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Myogenesis in adult mammalian skeletal muscle *in vitro*

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INTRODUCTION

It is now fairly well established that the adult skeletal muscle can undergo regeneration (or extensive replacement) after an injury (Carlson, 1973). The origin of the myogenic cells that participate in regeneration or extensive replacement (Carlson, 1972) of injured muscle is conjectural. Two hypotheses have been formulated to explain the origin of these cells. One hypothesis suggests that the uninjured myonuclei, surrounded by cytoplasm of the damaged muscle, form a new plasma membrane and break away from the muscle, giving rise to myogenic cells (Hay, 1959; Lee, 1965; Reznik, 1969; Teravanien, 1970). The other hypothesis (Mauro, 1961; Church, Noronha & Allbrook, 1966; Church, 1970; Shafiq, 1970; Bischoff, 1975; Konigsberg, Lipton & Konigsberg, 1975) suggests a reserve population of cells, called satellite cells and located between the sarcolemma and external lamina on basal lamina of the mature muscle cell, possess myogenic potentiality, and differentiate into fusion-competent myoblasts after injury to the parent fibre.

Recently, Bischoff (1975) dissociated mononucleated cells from adult skeletal muscle and tested their myogenic capacity in culture, using procedures known to support differentiation of embryonic rat muscle. Myogenesis was observed in the culture only from cells liberated by trypsin or pronase. These myogenic cells were reported to be satellite cells, on the basis of their frequency and the conditions required for their release from the basement lamina surrounding the fibres. Although the culture of the adult muscle tissue gave rise to myogenic cells capable of differentiation into fully formed fibres (Pogogeff & Murray, 1946), the origin of myogenic cells in such cultures was not well understood. Konigsberg *et al.* (1975) examined the regenerative response of single mature muscle fibres from juvenile Japanese quail, culturing the segments of individual fibres. They observed that approximately 22 % of fibres gave rise to a network of long cross striated multinuclear cells. In the majority of fibres a colony of fusion-competent bipolar cells arose from a few proliferation-competent fibre nuclei. These nuclei, which survived after degeneration of parent fibres, were found within separate bipolar cells closely applied to the fibre. These cells are interpreted to be satellite cells, on the basis of their morphology and location.

The present study has been undertaken to examine in detail the myogenic competence of the mononucleated cells (satellite cells) released from the adult rat skeletal muscle. It has characterized, with scanning and transmission electron microscopy, the freshly dissociated mononucleated cells and has followed the sequences of differentiation of the myogenically competent and non-competent cells in culture. It has also involved the study of DNA synthesis and the proliferation of the myogenically competent and non-competent cells in culture.

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MATERIALS AND METHODS

Enzyme incubation

Twenty adult rats (Sprague–Dawley) of both sexes, weighing approximately 200 g, were used for this study. The animals were anaesthetized with ether and the leg and thigh muscles were immediately excised and placed in a dish containing Tyrode's solution. The animals were later killed with a high dose of ether. The muscles were rinsed with Tyrode's solution several times and as much as possible of the connective tissue material cleaned off. The muscles were then minced and rinsed with calcium and magnesium-free Tyrode's solution (CMF). The muscle mince in CMF was aerated with balanced air (5 % CO_2 and 95 % air) and incubated at 37 °C for 15 minutes on a gyratory water bath shaker at 70 rev/min.

Four enzymes were tested to liberate myogenic cells from the mince: collagenase (Worthington Chemical Co.), hyaluronidase (Sigma), tryptar (Armour Pharmaceutical Co.) and pronase (Sigma). Of four enzymes tested, 1 % pronase (Bischoff, 1975) was found to be the most effective in the digestion of basement membrane and release of uninucleated cells from muscle mince.

The muscle mince was incubated in 1 % pronase with CMF for 50–60 minutes at 37 °C in a gyratory water bath shaker at 70 rev/min. The enzyme was then removed by centrifugation and the mince was washed several times with 4–5 volume aliquot of complete culture medium. Cells were released from the digested mince by repeated passages through a wide mouthed (2–3 mm) pipette. The yield of cell suspension thus obtained was not satisfactory. In order to increase the yield, the digested mince with an aliquot of culture medium was again dissociated by agitating with a wide mouthed pipette. Subsequently, the residual mince was removed by low speed centrifugation (500 g for 2 minutes) and transferred to a fresh medium for further dissociation. Altogether three sequential dissociation steps were involved. Each dissociation supernatant with released cells was pelleted by centrifugation, leaving most of the myofibrils and connective tissue elements in suspension. The pellet was suspended in fresh medium and filtered through three layers of nitex (Tetko Inc.). The cells were counted on the haemocytometer, although some myofibrillar debris was present in the preparations.

Cell culture

The cells were cultured in 60 mm Falcon petri dishes or in screw cap dishes (25 cm^2 growth area). The plates were pre-coated with a 1 % gelatin solution and subsequently washed with cold Tyrode's. Falcon screw cap dishes without gelatin coating also gave satisfactory results. For cultures grown on glass (Corning) coverslips, the coverslips were acid cleaned and coated with carbon before gelatin addition to the culture dishes. The culture medium consisted of 88 % Eagle's basal medium (with Earle's salts), 10 % horse serum, 1 % L-glutamine, and 1 % penicillin–streptomycin. Initially we used 10 % embryo extract in our medium. Later, when the embryo extract was discontinued we could find no significant difference between the two cultures. Each culture plate containing 3 ml of medium was inoculated with 10^5–10^6 cells. All cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. The medium was changed twice a week.

