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AN ELECTRON-MICROSCOPE STUDY OF CELL DELETION IN THE ANURAN TADPOLE TAIL DURING SPONTANEOUS METAMORPHOSIS WITH SPECIAL REFERENCE TO APOPTOSIS OF STRIATED MUSCLE FIBRES

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SUMMARY

The mechanism of cell deletion responsible for involution of the anuran tadpole tail during spontaneous metamorphosis was studied by light and electron microscopy, attention being focused on epidermis and striated muscle.

The earliest indication of pending dissolution of epidermal cells was found to be aggregation of condensed chromatin beneath the nuclear envelope. This is followed by breaking up of the nucleus, and cytoplasmic condensation and budding with the production of a number of compact, membrane-bounded cell fragments with relatively well preserved organelles. These are then ingested and degraded by nearby viable cells, the majority by distinctive macrophage-like cells, which are scattered throughout the epidermis, and a few by epithelial cells. The morphological changes observed in the dying epidermal cells are the same as those described both in the 'programmed cell death' that plays an important role in the normal development of vertebrate embryos and in the type of cell death that has been shown to be involved in regulating the size of tissues in adult mammals under normal as well as pathological conditions; it has been suggested elsewhere that apoptosis might be a suitable name for the phenomenon.

Deletion of striated muscle fibres in the tadpole tail is accomplished by a process that appears to be a modification of classical apoptosis, in which dilatation and confluence of elements of the sarcoplasmic reticulum lead to internal fragmentation, the usual surface budding presumably being precluded by the large volume and specialized structure of these cells. The early and late nuclear changes, and the apparent ultrastructural integrity of organelles in the membrane-bounded muscle fragments are typical of apoptosis, and subsequent degradation within macrophages follows the standard stereotyped pattern. An essentially similar process has been described by others in the muscles of metamorphosing insect larvae, but whether striated muscle cells in adult higher vertebrates can undergo apoptosis is still uncertain.

INTRODUCTION

The rapid involution of the anuran tadpole tail that takes place during metamorphosis provides a striking example of decrease in the size of vertebrate tissues under normal conditions. Whilst a number of studies have attested to involvement of lysosomal hydrolases in the regressive process (for review, see Weber, 1969), it seems likely that these enzymes are concerned, not with initiation of degeneration of the cells to be eliminated, but with digestion of their remnants within macrophages (Weber, 1963): in fact, there appears to have been relatively little detailed investigation of changes in the doomed cells prior to their engulfment.

The role of focal, precisely controlled, and often massive cell death in the fashioning of organs and limbs during the embryonic development of vertebrates has long been appreciated (Glücksmann, 1951; Saunders, 1966; Ballard & Holt, 1968; Menkes, Sandor & Ilies, 1970). Electron microscopy of various species (Bellairs, 1961; Saunders & Fallon, 1966; Farbman, 1968; Manasek, 1969; Webster & Gross, 1970; Hammar & Mottet, 1971; Mottet & Hammar, 1972) has disclosed a constant pattern of morphological change in the dying cells, characterized by gross condensation of cytoplasm and nuclear chromatin; the appearances are fundamentally different from those observed in coagulative necrosis (Trump, Goldblatt & Stowell, 1965*a*, *b*; Trump & Ginn, 1969), which is found under pathological conditions in tissues damaged by noxious stimuli. It has recently become clear, however, that the distinctive type of cell death that is so conspicuous at certain stages of normal vertebrate ontogeny also accounts for continuous, albeit less massive, dropout of cells in the tissues of mature animals (Kerr, Wyllie & Currie, 1972), and, in at least some of the organs of adult mammals, its occurrence is augmented and inhibited by hormones (Kerr *et al.* 1972; Kerr & Searle, 1973; Wyllie, Kerr, Macaskill & Currie, 1973). Indeed, it now appears probable that it constitutes a general mechanism of controlled cell deletion, which complements mitosis in regulating the size of vertebrate tissues and organs throughout life: the term apoptosis has been suggested to highlight this role in cell population kinetics (Kerr *et al.* 1972).

The present paper reports a light- and electron-microscope study of the mechanism of cell deletion in the anuran tadpole tail during spontaneous metamorphosis, attention being focused on epidermis and striated muscle. It was found that epidermal epithelial cells undergo ultrastructurally typical apoptosis, but that the process in striated muscle fibres is modified by the large size and specialized structure of these cells.

MATERIALS AND METHODS

Tadpoles of the dwarf tree frog, *Litoria glauerti*, were chosen for study because of the relatively small amount of pigment in their tails: it was found that abundant pigment granules present in other locally-available species tended to obscure some ultrastructural details. The tadpoles were collected from their natural habitat and kept at summer room temperatures (25–35 °C) in a large water-filled glass tank containing water-plants. Tissue from the distal half of their tails was processed for light and electron microscopy when they reached the stage of metamorphosis characterized by advanced forelimb development and regression of the tail to about half its original dimensions; the length of the tail at this time was approximately equal to that of each extended hind limb.

