ARTICLE I

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ELECTRON MICROSCOPY OF INDUCED HETEROTOPIC BONE FORMATION IN GUINEA PIGS

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Summary—A high yield of osteoid and some mineralized bone was induced in the abdominal muscles following implantation of allogenic demineralized and lyophilized dentine. The following types of cells and reactions were found: A. Resorptive reaction mediated by monocytes, macrophages and dentinoclasts. B. Fibroblastic reaction, as an unspecific capsulation process. C. Osteoblastic reaction with osteoid formation.

Dentinoclasts were observed before remineralization of the implanted dentine. Macrophages were active in the resorption of dentine, especially in the earliest stages. In dentinoclasts near remineralized dentine, crystals were observed in the ruffled border. Channels to the surface were found in osteoblasts, possibly originating from endoplasmic reticulum.

INTRODUCTION

The fine structure of bone and bone formation has been examined by many workers (Cameron, 1963; Scott, 1967; Gøthlin, 1973; Luk *et al.*, 1974; Lucht, 1972a), but ultrastructural studies do not appear to have been made on experimentally induced heterotopic bone. Using light microscopy, Bang (1972a,b,c, 1973a,b,c) showed the ability of demineralized dentine to induce heterotopic bone formation.

Two theories prevail regarding the origin and differentiation of osteoblasts and osteoclasts. Tonna (1960); Young (1963, 1964); Kember (1960); Mills *et al.* (1972); Rasmussen and Bordier (1974) and Hall (1975) proposed the hypothesis that one basic cell gives rise to both osteoblasts and osteoclasts. Scott (1967), using transmission electron microscopy (TEM), concluded that two separated cell populations differentiate either to osteoblasts or osteoclasts, a view confirmed by several workers (Gøthlin and Ericsson, 1973; Büring, 1975; Thyberg, Nilsson and Friberg, 1975).

My aim was to examine the ultrastructure of tissue changes taking place during the induction of heterotopic bone formation by dentine.

MATERIALS AND METHODS

Technical procedures

Ten randomly-bred male albino or pigmented guinea pigs, two to four months old, were divided into 5 groups of 2 animals and maintained on a mixed diet (Norwegian standard for guinea pigs and rabbits, Statens Institutt for Folkehelse, Oslo, Norway) supplemented with swedes and hay with tap water *ad libitum*. The animals were weighed before the operative procedure and again before being killed. The implanted material was allogenic dentine prepared from freshly extracted guinea pig molars. The teeth were demineralized at 4°C using 0.2 M HCl, lyophilized and sterilized in ethylene oxide gas for 3 h. All implants were stored in sterile glass vials at 4°C for a maximum of one week.

The animals were anaesthetized by intraperitoneal injection of 40 mg/kg of Nembutal® (sodium pentobarbital 50 mg/ml). Each animal received 4 implants in the abdominal muscles. A detailed description of the treatment of the implants and the operative procedure has been given by Bang (1973b). At the end of each experimental period, i.e. after 7, 10, 14, 16 and 22 days, one group of two animals was killed by an overdose of Nembutal® and the implants were dissected free with some of the surrounding soft tissue and immediately placed in 3 per cent purified glutaraldehyde buffered with 0.1 M cacodylate buffer (300 mosmol) at 4°C, divided into small pieces (1 × 1 mm) and stored overnight. After being washed thoroughly in cacodylate buffer, post-fixation was performed at 4°C in 1 per cent cacodylate buffered OsO_4 for 2 h. The specimens were then dehydrated in alcohol solutions of increasing concentration and, after overnight infiltration, in a 1:1 mixture of Epon and propylenoxide and embedded in Epon 812 (epoxy resin with a ratio of A:B solution of 7:3).

Sections (1 μm thick) cut on a LKB pyramitome using glass knives were stained with toluidine blue. Various areas of dentine and surrounding tissue were then prepared for thin sections, cut at about 70 nm (700 Å) on a Reichert OM2 ultramicrotome or a LKB Ultratome with glass or diamond knives and stained with lead citrate and alkaline uranyl acetate after being mounted on formvar- and carbon-coated 100-mesh copper grids. The sections were studied in a Philips 300 electron microscope at 80 kV.