Autoradiography

The cultures were continuously labelled for 24 hours with 1 μCi/ml of [^{3}H]-thymidine (^{3}H-TdR) prior to fixation, fixations being done after culturing for 1–7 days inclusive. Following incubation, the culture plates were rinsed with Tyrode's and fixed with 3 % glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) for 3 hours at room temperature. Dried culture plates were coated with Kodak NTB-2 photographic emulsion and stored for 2 weeks in a light-tight black box with desiccants at room temperature (21–22 °C). The plates were then developed in Microdol-X for 3 minutes, fixed in 20 % sodium thiosulphate for 10 minutes, and examined under phase contrast and bright-field light microscopes. The labelled nuclei in the large flattened cells (non-myogenic), bipolar cells (myogenic) and myotubes with labelled nuclei were counted.

Electron microscopy

Transmission electron microscopy

The freshly dissociated cells and the cell cultures were fixed in a modified Karnovsky's fixative (1965), for 1½–2 hours at room temperature. The fixative consisted of 4 % paraformaldehyde and 4 % glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4). Dissociated cells were processed in suspension with centrifugation at 37 g for 3 minutes between steps of the procedure. The dissociated cells and the cultures were rinsed with 0·1 M cacodylate buffer (pH 7·4), post-fixed with 1 % osmium tetroxide and dehydrated in graded alcohol and propylene oxide. Some of the cultured cells at the dehydration step of 70 % alcohol were gently scraped out of the culture dish with a rubber 'policeman,' pelleted and subsequently processed by centrifugation. like those of the freshly dissociated cells. The freshly dissociated and scraped cells were embedded in Araldite after pelleting them in the embedding capsule. The cells in some of the culture dishes were embedded *in situ*. For examination of the orientation of the cells, 0·5 μm Araldite sections were stained with 1 % toluidine blue and examined under a Zeiss light microscope. For electron microscopy thin sections were stained with uranyl acetate and lead citrate, examined and photographed in a Philips 200 electron microscope operated at an accelerating voltage of 60 kV.

Scanning electron microscopy

The cultured cells in the plastic dish as well as those on glass coverslips were fixed in modified Karnovsky's fixative, as before, and were post-fixed in 1 % osmium tetroxide in cacodylate buffer for 1–2 hours at about 4 °C. They were then rinsed several times with cacodylate buffer. A saturated aqueous solution of thiocarbohydrazide (TCH; Eastman Organic Chemicals) was prepared and the cells were incubated in an excess of TCH at room temperature for 15 minutes (Kelly, Dekker & Bluemink, 1973). The cell preparations were then rinsed several times in distilled water and treated with 1 % OsO_4 for 1 hour. A second osmication was followed by an additional treatment of TCH for 10 minutes when the cells were rinsed and kept in aqueous 1 % OsO_4 for 1 hour. A third osmication was followed by several rinses in distilled water and the cells in the plastic dishes were dehydrated through graded alcohol, whereas those on the coverslips were dehydrated through graded acetone. Both preparations were dried from liquid CO_2 by the critical point method (Anderson, 1956). The dried cell culture in the petri dish was mounted on the

aluminium specimen (along with a plastic piece of the petri dish) stub with silver paint. The coverslips containing cell cultures were also mounted on the specimen stub with silver paint. The mounted samples were lightly coated with carbon, then with golden-palladium by the DC sputtering device of Hummer (Technics, Inc.). The samples were then examined with a Colter or ETEC scanning electron microscope operated at 5–20 kV.

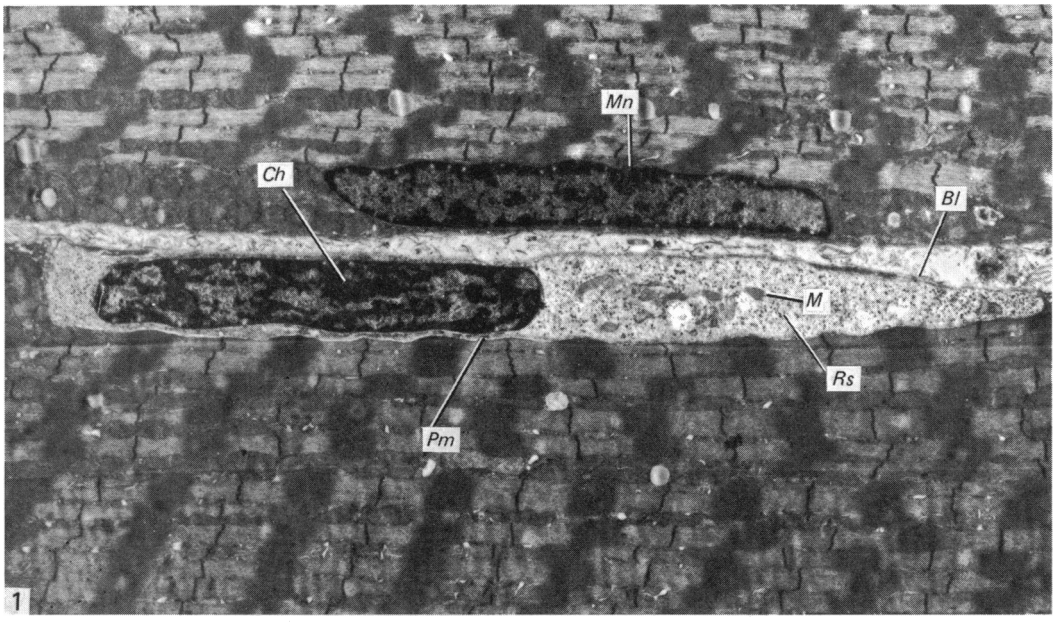
RESULTS

Satellite cells were easily identified by their unique positions on the surface of the adult muscle fibres. Previous studies (Mauro, 1961; Katz, 1961; Ishikawa, 1966; Church, 1970; Bischoff, 1975; Konigsberg *et al.* 1975; Schultz, 1976) are consistent with our present observations that the satellite cells lay between the basal lamina of the muscle fibres and the fibre plasma membrane. In addition, the satellite cells had certain characteristic features such as abundant clumped chromatin in the nucleus, numerous free ribosomes, polysomes, scanty rough endoplasmic reticulum, small and few mitochondria in the cytoplasm (Fig. 1).