For light-microscope studies, 10 tadpoles were killed by decapitation, and transverse slices of their tails were fixed in 4% formaldehyde solution. Paraffin sections of this tissue were stained with haematoxylin and eosin. For electron microscopy, it was found that preliminary perfusion of the blood vascular system of intact animals with glutaraldehyde solution and subsequent immersion fixation of small blocks resulted in better preservation of the soft connective tissues than did dicing of fresh tissue in fixative. Each of 10 tadpoles was narcotized, the heart exposed and the ventricle pierced with the tip of a fine pipette, and the vascular system gently perfused for 4 min with ice-cold 5% cacodylate-buffered glutaraldehyde solution (pH 7·2). This procedure was followed by appreciable hardening of the tail, the distal half of which was then amputated, cut into small cubes and fixed for 2 h in the same solution. The blocks were postfixed for 90 min in 1% cacodylate-buffered osmium tetroxide solution, stained for 30 min in 5% uranyl acetate solution, dehydrated in ethanol, and embedded in Epon 812. Thin sections

were cut, stained with lead citrate, and examined in a Hitachi HS7S electron microscope. One-micrometre sections were also cut from some blocks, stained with toluidine blue, and examined with a light microscope.

RESULTS

Examination of histological sections of the epidermis covering both the main body of the tail and the tail fins disclosed many clearly delineated spherical and ovoid cytoplasmic fragments scattered amongst the intact epithelial cells. They vary considerably in size, and often contain one or more pyknotic remnants of nuclei. Their precise topographical relationship to surrounding viable cells was usually difficult to determine with the light microscope. These structures are identical in appearance with the dying cells observed in normal embryos (Glücksmann, 1951); their development constitutes the cardinal histological feature of apoptosis (Kerr *et al.* 1972), and it is convenient to refer to them as apoptotic bodies.

Electron microscopy revealed 2 distinctly different cell types in the epidermis. Epithelial cells, which comprise the bulk of the population, are characterized by the presence of desmosomes, cytoplasmic tonofibrils, and numerous microvilli (Figs. 1, 3, 5); mucous vesicles, similar to those illustrated by Michaels, Albright & Patt (1971), were seen in surface cells. Cells of the second type are only sparsely distributed amongst the epithelial cells, and they never extend to the external surface. Their cytoplasm is rather less densely stained than that of the epithelial cells (Figs. 1, 4), they do not contain tonofibrils, and desmosomes were not detected at their points of contact with adjoining cells. Since these latter cells appear to be largely responsible for the phagocytosis and degradation of apoptotic bodies in the epidermis (Figs. 1, 2, 4), they will be called macrophage-like cells.

The earliest indications of apoptosis of epithelial cells are aggregation of chromatin near the nuclear membrane and cytoplasmic condensation, the appearances being the same as those described in the regressing external gills of *Rana pipiens* (fig. 1 in Michaels *et al.* 1971), in vertebrate embryos (Bellairs, 1961; Manasek, 1969), and in a variety of adult mammalian tissues (Kerr, 1971; Helminen & Ericsson, 1972; Kerr *et al.* 1972; Kerr & Searle, 1973). The nuclei then fragment, and the condensing cells bud to produce extremely compact apoptotic bodies of various sizes, which are probably rapidly phagocytosed, for they were infrequently observed in the extracellular space (Fig. 1). Though the majority are taken up by macrophage-like cells (Figs. 1, 2, 4), a few are ingested by epithelial cells (Fig. 3). Well preserved organelles and nuclear fragments can still be identified in recently phagocytosed bodies (Figs. 1, 2), but progressive degradation then ensues (Figs. 1, 3, 4), and they are soon reduced to lysosomal residual bodies (Fig. 4). Some of the macrophage-like cells were found to be packed with such telolysosomes (Fig. 5), whereas organelles with the ultrastructural features of secondary lysosomes are relatively uncommon in epithelial cells (Fig. 5).

The earliest histological change indicative of dissolution of muscle fibres is the occurrence of longitudinal clefts between myofibrils, best seen in toluidine blue-stained sections of Epon-embedded tissue. This is followed by fragmentation of the cytoplasm into a number of so-called sarcolytes with well preserved cross-striations, the

appearances being the same as those figured by Weber (1969). The clusters of sarcolytes are then invaded by macrophages, and their phagocytosis is succeeded by progressive loss of their organized structure. At a late stage of the process, the cylindrical space previously occupied by each fibre was seen to be filled with residual body-laden macrophages, in which myofibrils could no longer be identified.

In electron micrographs, the nuclei of striated muscle fibres that display cytoplasmic changes interpreted as being indicative of pending dissolution show the same peripheral aggregation of condensed chromatin (Fig. 6) as is observed in other types of cell undergoing apoptosis. The cytoplasm and myofibrils of such fibres are rather condensed, and there is always widespread dilatation of the sarcoplasmic reticulum (Figs. 6, 7); in places the membranes between the dilated sacs were seen to have disappeared so that adjacent myofibrils become separated from one another by fissures (Fig. 8). At the same time, bundles of myofibrils often slide over one another, as evidenced by relative longitudinal displacement of their bands (Fig. 6). Fragmentation of fibres into a number of sarcolytes or apoptotic bodies (Figs. 9, 10) appears to be accomplished by extension of the longitudinal fissures, together with transverse fracture of the bundles of myofibrils. There seems to be no consistent tendency for the fibrils to fracture at a particular point (Figs. 9, 10, 11, 12), though it is conceded that such a tendency might be obscured by varying planes of section.