Classification of cells

The cells involved in the reaction were grouped as follows: A-cells: cells associated with matrix production, including osteoblasts, chondroblasts, fibroblasts and mature osteocytes, chondrocytes and fibrocytes, and their precursors (Cameron, 1963; Scott, 1967; Gøthlin, 1973; Luk *et al.*, 1974). The osteocytes included those within osteoid (Dudley and Spiro, 1961) and not only cells surrounded by mineralized collagen. The matrix near these cells contain vesicles classified by Anderson (1969) as vesicles I and II.

Type I resemble intracellular dense bodies and type II are like cytoplasmic extensions. B-cells: cells with matrix-resorbing properties and phagocytic activity, including monocytes, macrophages and multinucleated matrix-resorbing giant cells such as dentinoclasts and osteoclasts (Scott, 1967; Furseth, 1968; Fedorko and Hirsch, 1970; Gøthlin, 1973; Lucht, 1972a,b). C-cells: endothelial cells and inflammatory cells (Schroeder and Munzel-Pedrazzoli, 1973).

RESULTS

Except for one which died during the operative procedure, all the guinea pigs gained weight during the experimental period and showed only a slight local reaction to the implants.

With light microscopy, the implanted dentine was found to induce heterotopic osteoid formation after 14 days, but with TEM osteoid was seen at 10 days. The amount of osteoid increased with time but mineralization occurred only in a few areas even after 22 days. After 7 days, monocytes and macrophages, and after 14 days, multinucleated giant cells (dentinoclasts) were resorbing dentine. Remineralization of the implanted dentine was first noticed at 14 days but remained sparse throughout the experimental period.

Matrix-producing cells (A-cells)

Three subgroups of the A-cells were observed: perivascular cells not evidently associated with matrix production, matrix-producing cells without the characteristics of mature osteoblasts, and highly differentiated matrix-producing cells (osteoblasts). At seven days, the most frequent A-cells were near capillaries and were immature, round and spindle-shaped cells with a high nuclear-cytoplasmic ratio, few organelles, only a few flattened lamellae of the rough endoplasmic reticulum (RER) and mitrochondria.

The more mature A-cells had a well-developed RER and collagen production was well established. No intracellular collagen fibres were observed. These A-cells varied from elongated cells with an appearance like fibroblasts to more cuboidal cells with a cross-section of 15–25 μm, resembling osteoblasts. After 10 days, they were observed along the dentine surface and in the vicinity of osteoid.

The fibroblasts were elongated cells with abundant RER, close contact to collagen and a well-developed Golgi apparatus. In areas with such a fibroblastic reaction, osteoblasts were absent and the dentine surface was not so irregular and eroded as in areas of osteoblastic reaction. In some places, however, cell extensions from macrophages were seen between the fibroblasts and the dentine. The highly differentiated matrix-producing cells had the appearance of osteoblasts (Fig. 1), and only seldom of chondroblasts. Osteoblasts were first seen after 10 days and predominantly close to the dentine (Fig. 1); later they were in the outer layer of osteoid. Their nuclei were eccentrically situated and often with 1–2 nucleoli. The Golgi apparatus was well developed and the pole opposite to the nucleus was filled with RER. The cell membrane was distinct with cell projections mainly on the dentinal side of the cell. An organelle-free zone of 0.5–1.0 μm closely packed with microfilaments with a diameter of 5–7 nm was often seen. The microfilaments extended into the cellular projections.

In the Golgi area, there were many vacuoles measuring 50–300 nm and limited by a triple-layered membrane about 9 nm thick. Most contained an amorphous, homogeneous material but some appeared empty. Dense bodies, 0.1–1.0 μm in diameter, seemingly budding from the Golgi apparatus, were present in constant numbers (3–4 per cell). Filamentous material was occasionally seen in some larger Golgi vacuoles (Fig. 2).