Phase contrast microscopic examination of the freshly dissociated cell suspension from the digested muscle mince showed the presence of spherical to ovoid cells (Fig. 2). Transmission electron microscopy allowed the identification of presumptive satellite cells in this cell suspension. The characteristic features of the satellite cells, mentioned above, were observed in these cells, although there was some vesiculation in the cytoplasm after enzyme treatment during dissociation (Fig. 3). Fibroblastic and blood cells were also observed in the cell suspension. When this cell suspension was plated for culture, the satellite cells and the fibroblastic cells were differentiated and exhibited characteristic features which were sequentially followed and examined by parallel scanning and transmission electron microscopy.

Scanning electron micrographs revealed a considerable number of cells which appeared to be attached to the floor of the culture dish after 24 hours in culture. Two types of cell became evident in the cultured cell population between 24 and 48 hours: in one the cell was flattened and was presumably a non-myogenic fibroblast; in the other the cell was round to spindle-shaped, and interpreted as being a myogenic cell or a satellite cell (myoblast) (Fig. 4). Some cells were found to be in G_1 stage as determined by the surface morphology of the cell (Porter, Prescott & Frye, 1973) whence there were large numbers of microvilli and blebs, as shown in Figures 4 and 5. Autoradiography studies indicated that approximately 15 % of the mononucleated, spindle-shaped cells (presumably satallite cells) and 16 % of the flattened cells (presumably non-myogenic cells) had incorporated [^{3}H]-thymidine into their nuclei in the 1 day old culture (Fig. 6).

By transmission electron microscopy the 2 days old culture showed two types of cell with different cellular morphology. One type of cell contained abundant clumped chromatin in the nucleus, and the cytoplasm exhibited organelles as observed in the freshly dissociated as well as *in vivo* satellite cells (Fig. 7). Another type of cell possessed numerous filopodia, a characteristically lobulated nucleus and rough endoplasmic reticulum; this cell type was interpreted as being non-myogenic. The quantitation of autoradiographs indicated that 70 % of the mononucleated, spindle-shaped cells (satellite cells) and 79 % of the flattened cells (fibroblastic cells) had incorporated [^{3}H]-thymidine into their nuclei in the 2 days old culture (Fig. 6).



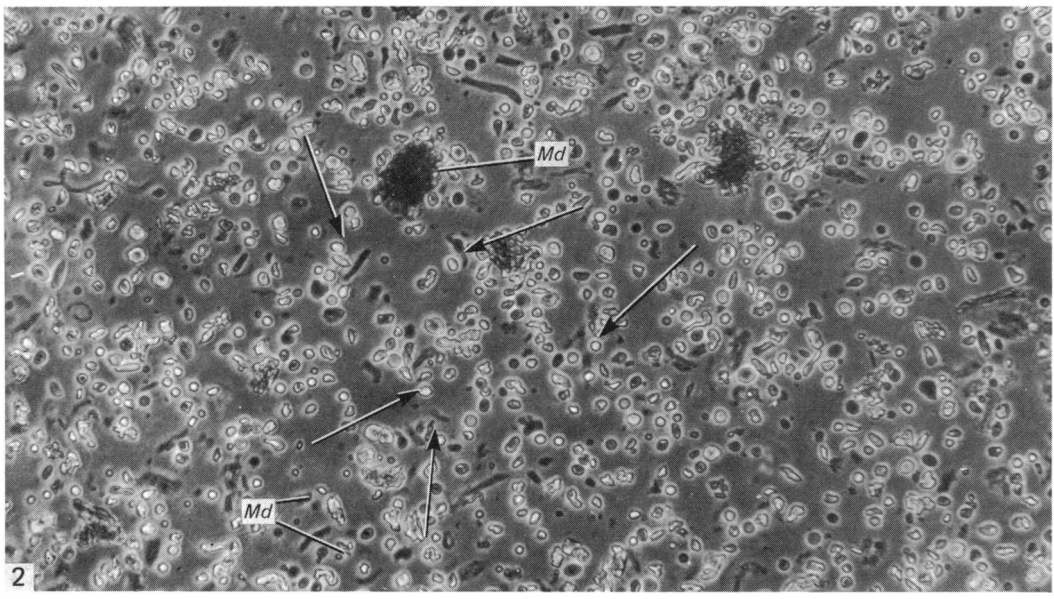


Fig. 1. Transmission electron micrograph of an *in vivo* satellite cell of an adult rat skeletal muscle. *Bl*, basal lamina; *Ch*, clumped chromatin of satellite cell; *M*. mitochondria; *Mn*, myonucleus; *Pm*, plasma membrane. × 5200.

Fig. 2. Phase contrast light micrograph of freshly dissociated cells. Note the presence of spherical to ovoid cells (arrows) in the cell suspension. Some myofibrillar debris (Md) is present in the field. × 500.