Extracellular muscle apoptotic bodies are invariably surrounded by membranes (Figs. 9, 10, 11). As might be expected from the great cytoplasmic bulk relative to nuclear volume in striated muscle fibres, nuclear remnants were observed much less frequently than in apoptotic bodies derived from epidermal cells: a typical nuclear remnant in a phagocytosed body is illustrated in Fig. 12. Prior to their ingestion by macrophages, the myofibrils of apoptotic bodies always remain structurally intact (Figs. 9–12), as do organelles such as mitochondria (Fig. 10).

The development of surface clefts in muscle fibres is rapidly followed by insinuation of long cytoplasmic processes of macrophages into their interior, and the membrane-bounded fragments that subsequently form are ingested by these cells (Fig. 9). Well preserved muscle apoptotic bodies within macrophages are surrounded by narrow spaces (Fig. 11), the boundaries of which comprise the membranes of body and phagosome respectively. Degradation of bodies to electron-dense debris (Fig. 9) is presumably brought about by the action of lysosomal enzymes. The eventual fate of the residual body-laden macrophages was not investigated.

DISCUSSION

Deletion of epidermal epithelial cells during spontaneous regression of the tadpole tail is accomplished by ultrastructurally typical apoptosis, the morphological appearances being essentially the same as those described in vertebrate embryos (Glücksmann, 1951; Bellairs, 1961; Saunders & Fallon, 1966; Farbman, 1968; Manasek, 1969; Webster & Gross, 1970; Mottet & Hammar, 1972), in adult mammals (Kerr, 1971, 1973; Kerr *et al.* 1972; Kerr & Searle, 1973), and in metamorphosing insects (Goldsmith, 1966). The phenomenon involves a stereotyped sequence of changes, and

begins with peripheral aggregation of nuclear chromatin; this is followed by nuclear fragmentation, and cytoplasmic condensation associated with prolific budding to produce membrane-bounded apoptotic bodies of varying size. As is the case in other solid tissues (Kerr, 1971, 1973), the actual process of budding is rarely observed, possibly because of the avidity with which viable cells engulf the condensing ones (Kerr, 1973); it is much more frequently seen in apoptosis of mouse ascites tumour cells induced by actinomycin D, where all cells are separated from one another by abundant plasma and phagocytosis is long delayed (authors' unpublished observations).

A few of the apoptotic bodies in the tadpole skin are ingested by epithelial cells. However, the paucity of secondary lysosomes in these cells at a fairly advanced stage of tail regression suggests that they play a relatively minor role in disposing of cell fragments: epithelial cells are sometimes more active in this regard in the embryo (Farbman, 1968), and under pathological conditions in adult mammals (Kerr, 1971, 1973; Kerr & Searle, 1972). The majority of the bodies in the tail epidermis are ingested and degraded by specialized cells, whose cytoplasm becomes progressively laden with telolysosomes. Very similar cells in the epithelial lining of adult rat prostatic acini dispose of apoptotic bodies both in the normal gland and during the rapid atrophy that follows castration (Kerr & Searle, 1973); in grossly atrophic glands they display the same packing with lysosomal residual bodies as is illustrated in Fig. 5. Further study of epithelial surfaces is needed to determine whether such cells are of common occurrence.

In an electron-microscope study of epidermal changes in thyroxine-induced tadpole tail involution carried out by Gona (1969), a clear distinction was not made between autophagy on the one hand and heterophagy of cell remnants on the other. Autophagic vacuoles may indeed be difficult to distinguish from ingested apoptotic bodies that do not contain nuclear remnants (Kerr, 1973), but differentiation between these two processes is important, for the former merely involves segregation and degradation of part of the cytoplasm of a viable cell, whilst the latter is an indication of cell death. Autophagy undoubtedly occurs in the tadpole tail, but our findings do not suggest that it plays a major part in the regressive process.

It was noted in passing that several other types of cells in the tail undergo typical apoptosis, but most interest centred on the mode of deletion of striated muscle fibres. *A priori* it might be expected that the relatively huge mass-to-surface ratio of these cells would preclude their condensing and budding to produce apoptotic bodies in the usual way, but it would, nevertheless, seem unlikely that a different and unique mechanism should have evolved for the controlled deletion of muscle. It is thus of interest that the nuclear changes in the early stages of muscle fibre dissolution are typical of apoptosis. Further, the extracellular muscle fragments are membrane-bounded, and show the same striking preservation of the fine structure of organelles as is apparent in apoptotic bodies of diverse origins (Kerr *et al.* 1972); the pyknotic nuclear remnants, though rare, are morphologically typical of apoptosis (compare Fig. 2 with Fig. 12). Lucent vacuoles, possibly derived from the endoplasmic reticulum, have been observed in apoptotic bodies by several workers (Manasek, 1969; Kerr *et al.* 1972; Mottet & Hammar, 1972; Kerr, 1973), and the dilatation of the sarcoplasmic reticulum that accompanies apoptosis-like nuclear change in muscle is probably an analogous process.