The osteoblasts showed close intercellular contacts interpreted as tight junctions. In some of the osteoblasts and osteocytes, channels to the surface were observed (Fig. 3). The cells embedded in collagen, the osteoid osteocytes, usually resembled osteoblasts (Fig. 4). In some deeply seated cells within mineralized matrix, however, the cytoplasm was more condensed and contained myelin bodies, dense bodies and fissured nuclei, indicating degenerative changes. Cilia and centrioles were seen occasionally (Figs. 4 and 5) in osteoblasts and osteoid osteocytes.

In the collagen matrix surrounding the osteoblasts and osteoid osteocytes, two types of membrane-bound vesicles ranging from 40–100 nm were observed. The contents of the matrix vesicles of type I resembled intracellular dense bodies (Fig. 6), but those of type II vesicles resembled cytoplasmic extensions and sometimes contained ribosomes (Fig. 7). In contrast to the small number of type I vesicles, type II vesicles were common in the osteoid. The few chondroid areas contained more matrix vesicles I.

The collagen had a periodic banding of 60–65 nm and a diameter about half that of the implanted dentine collagen (Fig. 1).

Matrix-resorbing cells (B-cells)

These cells varied from immature monocytes (Fig. 8) to highly differentiated dentinoclasts (Fig. 9). The immature cells were regularly found near capillary sprouts and had an eccentric nucleus with abundant condensed chromatin along the nuclear membrane. In general, the B-cells were a little darker than A-cells, and the cytoplasm of macrophages contained more dense bodies and mitochondria than the monocytes and had a higher nuclear:cytoplasmic ratio. In complex macrophages, the Golgi region was larger, and the cell membrane more irregular. The RER was sparse but free ribosomes and polysomes were abundant in the cytoplasm, in contrast to A-cells. Macrophages containing erythrocytes were frequent. At 7 days, monocytes dominated but at later stages macrophages increased in number and multinucleated giant cells appeared after 14 days. In the vicinity of monocytes and macrophages, splitting of dentine collagen seemed to have occurred (Fig. 8).

At 14 days after implantation and later, multinucleated dentinoclasts were found close to the dentine surface (Fig. 9). Near the dentine, the cells showed finger-like projections in an organelle-free border 2–3 μm thick (Fig. 9). Next to this ruffled border, there was a zone rich in mitochondria, membrane-bounded vacuoles and dense bodies. Well-developed Golgi apparatus was close to the nuclei, consisting of four to six parallel cisternae with many small

vacuoles and dense bodies. Near the ruffled border were larger vacuoles, up to 2 nm in diameter, some coalescing with dense bodies. In sections with remineralized dentine, some vacuoles as well as the channels of the ruffled border apparently contained crystals (Figs. 9 and 10). No collagen could be seen in the cytoplasmic vacuoles. In the invaginations, however, collagen with cross-striation (Fig. 11) was observed, but only in areas close to unremineralized dentine. Diffusely outlined dense bodies, 60–100 nm across (Fig. 10) were sometimes observed in dentinoclasts next to remineralized dentine. In the cytoplasm of these giant cells, there were many free ribosomes, often as polysomes, and many mitochondria closely related to the lamellae of RER. The ruffled border was only observed at the side facing the dentine collagen. The other side was much more smooth surfaced and showed pinocytosis. The ruffled border only seldom exhibited a clear zone sealing off the periphery of the cell. The B-cells, like the A-cells, contained no intramitochondrial granules.

Intimate contact between osteoblasts and macrophages, as well as between less differentiated A and B cells was found regularly (Fig. 1).

Other cells (C-cells)

Endothelial cells of sprouting capillaries and occasional lymphocytes and neutrophilic granulocytes were noted close to the dentine.

DISCUSSION

The observations were limited to the interval between 7–22 days. Before this time, the tissue reaction consisted mostly of a mild inflammatory reaction and, after three weeks, the intensity of the cellular reaction in connection with the implanted dentine seems to have reached its maximum (Bang, 1973c).