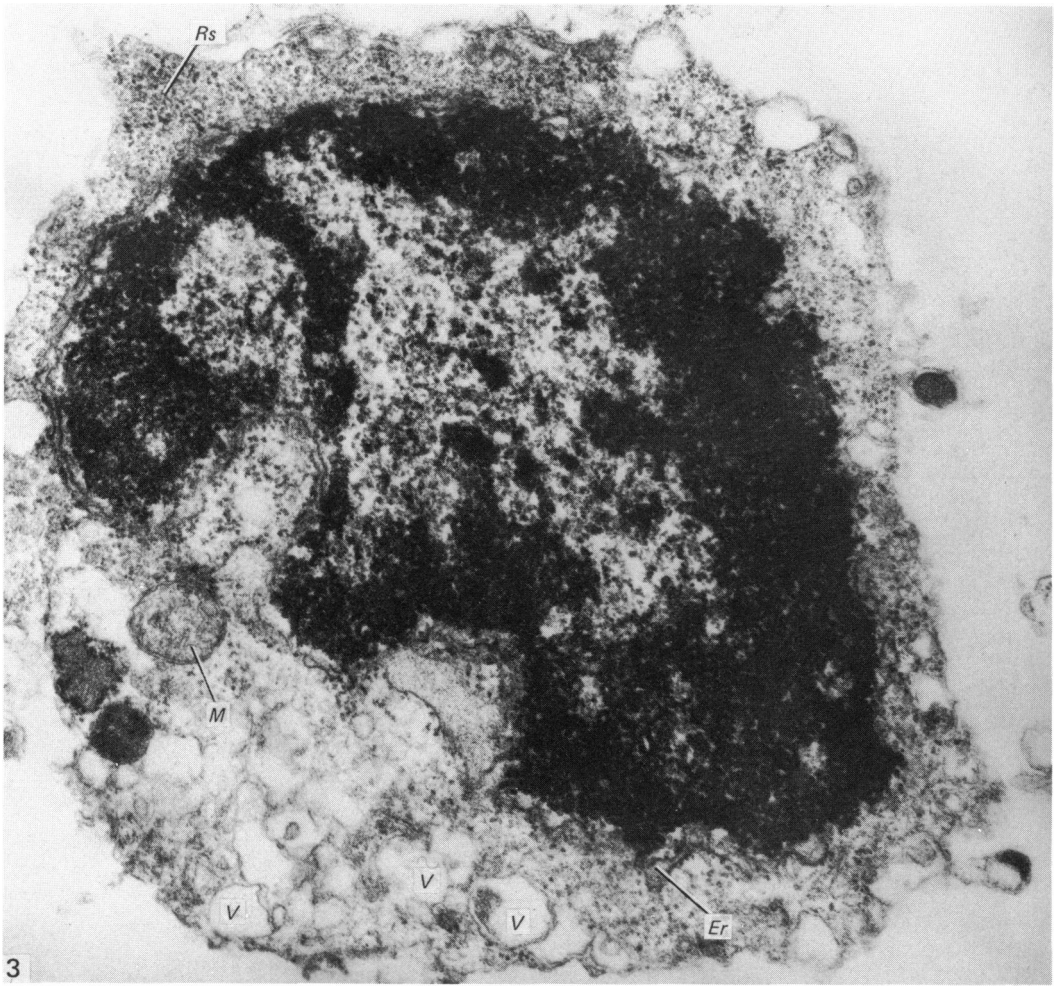
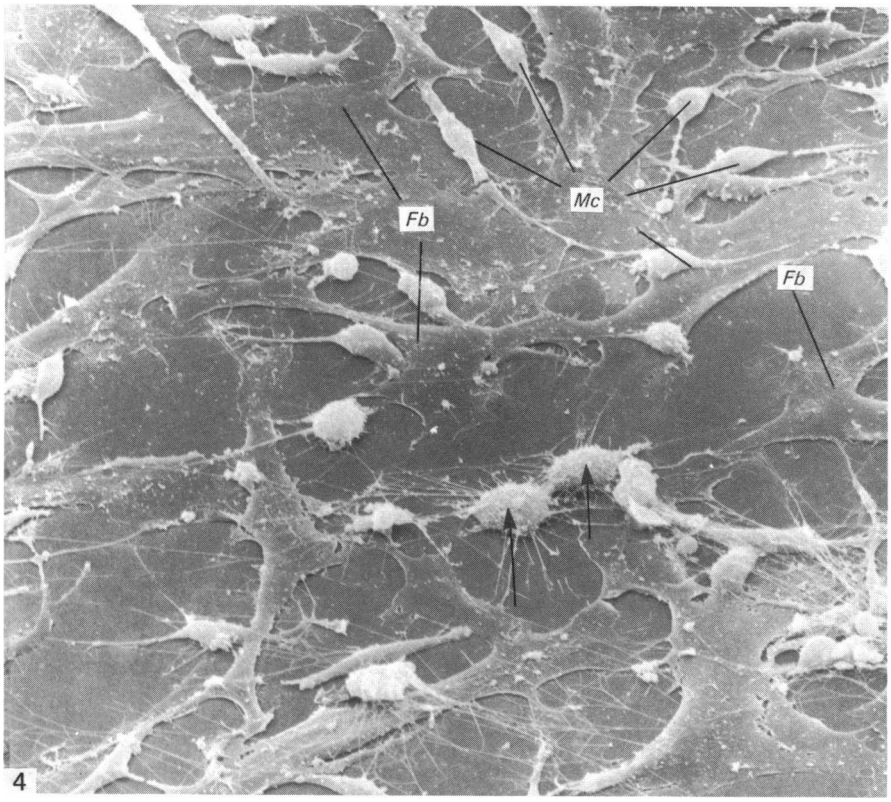


Fig. 3. Transmission electron micrograph of a freshly dissociated satellite cell. Note the presence of characteristic clumped chromatin of the nucleus. Free ribosome (*Rs*)-rich cytoplasm of the cell exhibits few mitochondria (*M*) and little rough endoplasmic reticulum (*Er*). Vesiculation (*V*) is evident in the cytoplasm of the cell. × 31 250.