Just how confluence of the resulting spaces leads to the formation of membrane-bounded apoptotic bodies is not yet clear, but estimates of the surface area of the sarcoplasmic reticulum (Peachey, 1965) indicate that ample membrane is available to encompass the fragments. Indeed vacuoles, possibly representing redundant membrane, are seen at the surface of newly formed bodies (Fig. 9). Extension of fissures between myofibrils to the cell surface is presumably associated with fusion of their bounding membranes with the sarcolemma. The T-system does not appear from the micrographs to be involved, in spite of its known normal communication with the exterior (McCallister & Hadek, 1970). In summary, apoptosis of muscle fibres appears to represent a modification of the basic pattern, in which internal cell membranes participate to a large degree in the formation of membrane-bounded cytoplasmic fragments. Once ingested by macrophages, the muscle apoptotic bodies go through the usual series of degradative changes within phagolysosomes.

Finally, it is significant that the ultrastructural events described in breakdown of larval intersegmental muscles during metamorphosis in Diptera (Crossley, 1968) appear to be essentially the same as those observed in the tadpole. In particular, Crossley stressed that changes in the doomed muscles precede invasion by phagocytic haemocytes, and described partial breakdown of subdivisions of the sarcoplasmic reticulum with the creation of abnormal elongated vacuoles between myofibrils; he considered that fracture of the myofibrils takes place at the level of the I-bands.

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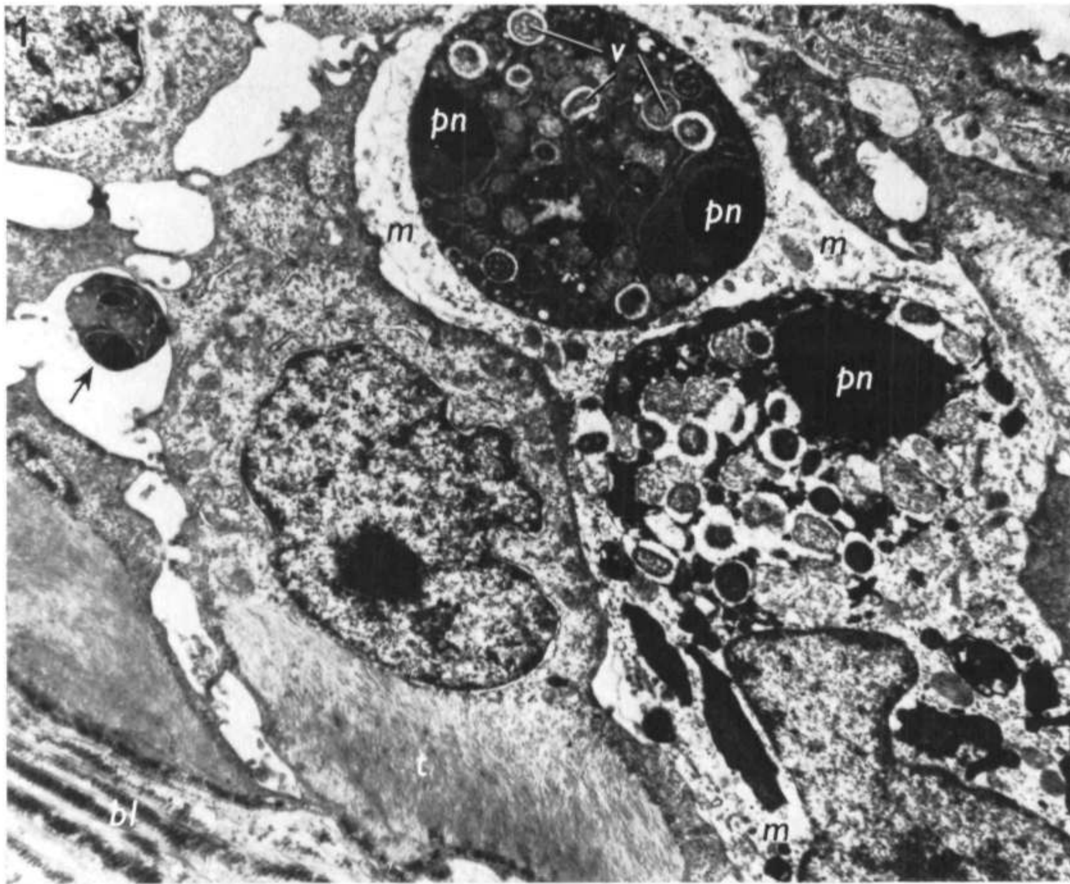
Figs. 1–3. Apoptosis of epidermal epithelial cells.

Fig. 1. The full thickness of the epidermis is included in this micrograph, the external surface being at top right and the basement lamella (*bl*) at bottom left. A moderately well preserved apoptotic body and a partly degraded body are seen within a large macrophage-like cell (*m*). Mucous vesicles (*v*) can be recognized in the former, indicating that it is derived from a superficial epithelial cell; both bodies contain pyknotic remnants of nuclei (*pn*). A third, extremely compact apoptotic body (arrow) is still extracellular. The wide space between epithelial cells may be a fixation artifact. It was, however, consistently observed in all animals. *t*, tonofibrils in epithelial cell. × 5300.