The results are in keeping with the theory of Scott (1967) that one cell-line is involved in matrix production and another in resorption of bone. Other workers have made similar observations in fracture healing (Gøthlin and Ericsson, 1973), induction of heterotopic bone (Büring, 1975) and metaphyseal bone (Thyberg *et al.*, 1975). Rasmussen and Bordier (1974) disagree with this view and stress origin from one common osteoprogenitor cell.

Frost (1965) and Rasmussen and Bordier (1974) suggested that osteoblasts develop from dentinoclasts, but in my study osteoblasts were observed prior to dentinoclasts.

Monocytes and macrophages as well as dentinoclasts were active in dentine resorption. Furth (1972) considered the two former to be part of a mononuclear phagocytic system and not granulocytes as Scott (1967) proposed. Resorption of bone and cartilage by macrophages has been reported by several other authors (Schenk, Spiro and Wiener, 1967; Irving and Bond, 1968; Thyberg *et al.*, 1975). Dentinoclasts were active in the resorption process and not only as cells in a second line of resorption as proposed by Horn, Dvorak and Szarka (1975).

Collagen of unmineralized dentine was found in invaginations of macrophages and sometimes the dentine near these cells seemed to be split. Only rarely was collagen observed intracellularly. The mechanism of collagen resorption could be explained by the excretion of hydrolytic enzymes, instead of engulfment and subsequent degradation of collagen as demonstrated in collagen resorption in hair follicles (Parakkal, 1969). The monocytes and the macrophages are thus important, not only as pre-dentinoclasts, but as active dentine-resorbing cells.

The structure of dentinoclasts was similar to that of osteoclasts (Cameron, 1963; Lucht, 1972a), odontoclasts (Furseth, 1968) and chondroclasts (Cameron, 1963; Thyberg *et al.*, 1975). Dentinoclasts resorbed unmineralized as well as mineralized dentine. According to Dudley and Spiro (1961), Schenk *et al.* (1967) and Thyberg *et al.* (1975), however, only osteoclasts and chondroclasts resorb mineralized tissue.

The monocytic cells dominated the resorption at the early stages although later the dentinoclasts increased. This supports Urago *et al.* (1975) who suggested that osteoclasts appear predominantly on the bone surface in the rapid bone resorption seen in growth and the active phase of repair and inflammation.

When dentinoclasts resorbed remineralized dentine, crystals were observed in invaginations and in vacuoles near their ruffled border. Schenk *et al.* (1967) suggested that these crystals are artifacts due to the phosphate buffer, but I used cacodylate buffer. Few remineralized areas were seen and the absence of intramitochondrial granules in the dentinoclasts may thus be due to the low mineral gradient. Such granules have been found regularly in mineralized bone (Martin and Matthews, 1969; Gøthlin, 1973; Holtrop, 1975). The lack of a high mineral gradient is also a possible explanation for the absence of a clear sealing zone at the periphery of the ruffled border. Schenk (1974) stressed the importance of this zone in producing a closed acid micromilieu. The diffusely outlined dense bodies in the ruffled border of dentinoclasts appear not to have been reported before. Their nature is obscure and artifact cannot be excluded.

The osteoblasts were characterized by an extensive RER with dilated cisternae. Cameron (1963) and Rohr (1965) consider that this, together with the well-developed Golgi apparatus, indicates active protein synthesis and not poor fixation. The fact that these dilatations are more numerous in osteoblasts than in less developed A-cells supports this view. The small amount of glycogen in the osteoblasts or less developed A-cells accords with the findings of Thyberg *et al.* (1975) rather than those of Scott and Glimcher (1971). This relative lack of glycogen may be explained by the high activity of the osteoblasts Bonucci (1965).

Exocytosis has been proposed as the main mode of releasing collagen precursors (Sheldon and Kimball, 1962; Weinstock and Leblond, 1971). Rohr (1965) thought that only a small part of the tropocollagen was secreted via the Golgi vacuoles. In the periphery of the mature A-cells, microfilaments with a diameter of 5–7 nm were observed in a band-like arrangement extending out into cell projections. This is in accord with the findings of Hancox and Boothroyd (1965) and Stanka (1975). Stanka proposed that these filaments play a role in the growth and differentiation of bone, although Chapman (1962) thought

them to be collagen precursors. Recently, King and Holtrop (1975) used a heavy merocycin which binds actin specifically to demonstrate that the peripheral filaments were actin-like.