The 3 days old culture exhibited large numbers of round to spindle-shaped and flattened cells which were presumably myoblasts (satellite cells) and fibroblasts, respectively (Fig. 8). A close examination of this culture with the scanning electron microscope revealed that a number of spindle-shaped cells were aligned with one another (Fig. 9). The regions of overlap between these cells were not discernible, which indicated a commencement of fusion as observed in embryonic myoblasts. The autoradiography studies showed that approximately 77 % of the mononucleated spindle-shaped cells (satellite cells) and 80 % of the flattened non-myogenic cells had incorporated [^{3}H]-thymidine into their nuclei. In addition, approximately 35 % of the multinucleated myotubes were labelled in the 3 days old culture (Figs. 6, 10, 11). The multinucleated state, which was indicative of fusion and formation of myotubes, was very common in the 4 days old culture. Although the incorporation of [^{3}H]-thymidine into the nuclei of the spindle-shaped myogenic cells in the 4 days old



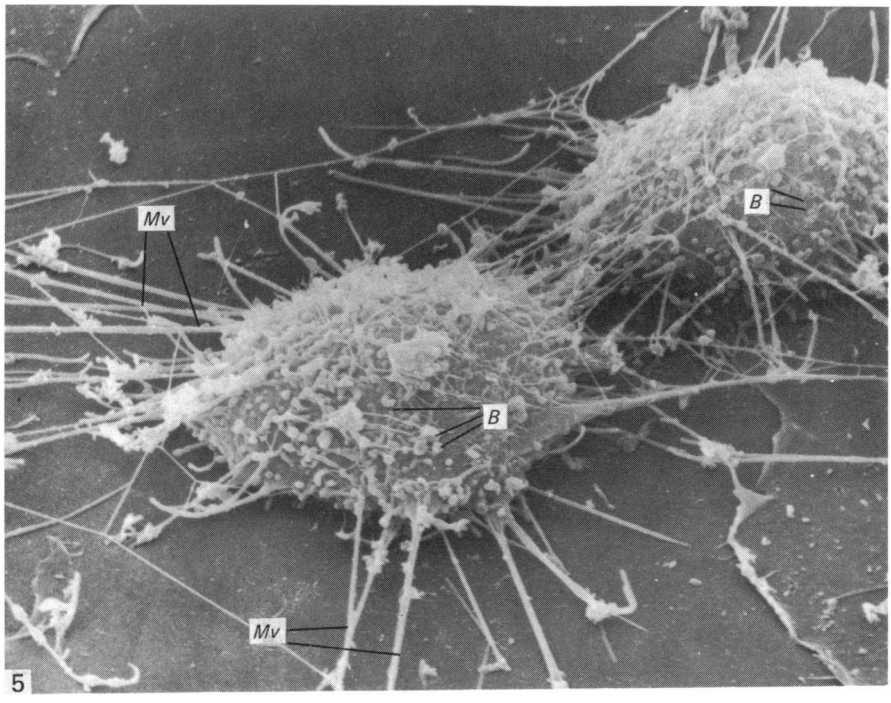


Fig. 4. Scanning electron micrograph of a 24 hours old culture established with the mononucleated cell suspension obtained from adult rat skeletal muscle. Two types of cell are visible: round- to spindle-shaped myogenic cells (satellite cells) and flattened non-myogenic fibroblastic cells (Fb). Mc, myogenic cells. The arrows show two cells in G_1 state (see text). ×530.

Fig. 5. Higher power scanning electron micrograph of two cells (arrows) in Fig. 4. The surfaces exhibit microvilli (Mv) and blebs (B). ×2660.

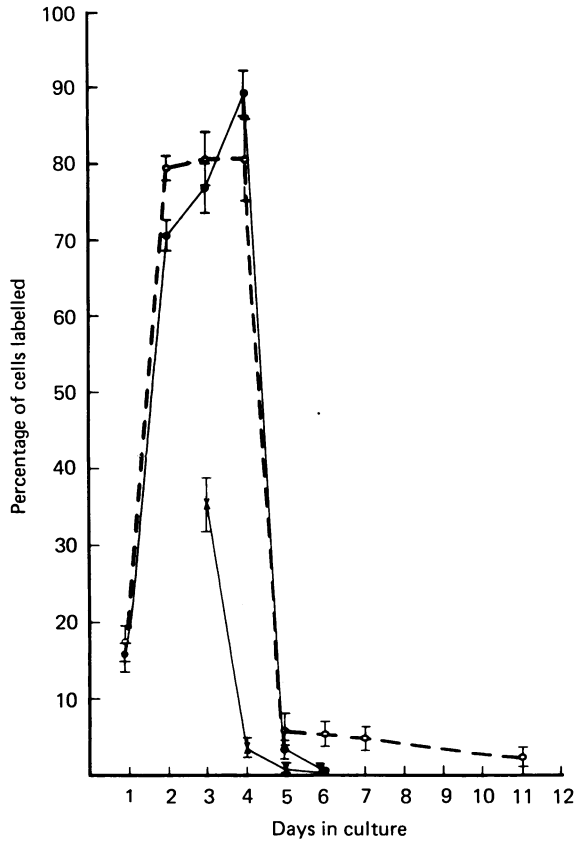


Fig. 6. Quantitation of the autoradiographs of cell cultures established with the enzyme-released cells from the adult rat skeletal muscle. Each culture was labelled with 1μCi/ml of [^{3}H]thymidine for 24 hours prior to its fixation and processing. Bars represent standard deviations. o—o, Non-myogenic cells; •—•, mononucleated myogenic cells; ×—×, myotubes.

culture attained a peak, showing that 89 % of the nuclei were labelled, the number of labelled myotubes in the culture had decreased, to approximately 3·5 %. The labelling of flattened non-myogenic cells in the 4 days old culture had levelled off (Figs. 6, 12, 13).

The multinucleated cylindrical cells were seen to contract spontaneously in the 5 days old culture. Transmission electron microscopy of this culture revealed the presence of differentiating myofibrils in the cells (Fig. 14) where a considerable number of myofilaments were found to be aligned with Z-lines which appeared as patches of electron-dense materials. In some myofibrils the dense patches of Z-line were arranged in rows but without forming continuous bands or lines as observed in the mature muscle cells. Lipid droplets were observed in these differentiating cells. The examination of the 6 days old culture showed the presence of a few newly formed myotubes and the participation of some myoblasts in the fusion process (Figs. 15, 16). The flattened fibroblastic cells and free myoblasts were also observed in the 6 days old culture. The examination of autoradiographs showed an overall drop in labelling index of all types of cells in the 5 and 6 days old cultures (Fig. 6).

