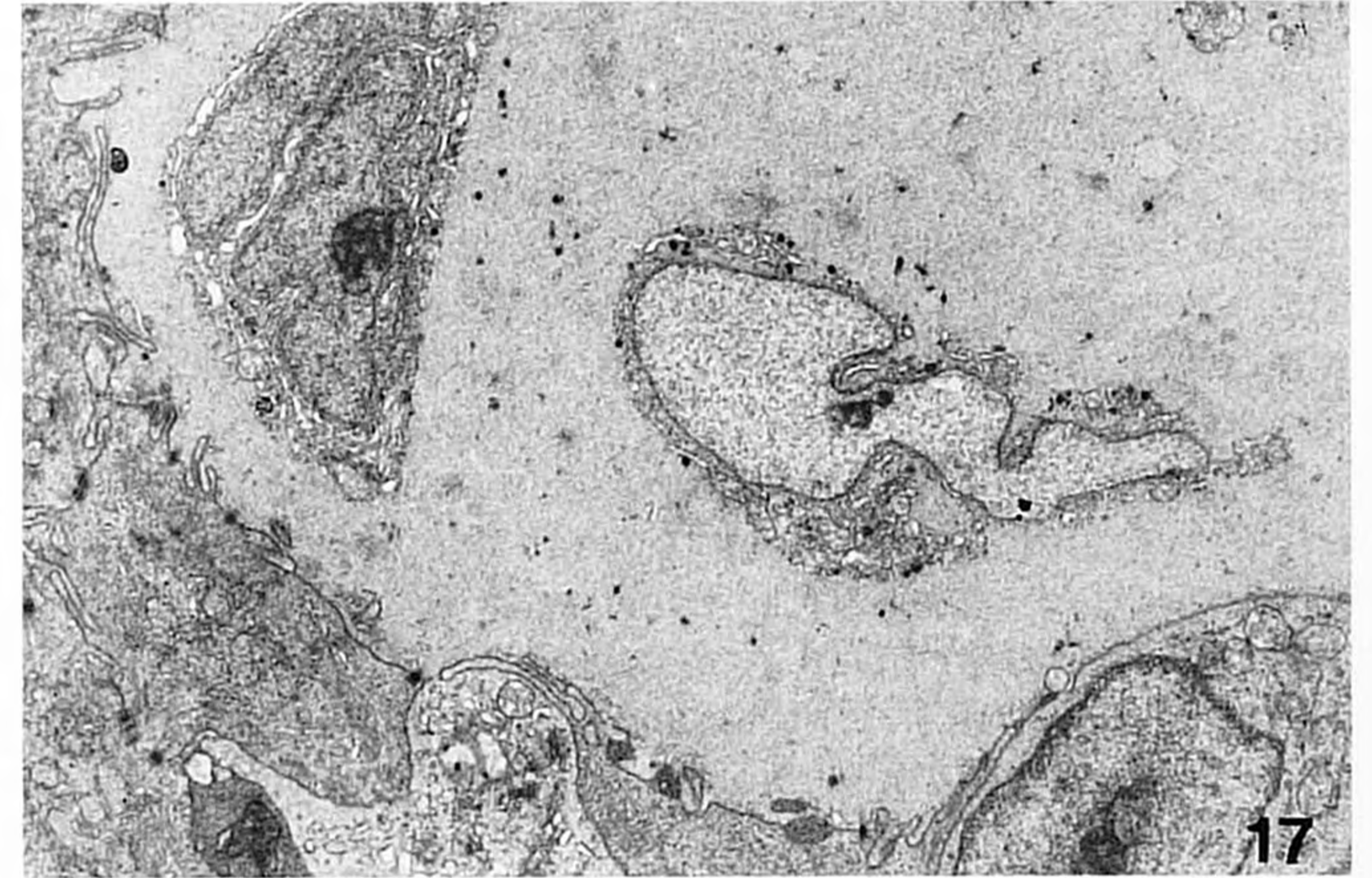
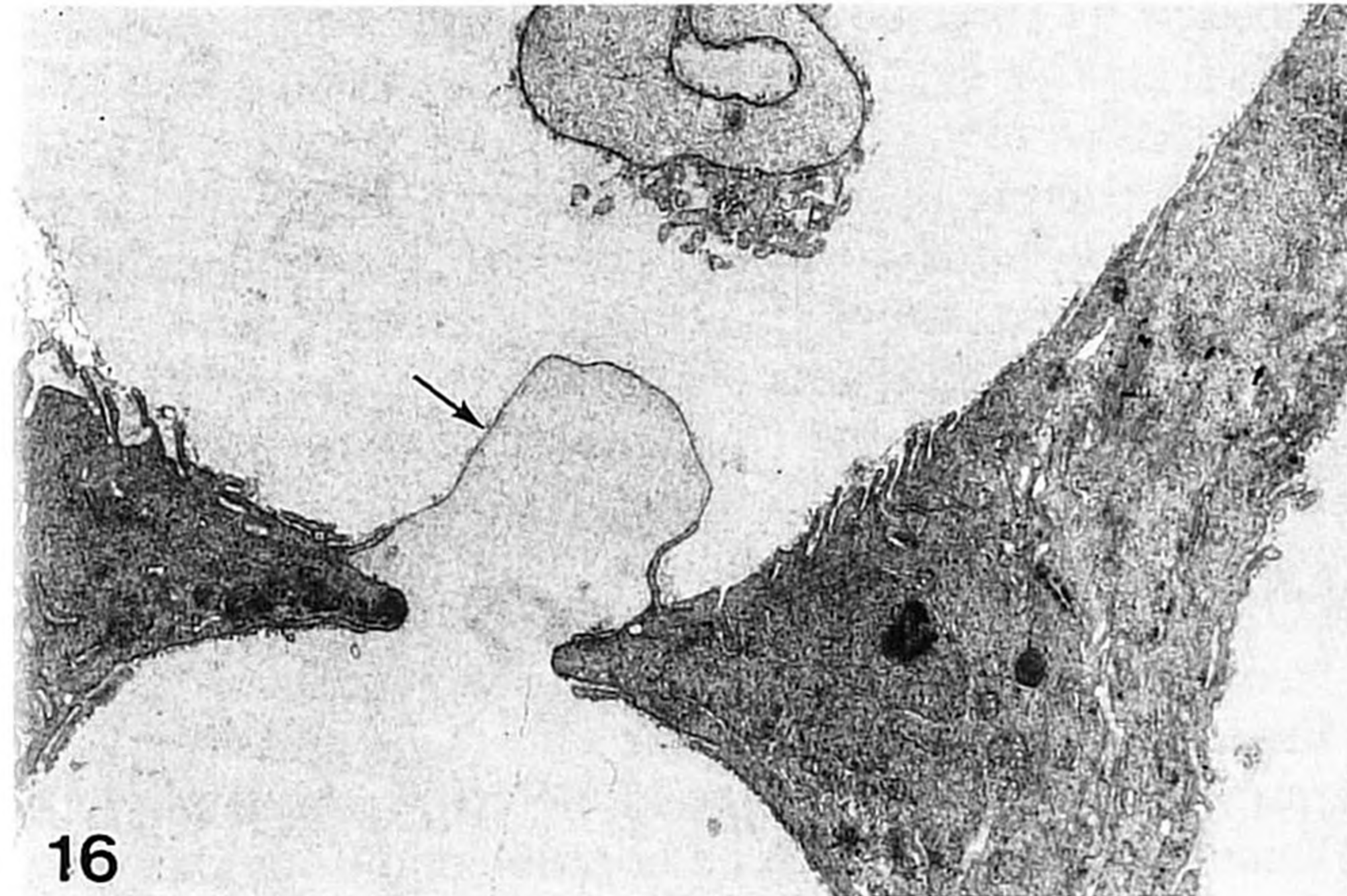
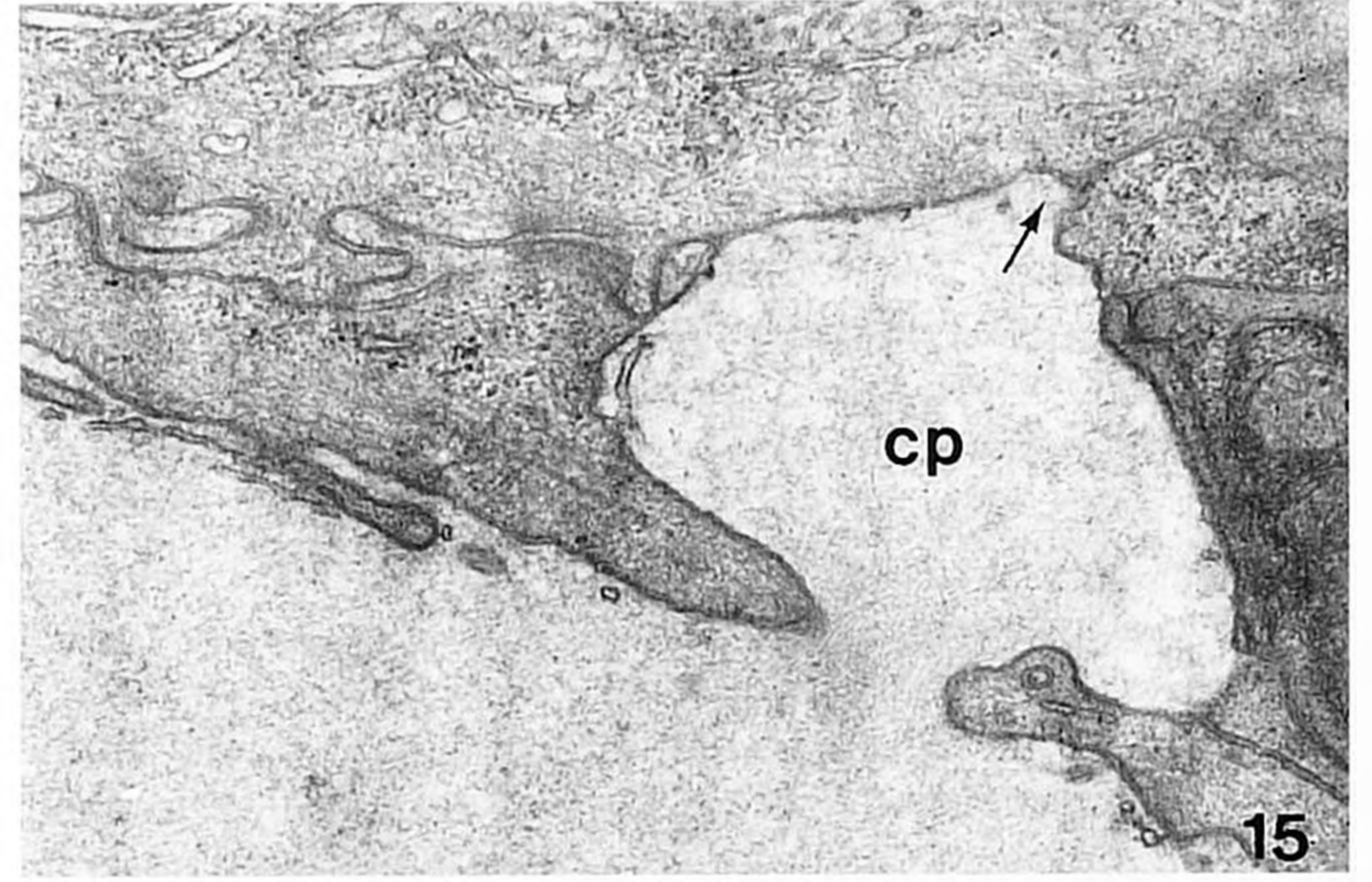
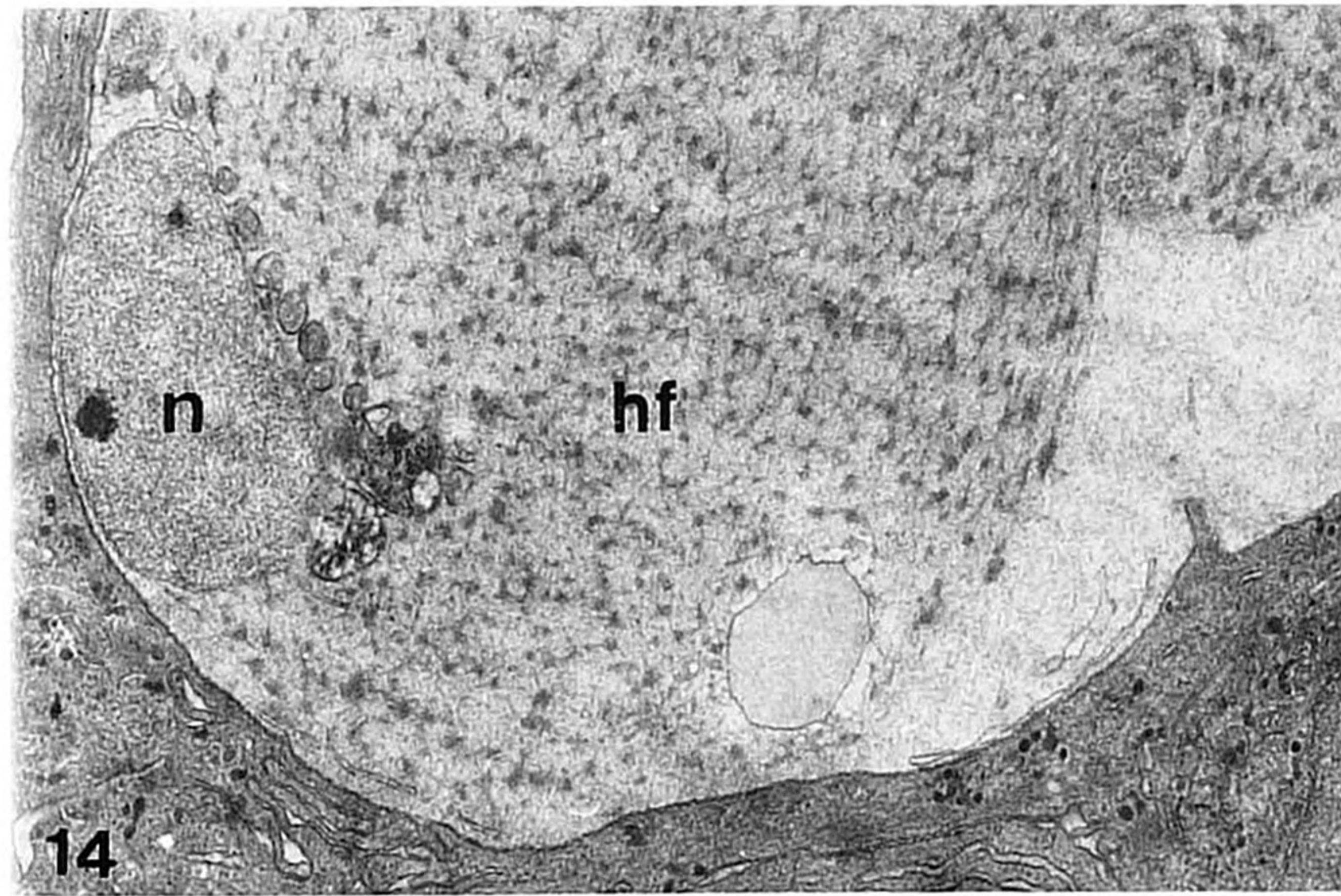
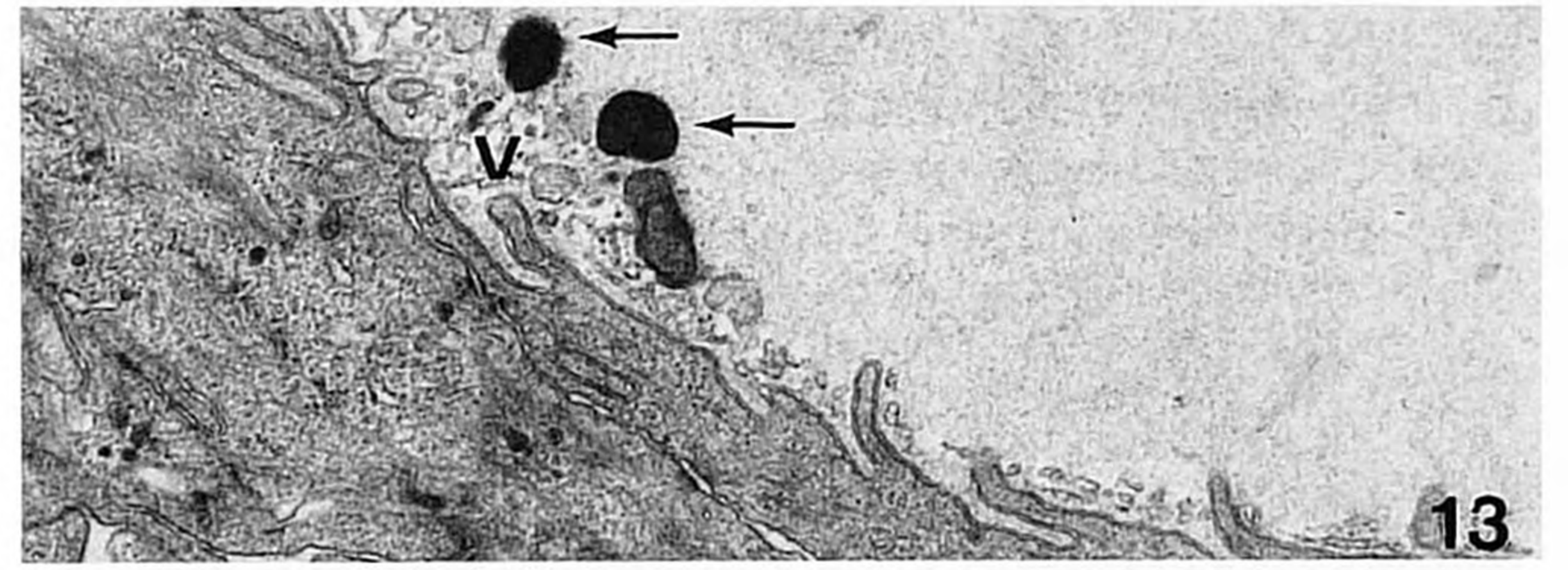
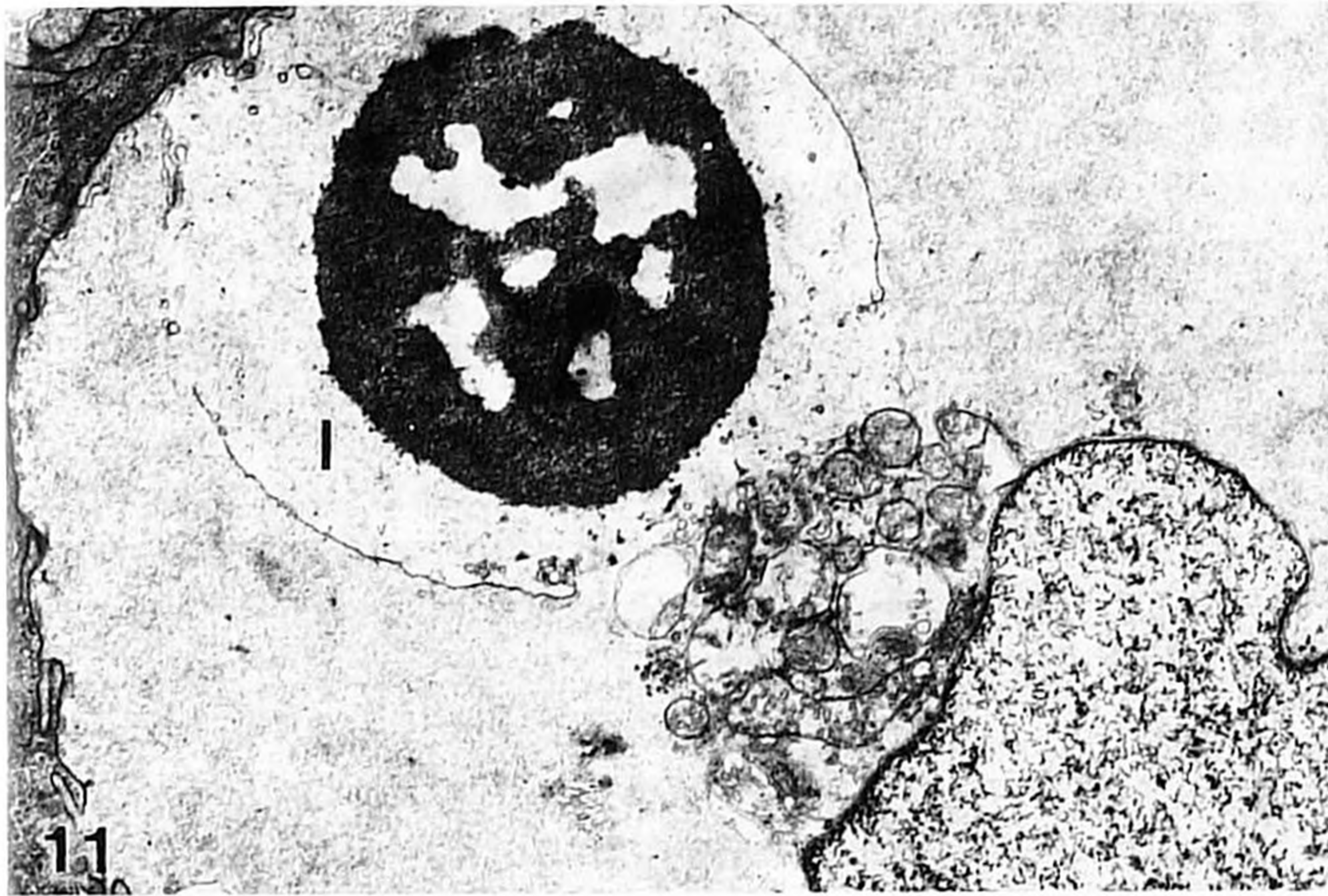
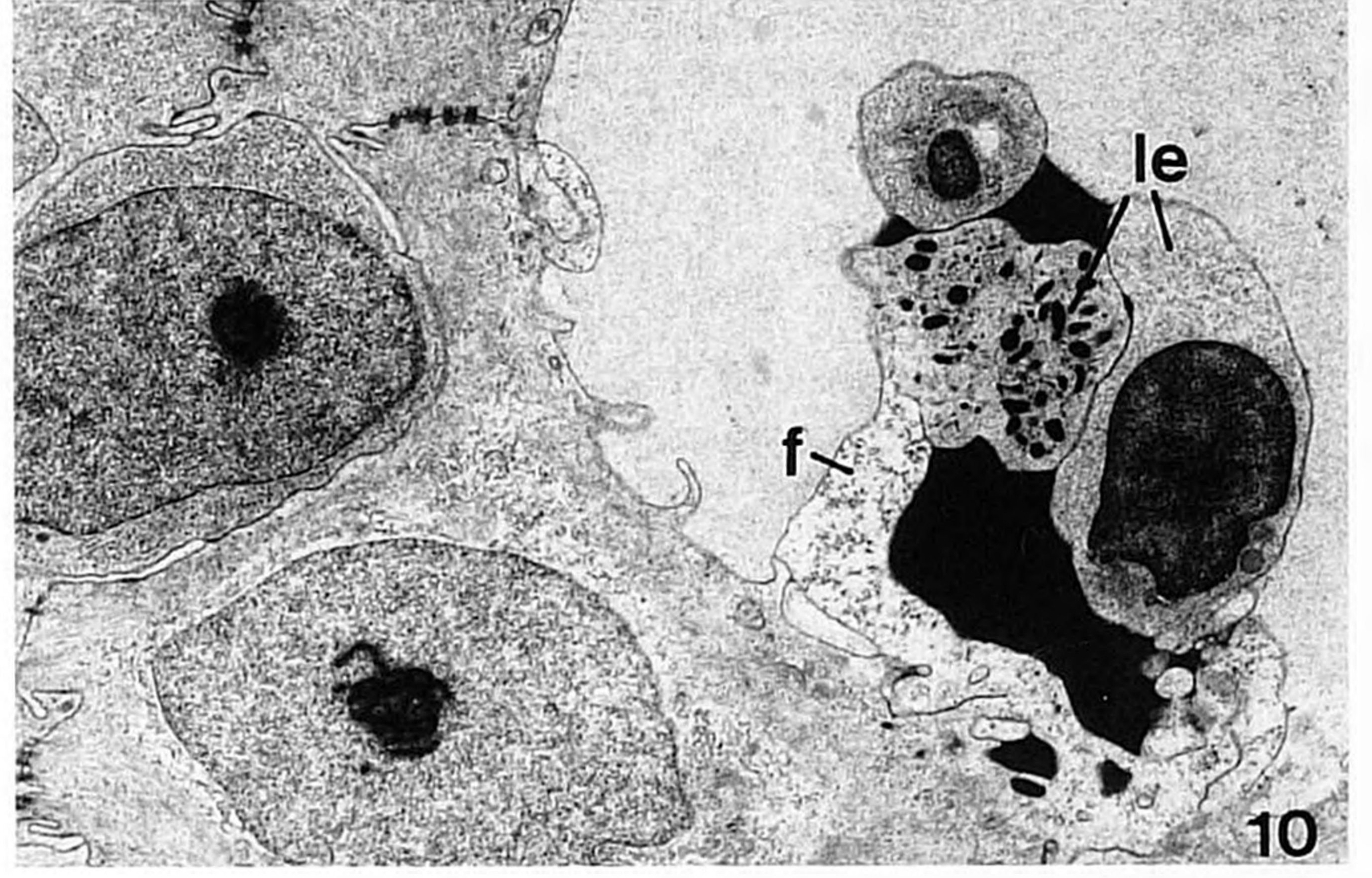
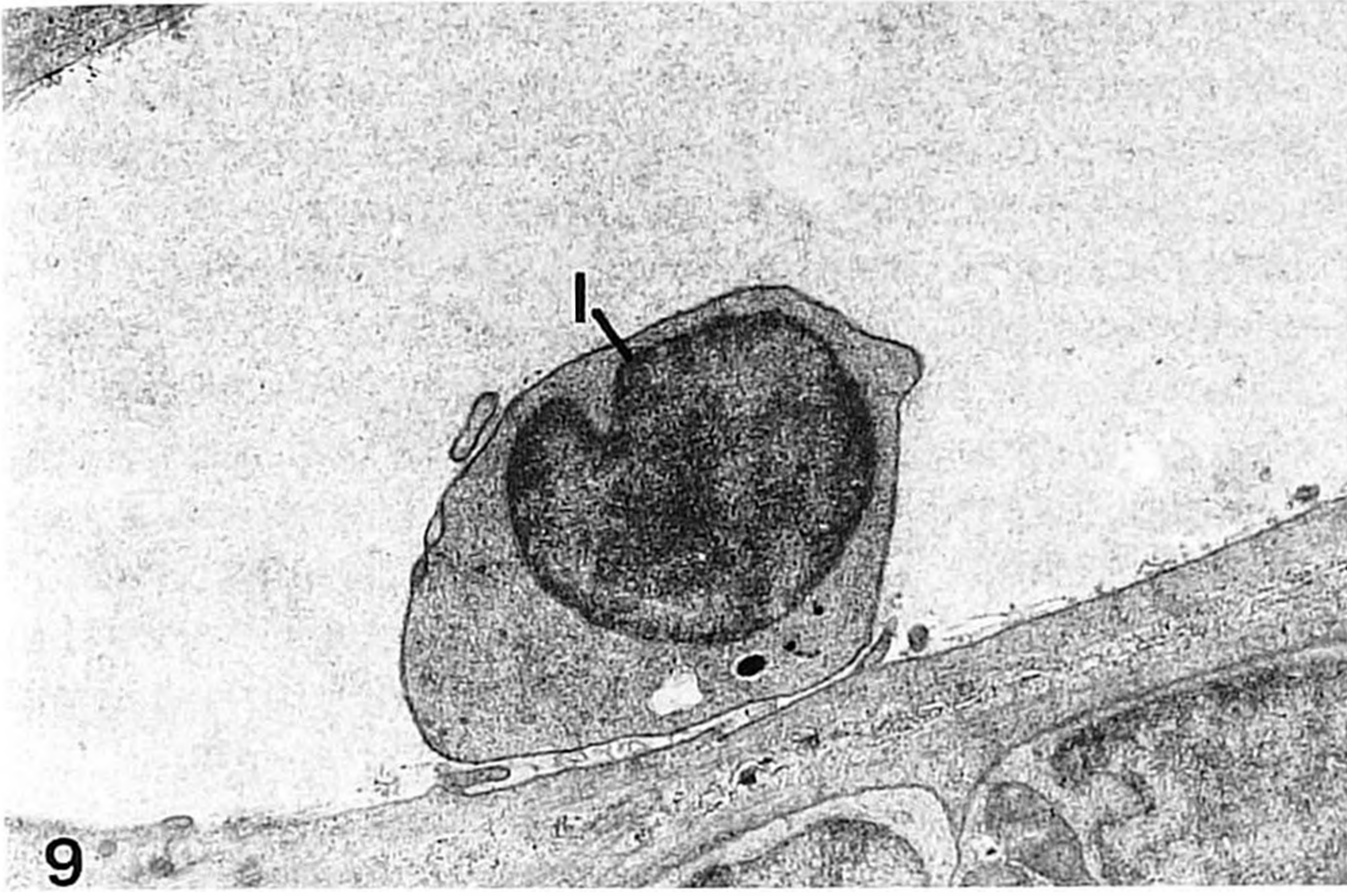