Some cells exhibited empty channels to the surface, possibly originating from the endoplasmic reticulum. Sheldon and Robinson (1961) reported similar communications in bone cells, possibility originating from dilated cisternae of RER, but Thyberg *et al.* (1975) could not find such channels in cartilage cells. Tonna and Lampen (1972) proposed a connection between centrioles and cilia like I have described. Federman and Nichols (1974) suggested that the cilia, by moving fluid, have a function in the calcium homeostasis.

The number of extracellular vesicles, mainly of type II, was highest in the area of bone formation. Alkaline phosphatase, demonstrated in the membrane of these vesicles by Thyberg *et al.* (1975) may help to initiate the mineralization of cartilage and bone (Anderson and Reynolds, 1973; Schenk, 1974) or may be simply concerned with collagen production (Hancox and Boothroyd, 1965). In contrast to the findings of Anderson (1969), free ribosomes were often present in the matrix of type II vesicles. The nature of these vesicles is not clear but they may be part of cell extensions. In cartilage, however, Schenk (1969) claimed that they were discrete vesicles. Matrix vesicles of type I were sparse where osteoid was being found. Thyberg *et al.* (1975) thought that this type of vesicle in cartilage represented extruded dense bodies as a lysosomal secretion.

Bang (1972a) showed that the implanted dentine has an antigenic effect. Many authors have stressed the importance of macrophages in mediation of the immune stimulus and in handling of the antigens (Fishman and Adler, 1967; Craddock, Longmire and McMillan, 1971a,b; LoBuglio, 1973; Unanae, 1975).

The importance of resorbing cells in the heterotopic bone formation process may explain the reduction of woven bone formation produced by indomethacin (Sudman, 1975), a potent prostaglandin inhibitor (Ferreira and Vane, 1974); Harris *et al.*, (1973) showed that prostaglandins stimulate bone resorption.

Only a few macrophages were found in the fibroblastic area and the dentinal surface was rarely eroded. The lack of resorption may be one factor leading to fibroblast proliferation instead of osteoblasts. Lindén (1975) proposed that both remineralization and surface erosion was a prerequisite for initiation of bone induction, while the work of Bang (1972b, 1973c) and the present results indicate that resorption of unremineralized dentine is the essential factor for osteoid formation.

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Plates 1–3 overleaf

Plate 1.

Fig. 1. 16 days. Osteoblast with close contact with a macrophage (m). Collagen of implanted dentine in the upper part (d), with contiguous newly formed collagen (coll.). Note the difference in thickness of the two collagens. × 122,220

Fig. 2. 16 days. Osteoblast. Golgi region with vacuoles containing microfilaments (arrows). Extracellular collagen (coll.). × 40,500

Fig. 3. 16 days. Osteoid osteocytes with a channel to the surface (arrow). × 31,280

Plate 2.

Fig. 4. 16 days, Osteoid osteocyte. Arrow indicates centriole in Golgi region. × 11,570

Fig. 5. 14 days. Part of osteoblast with a cilium. × 31,900

Fig. 6. 22 days. Part of chondroblast with cell projections, matrix vesicles I (arrow) and matrix vesicles II (arrowhead). × 29,215

Fig. 7. 16 days. Osteoblast, (obl.). with matrix vesicles II with free ribosomes (arrow). × 48,750

Plate 3.