Fig. 2. A well preserved apoptotic body lies within the cytoplasm of an epidermal macrophage-like cell (*m*). It contains multiple nuclear remnants (*pn*), in several of which both dense chromatin aggregates and relatively lucent areas are evident: the early nuclear changes of apoptosis that precede such fragmentation are illustrated in Fig. 6. Note the cluster of tonofibrils (*t*) and the marked cytoplasmic condensation. A second, partly degraded body is visible at the top right corner of the micrograph. The digestive vacuole at top left contains unrecognizable debris. *n*, nucleus of the macrophage-like cell. × 7400.

Fig. 3. A degenerate apoptotic body with a nuclear remnant (*pn*) is seen within a phagosome in the cytoplasm of an epidermal cell, which is clearly identified as epithelial by the presence of tonofibrils (*t*) and desmosomes (*d*). × 10200.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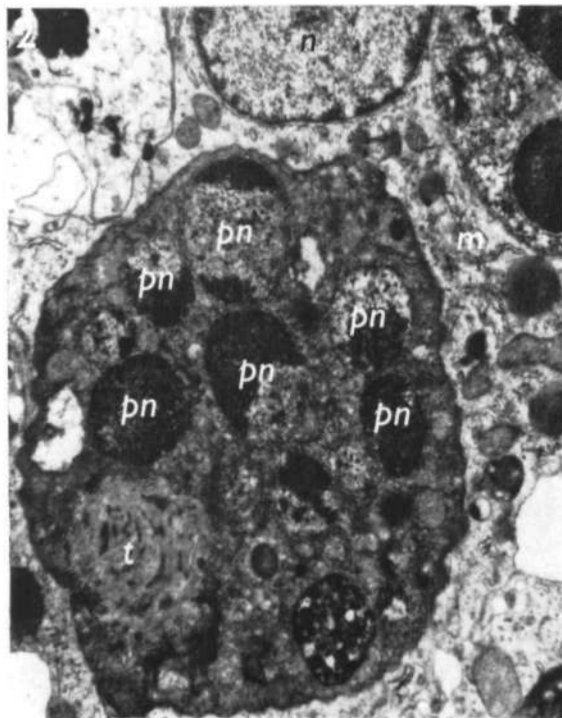
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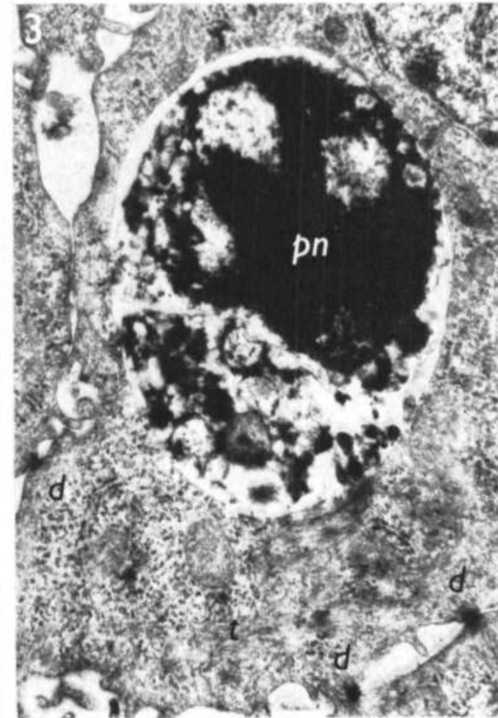
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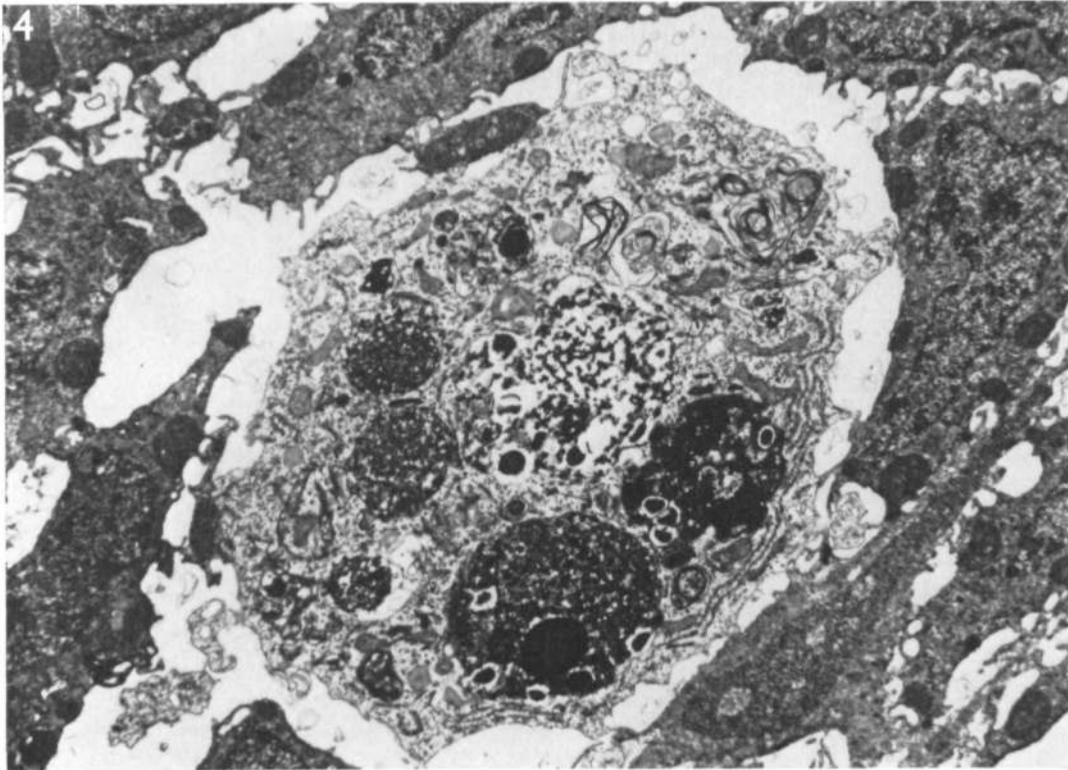
Figs. 4, 5. Large macrophage-like cells in epidermis.