Fig. 13. Cadmium contaminated water ($22 \mu\text{g}\cdot\text{l}^{-1}$, 24 h). Granular (*arrows*) and vesicular remnants (*v*) located at the apical area of a club cell, close to the cell membrane. $\times 10500$

Fig. 14. Cadmium contaminated water ($0.5 \text{ mg}\cdot\text{l}^{-1}$, 24 h). Degenerated club cell with dense aggregates of helical filaments (*hf*). *n* Nucleus. $\times 8200$

Fig. 15. Acid water (pH 5, 24 h). Cytoplasmic process (*cp*) of a club cell seems to penetrate between filament cells (*arrow*, direction of skin surface). $\times 14450$

Fig. 16. Cadmium contaminated water ($22 \mu\text{g}\cdot\text{l}^{-1}$, 24 h). Process of a club cell (*arrow*) invaginates another club cell. $\times 6400$

Fig. 17. Cadmium contaminated water ($0.5 \text{ mg}\cdot\text{l}^{-1}$, 7 days). Bi-nucleated club cell. The 2 nuclei have different electron density and are both surrounded by central cytoplasm. $\times 5100$



spaces (Fig. 15). The interdigitations of these cells with filament cells disappear, and the cells apparently migrate into the direction of the skin surface. Phenomena indicating fusion of club cells are occasionally observed (Fig. 16). Club cells with 2 nuclei (Fig. 17), which are occasionally found, may arise by this process. Finally, the club cells are seen close to or at the surface, and apparently they leave the epidermis. Only activated club cells are seen at this position.

Discussion

Three new observations are reported in this study. First, the carp club cells respond to stressors with increased activity, as concluded from the increase in cell size and the extension of endoplasmic reticulum and Golgi areas. Second, mitotic and apoptotic activity of the filament cells is mainly restricted to cells in the proximity to the club cells and, third, the club cells are involved in the lysis of leucocytes, probably after phagocytosis of these cells.