Fig. 8. 7 days. Macrophage close to dentine (d). Apparent splitting of collagen near the cell (arrow). × 10,880

Fig. 9. 16 days. Part of dentinoclast in area with remineralized dentine (d). Ruffled border (rb) with invaginations containing crystals (arrow). × 7575

Fig. 10. 16 days. Part of ruffled border of dentinoclast in area with remineralized dentine. Crystals in the invaginations (arrow) and diffusely limited, dense bodies in the organelle-free area of the cell (arrowhead). × 9430

Fig. 11. 22 days. Part of dentinoclast with collagen (coll.) from the dentine in invaginations. × 34,800

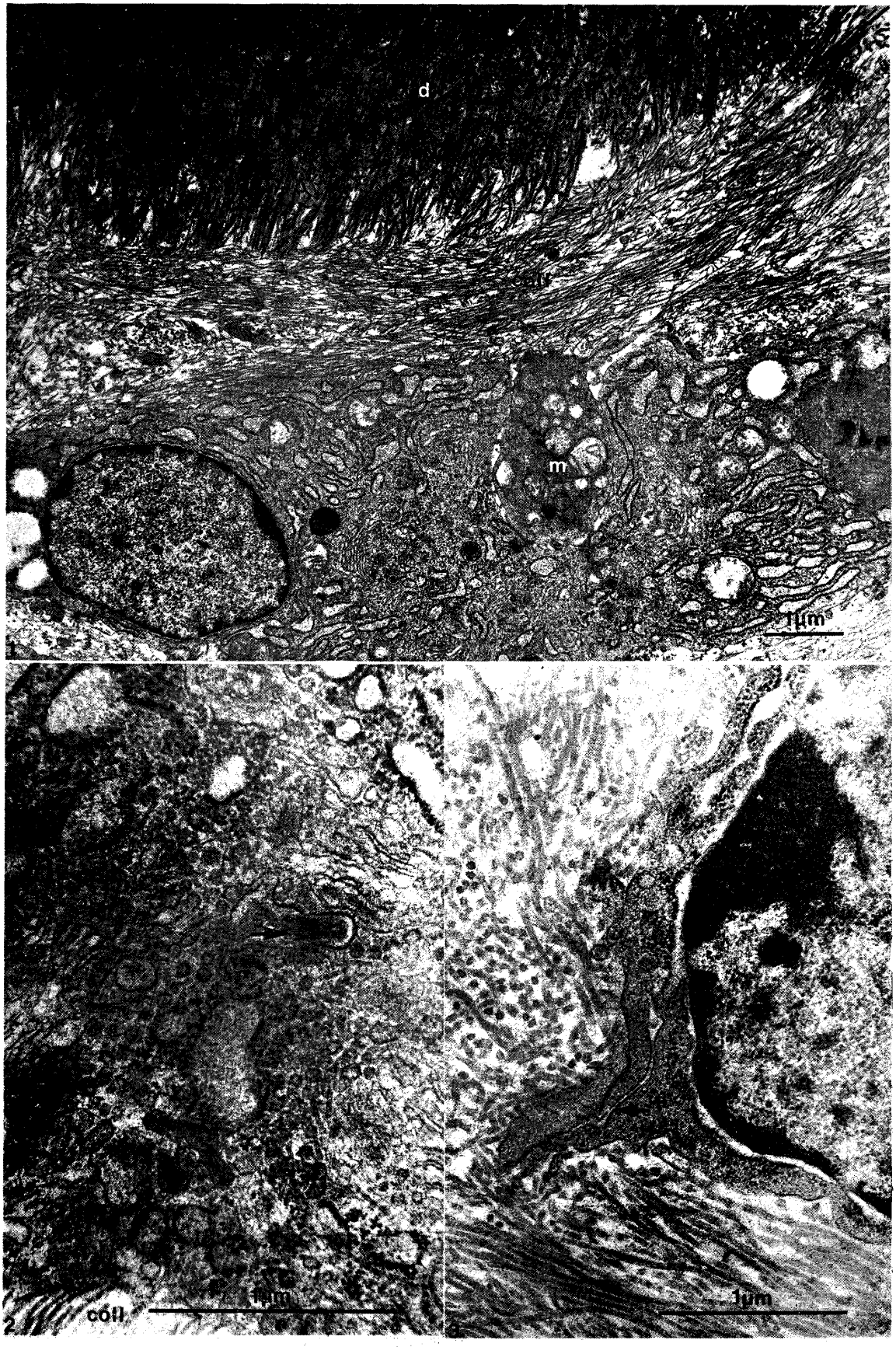

Plate 1.