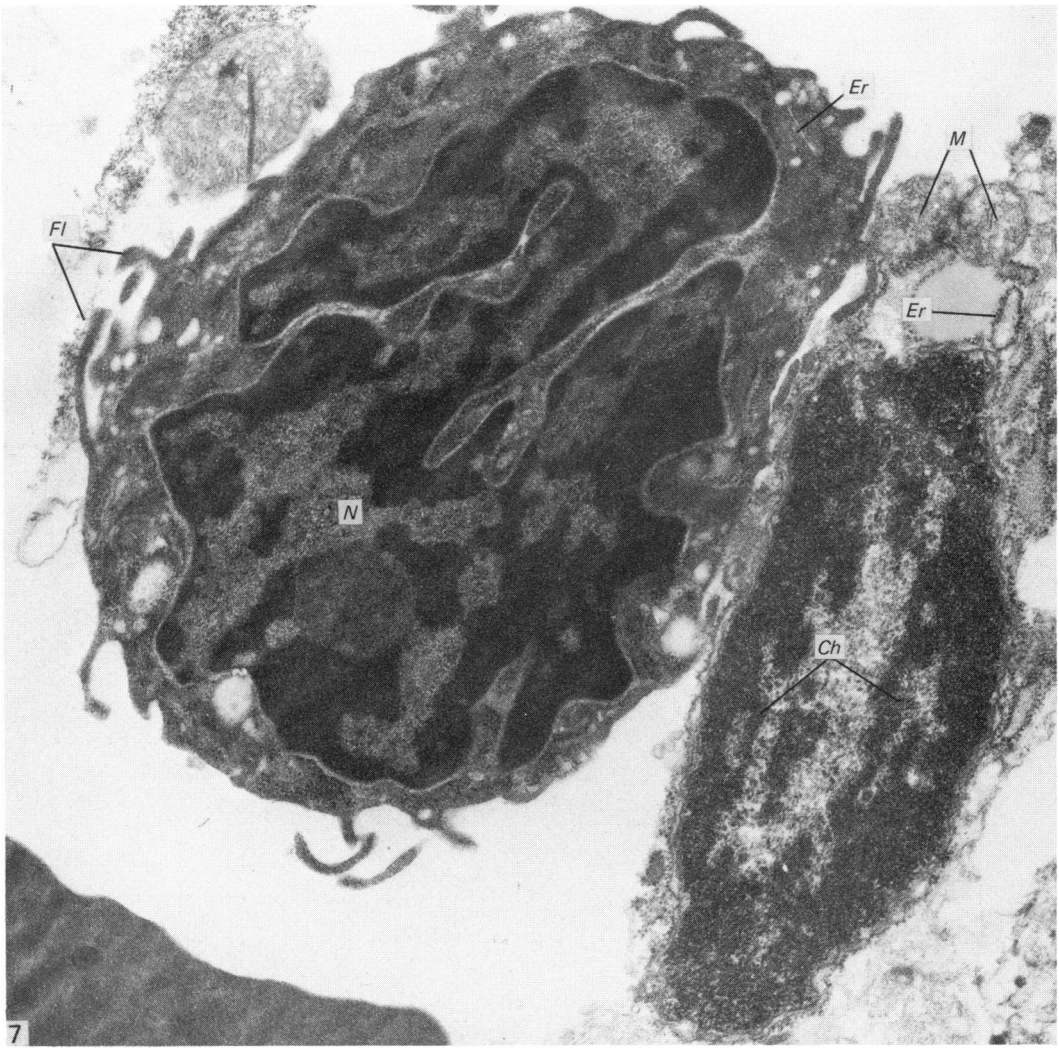
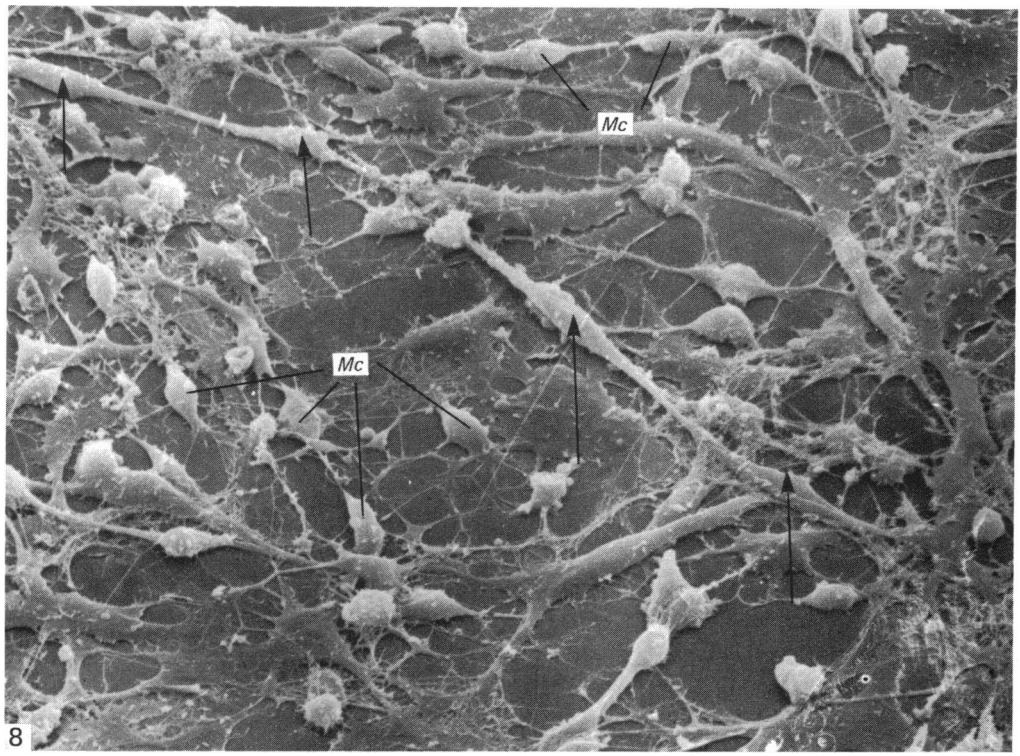