Fig. 4. The macrophage-like cell in the centre of the micrograph contains at least seven partly degraded apoptotic bodies, and several whorls of membranes. Its cytoplasm is more electron-lucent than that of the surrounding epithelial cells. × 4900.

Fig. 5. The cytoplasm of this macrophage-like cell is densely packed with membrane whorls, which probably comprise lipid-rich residues of apoptotic bodies within lysosomes. Lysosomes (*l*) are, by contrast, relatively sparse in the epithelial cells, which are characterized by the presence of tonofibrils (*t*), desmosomes (*d*), and surface microvilli. × 5600.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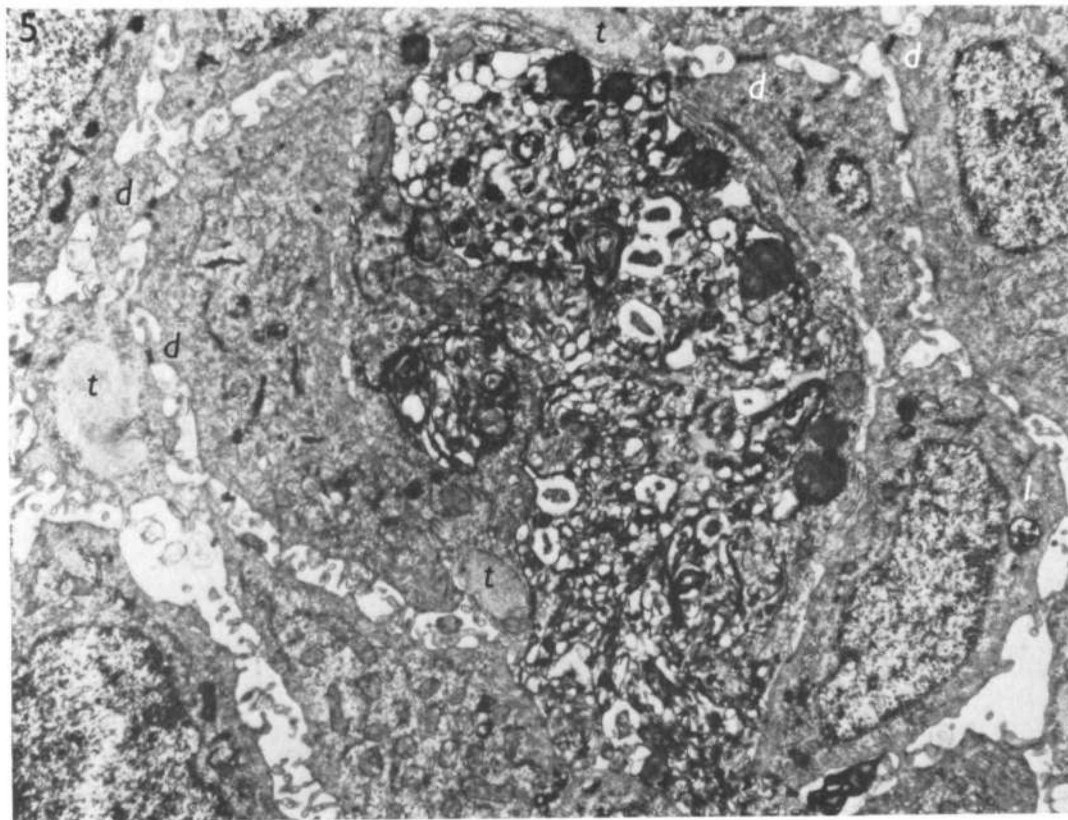
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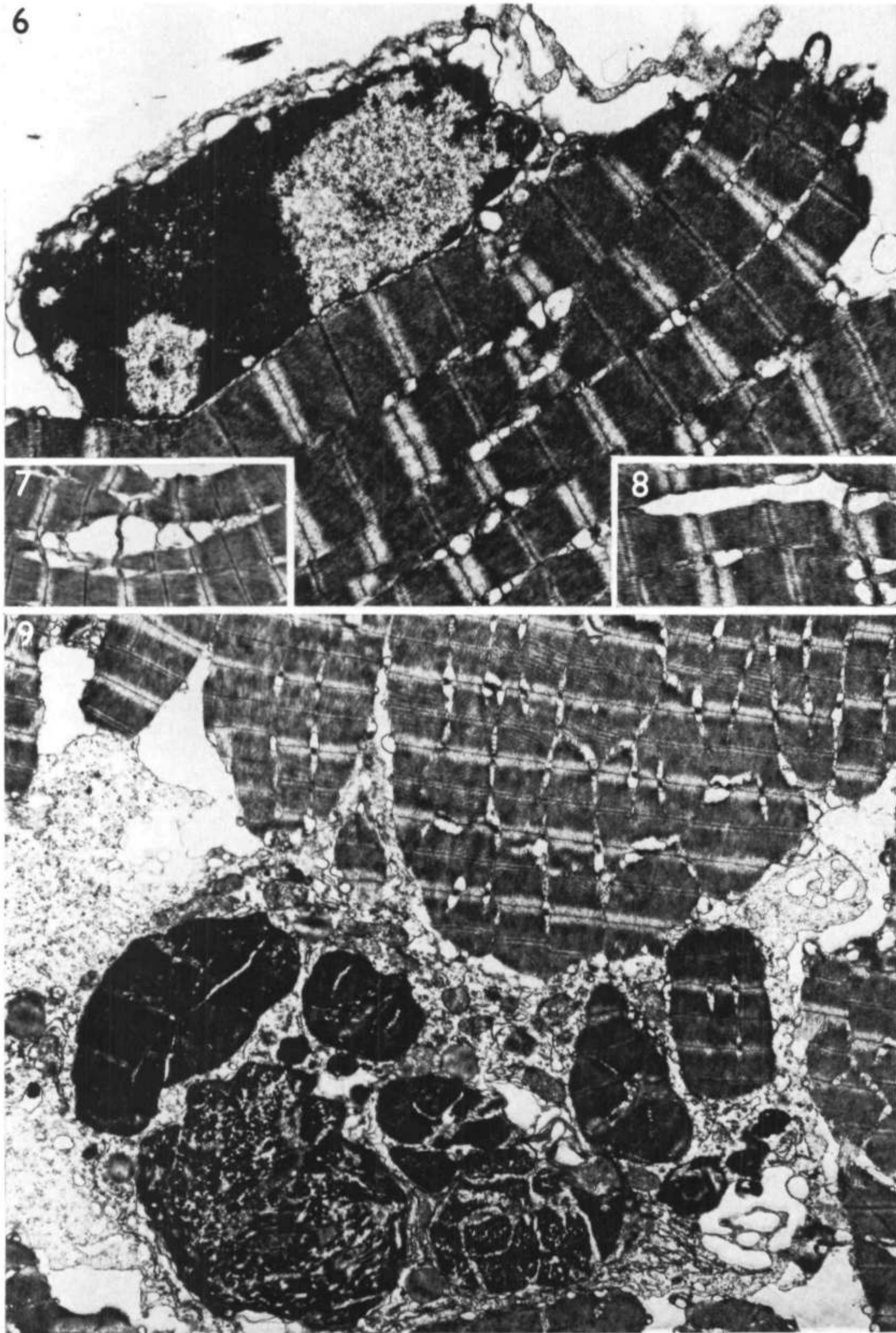
Figs. 6–9. Apoptosis of striated muscle fibres.