The high mitotic activity of filament cells of stressed fish has been reported earlier (Iger 1992; Iger and Wendelaar Bonga 1993). The high incidence of apoptosis of filament cells in close proximity of the club cells is remarkable, because in control fish apoptosis is limited to the upper layer of filament cells, the pavement cells. One could argue that the presence of mitotic and apoptotic cells adjacent to club cells is a coincidence because these cells are all located in the same epithelial layers. However, although the density of club cells is high, their uneven distribution over the epithelium allows us to conclude that there is a specific relationship between club cells and filament cells. Another argument in favour of this conclusion is the observation that mitotic and apoptotic filament cells are not found near immature club cells. Thus, our observations indicate that club cells are able to influence the turnover rate of the filament cells, possibly via paracrine interactions. That the effect is restricted to the mature club cells is consistent with the conclusion of Whitear (1986) that the secretory product of club cells changes as the cells mature. The paracrine factor involved in the effect on filament cell turnover might be serotonin. This bioamine has been localised in the club cells of some teleost fishes (Zacccone et al. 1990). In the teleost epidermis, serotonin is also present in different types of paraneurons, one of which is the Merkel cell (Fujita et al. 1988). Interestingly, Budtz and Spies (1989) noticed that apoptotic cells in the epidermis of a toad are often closely associated with Merkel cells. Gould et al. (1985) concluded that Merkel cells contribute to epidermal growth and differentiation by functioning as a pacemaker of this tissue. Our results suggest that club cells rather than Merkel cells, which also occur in carp epidermis (Iger 1992), have such an effect in the carp epidermis. However, more than one factor may be implicated in the control of the filament cell cycle in carp. Iger and Abraham (1990) showed that club cells are the last of the epithelial cells to differentiate after wounding, indicating that the filament cell cycle is under control of more factors than the presumptive factor of the club cells.

The increased incidence of mitosis and apoptosis in filament cells of fish exposed to stressors, reflects a higher turnover rate of the cells under these conditions. Parsons et al. (1983) considered increased apoptosis in the hyperplastic mouse epidermis as an effect of crowding of the epidermal cells by stimulated basal cell proliferation. Crowding of epidermal cells may have taken place in the carp epidermis of stressed fish by increased mitosis, the massive infiltration of leucocytes, and the increase in size of the club cells. Progressive apoptosis usually results in the formation of one or more large apoptotic bodies that are phagocytosed by macrophages or neighbouring epithelial cells (Wyllie 1981). Many of the apoptotic filament cells around the club cells are not phagocytosed by macrophages. Their apoptotic remnants may be removed by neighbouring filament cells, as we observed phagosomes in some of these cells. This is in contrast with the elimination of apoptotic pavement cells, which are shed into the water (Wendelaar Bonga and Van der Meij 1989; Iger 1992). It is possible that the remnants of filament cells have nutritional value for the adjacent cells. Bowen (1981) has suggested that apoptotic remnants may be utilized by neighbouring cells.

In a previous study on the skin of carp (Iger and Abraham 1990), we showed that both filament cells and mucous cells are capable of phagocytosis. In the present study we not only confirm the phagocytic activity of filament cells, but we also present indications that the club cells have the capacity to take up and destroy leucocytes. To our knowledge, phagocytic activity of club cells has only been ascribed to the club cells of catfish (Lufty, 1966). In our fish, lysis of leucocytes was not observed under control conditions, but was restricted to fish exposed to the different stressors. Thus, our observations indicate that club cells are engaged in the stress response of fishes, in addition to the production of pheromones reported in the literature. Whether the leucocytes penetrate into the club cells or the club cells actively phagocytose these cells remains unclear. Phagocytosis is indicated by the presence of 2 membranes around some of the leucocytes inside club cells. Although filament cells and leucocytes both show an increased degree of degeneration in the vicinity of club cells, they show a striking difference. While the former mostly display apoptosis, the leucocytes become necrotic after they appear inside the club cells. The necrotic lysis of leucocytes that seems to be induced by the club cells resembles the action of killer cells (Henkart 1985): the cellular organelles of these cells are oriented towards the target cell, and lysis is associated with rupture of the outer cell membrane of the latter. The "amorphous material" observed by Whitear et al (1991 b) in the periphery of the club cells of catfish might also represent remnants of necrotic leucocytes. Whether the interaction of the club cells with the leucocytes is limited to the destruction of the latter or also involves regulation of club cell function, for instance by cytokines (Whitear and Mittal 1983), remains to be established. Occasionally necrosis of phagocytosed leucocytes is followed by degeneration of the club cells, possibly as a result of the release of proteolytic factors from the lytic leucocytes (Suzuki 1986). In normal conditions club cells

show no degeneration of organelles (Whitear and Mittal 1983). Most phagosome-containing club cells finally migrate, actively or through the pressure of neighbouring cells, to the surface and apparently leave the epidermis.

In conclusion then, the present results indicate that club cells have important functions in addition to the production and release of alarm substances: they influence or regulate the cell kinetics of filament cells and are engaged in the elimination of leucocytes. These functions become noticeable under the influence of different types of stressors.

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