Fig. 7. Transmission electron micrograph of a 48 hours old culture which was sectioned *in situ*. The cell to the right exhibits characteristic clumped nuclear chromatin (Ch) found in the *in vivo* and freshly dissociated, presumptive satellite cells. The cell cytoplasm contains few mitochondria (M) and little rough endoplasmic reticulum (Er). The cell to the left possesses flattened body and the characteristically lobulated nucleus (N) of non-myogenic cells. The cell body has many filopodia (Fl). × 24850.

The mononucleated, spindle-shaped myogenic cells and flattened, non-myogenic cells exhibited approximately 3 % and 6 % labelled nuclei, respectively, in the 5 days old culture. Less than 1 % of the myotubes were labelled in the 5 and 6 days old cultures. No labelled mononucleated spindle-shaped myogenic cells were observed in the 6 days old culture, whereas 6 % of the flattened non-myogenic cells were labelled in this culture.

Transmission electron microscopy of 6 and 7 days old cultures indicated that the myofibrils of the myotubes were more differentiated than those of the 5 days old culture. Although ribosomes and polysomes were found in association with the free myofilaments (which were indicative of the synthesis of myofilaments), bundles



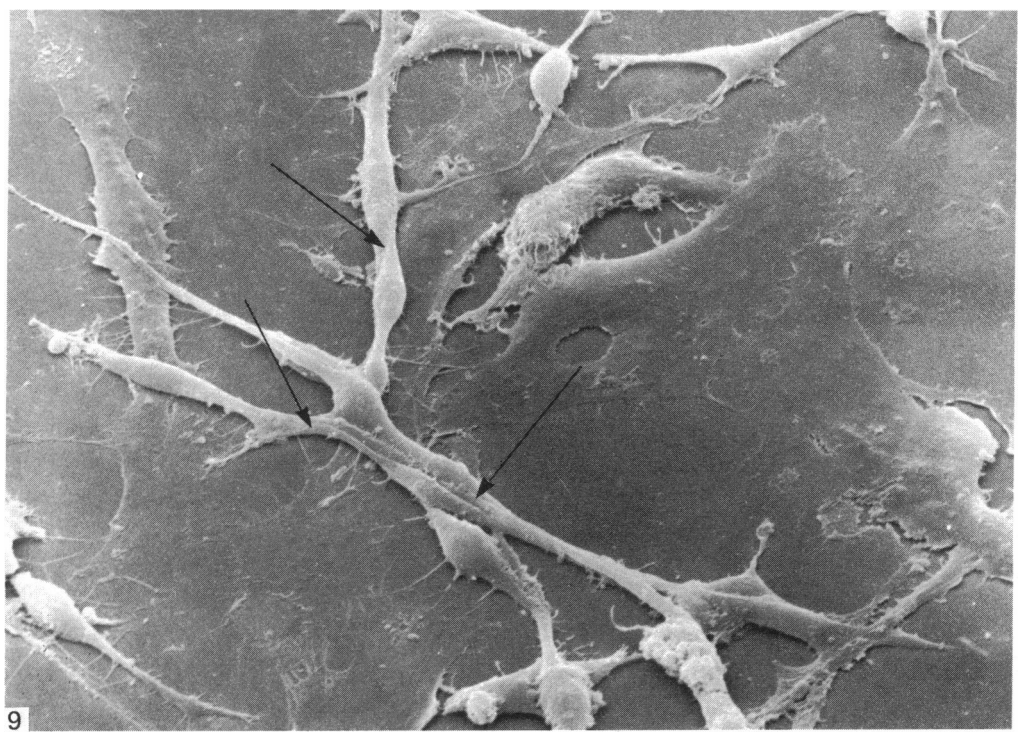
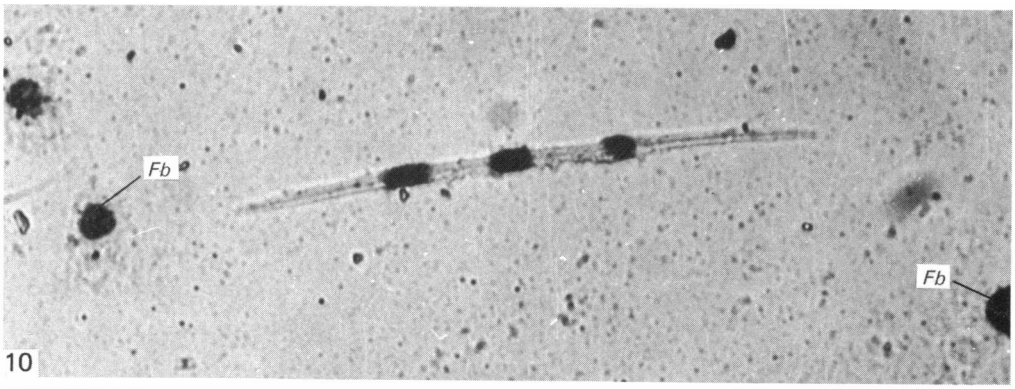
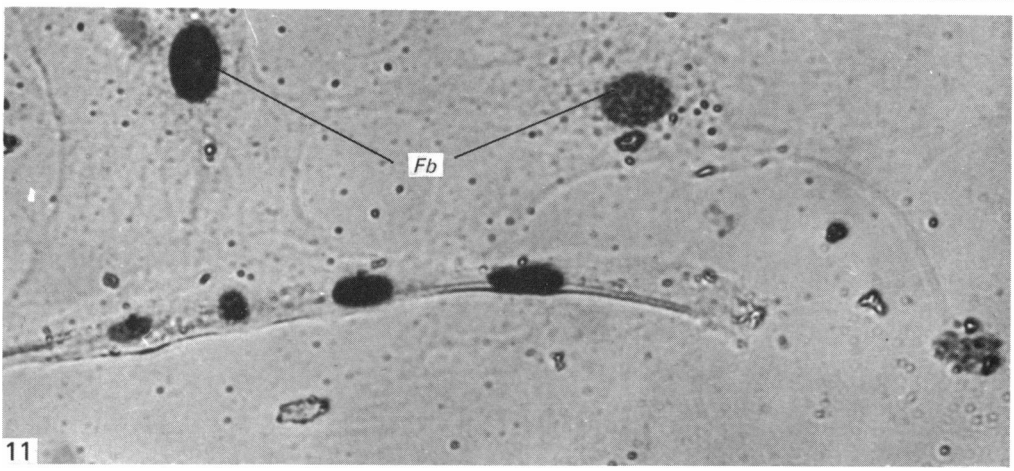