Fig. 6. The muscle fibre nucleus shows characteristic peripheral aggregation of condensed chromatin. Elements of the sarcoplasmic reticulum are dilated, and there is longitudinal displacement of myofibrils relative to one another. Note the surface cleft developing between myofibrils near the right-hand edge of the micrograph. × 10400.

Fig. 7. Part of a muscle fibre undergoing early apoptosis. Note the gross dilatation of the sarcoplasmic reticulum. × 5600.

Fig. 8. Part of another fibre displaying slightly more advanced changes. Septa between dilated elements of the sarcoplasmic reticulum have now disappeared resulting in the formation of a longitudinal fissure. × 11000.

Fig. 9. Macrophage lying within a muscle fibre undergoing apoptosis. It is insinuating processes of its cytoplasm into clefts that have developed between myofibrils. Apoptotic bodies, which are clearly of muscle origin and vary considerably in size, and which show different degrees of digestion, are seen within the macrophage. × 6000.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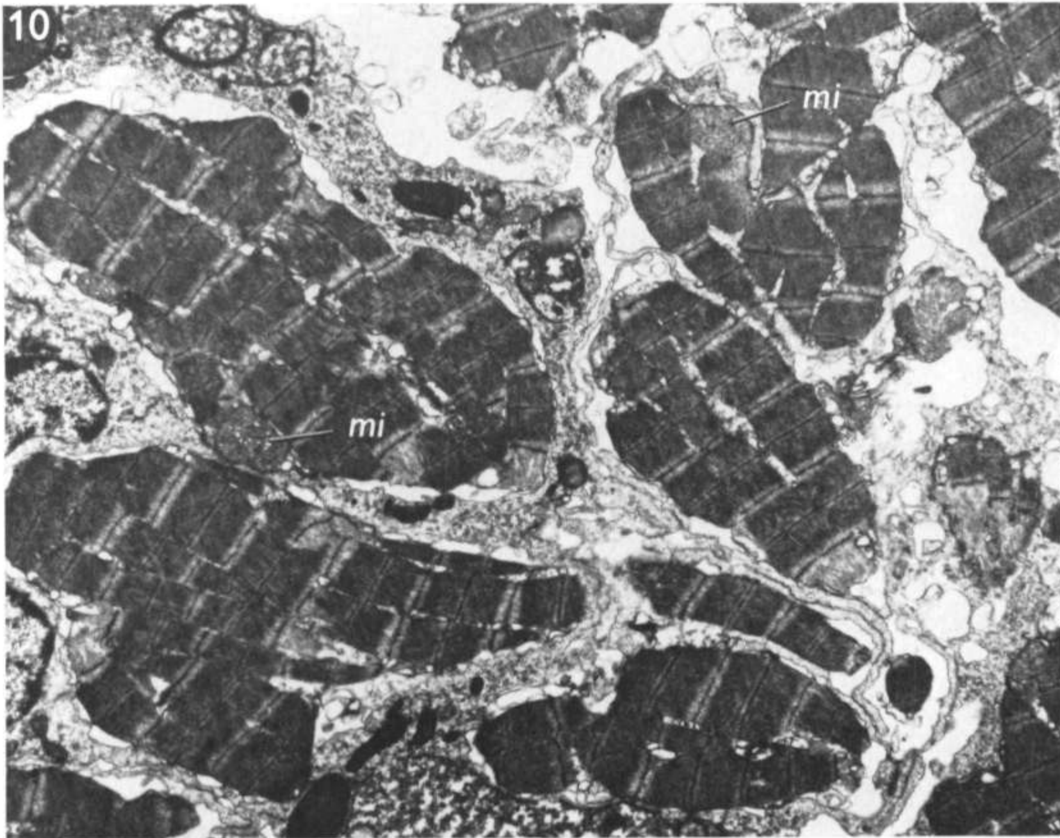
Figs. 10–12. Apoptosis of striated muscle fibres.

Fig. 10. Cluster of muscle apoptotic bodies. Some lie within macrophages; others are still extracellular. Note the well preserved mitochondria (*mi*) in 2 of the bodies. Even at this magnification, it is clear that the muscle fragments are bounded by membranes. × 6000.

Fig. 11. The extracellular apoptotic body at the bottom of the micrograph is bounded by a membrane; the ingested body within the macrophage is surrounded by a narrow, membrane-enclosed space, the outer limit of which comprises the phagosome membrane. × 16800.

Fig. 12. Extracellular and phagocytosed muscle apoptotic bodies, one with dense nuclear remnants (*pn*). × 5300.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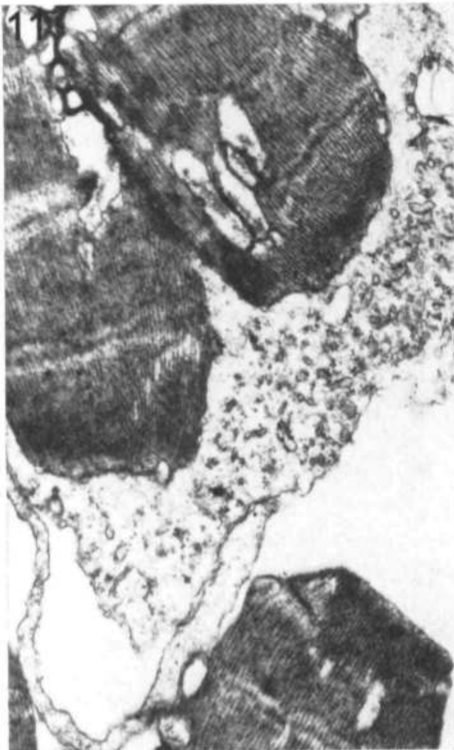
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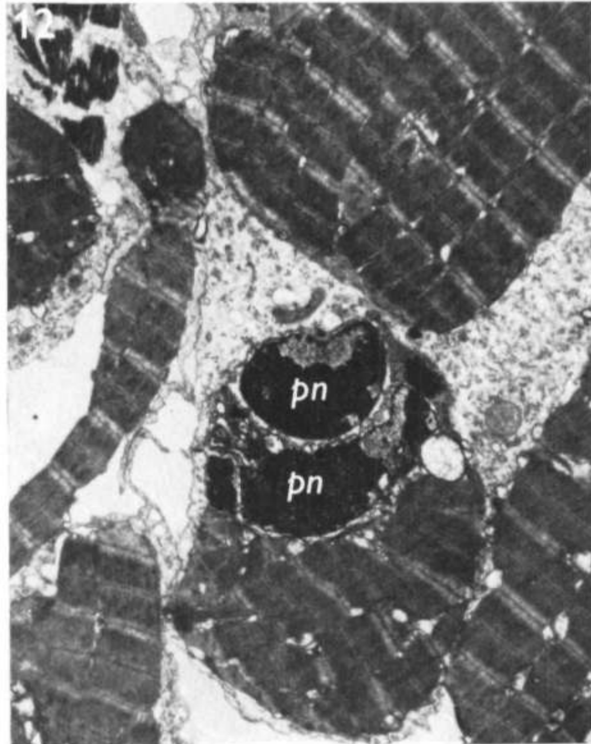
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