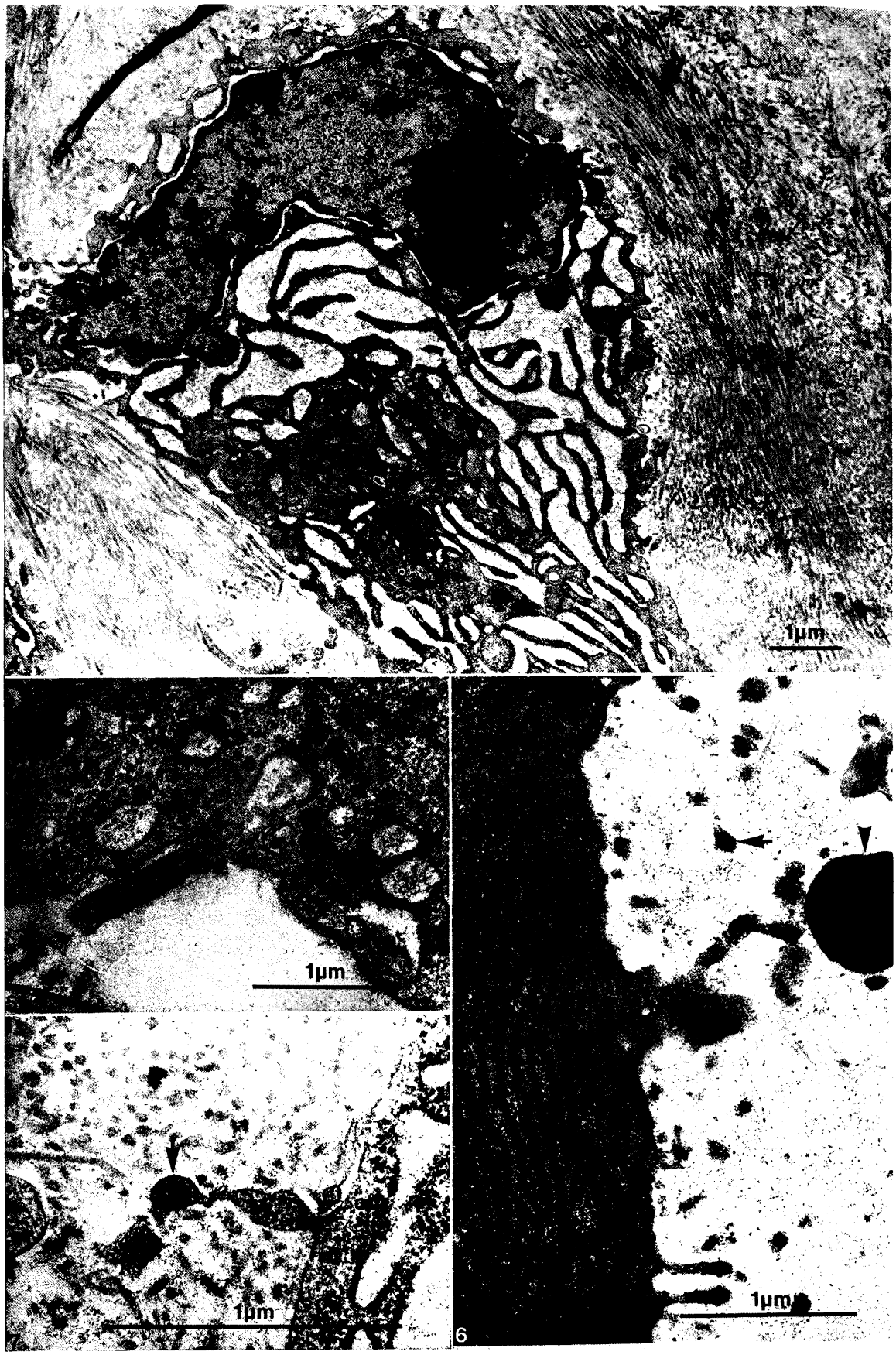

Plate 2.

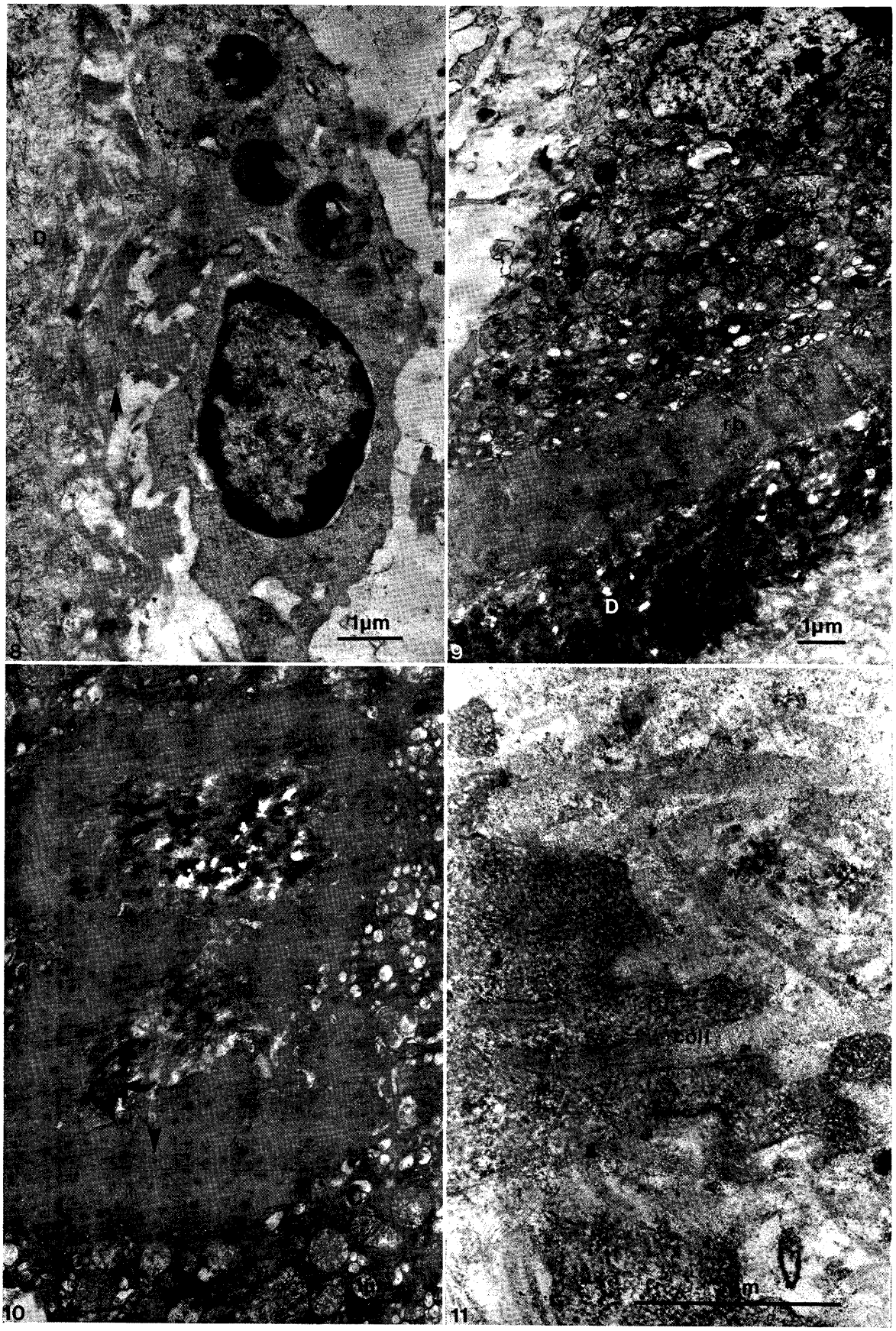

Plate 3.