Fig. 8. Scanning electron micrograph of a 3 days old culture, showing a large number of round to spindle-shaped myogenic cells (Mc) in the field. Flattened fibroblastic cells are visible in the background. The arrows show some lined-up spindle-shaped cells. × 620.

Fig. 9. Scanning electron micrograph of a 3 days old culture, showing particularly the alignment of the spindle-shaped myogenic cells during the formation of myotubes. Note that some of the regions of overlap are not discernible (arrows). × 620.



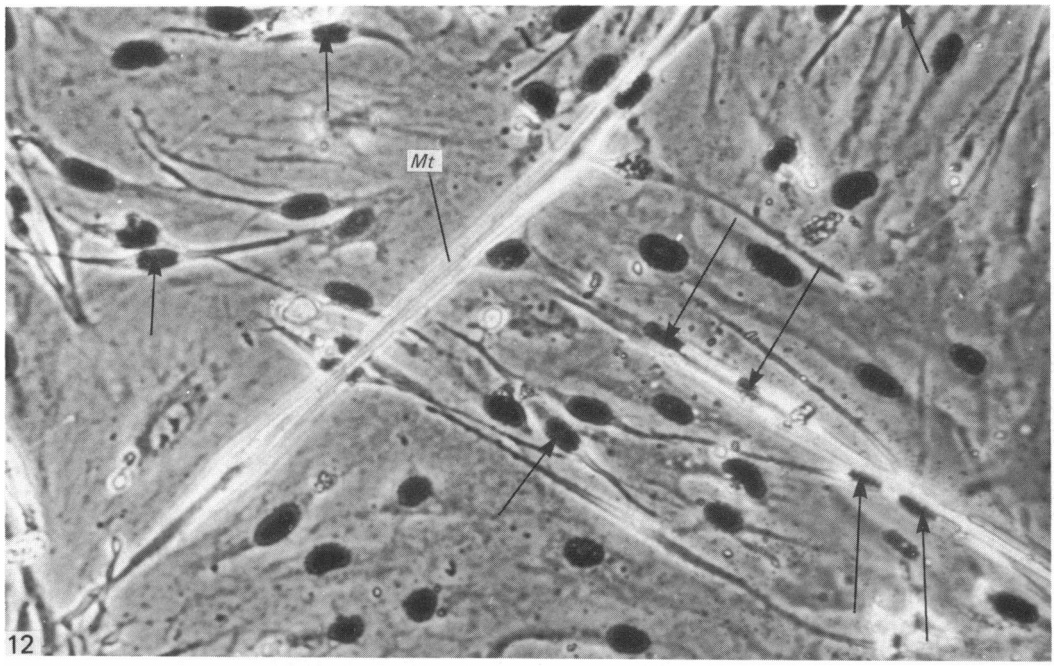


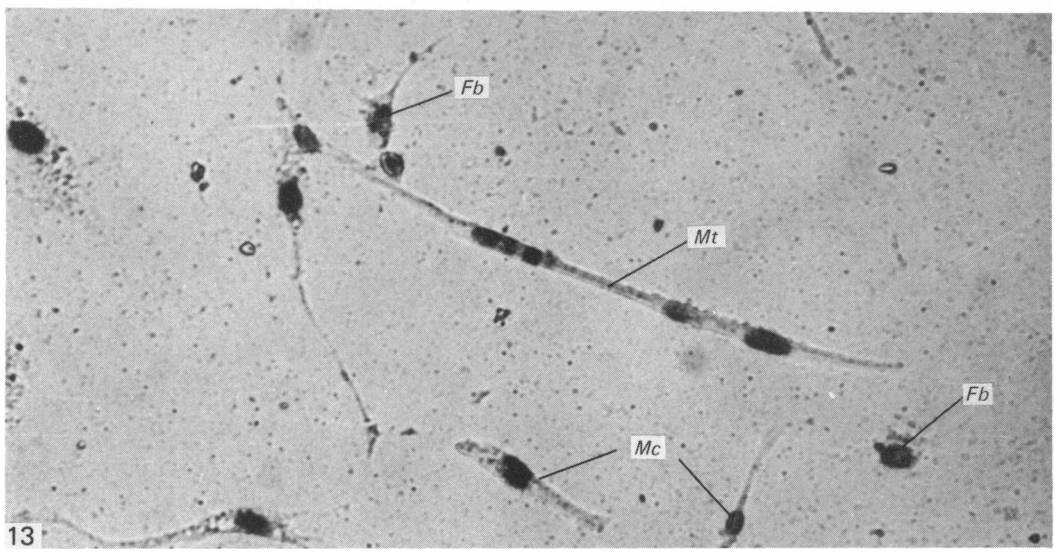
Figs. 10–11. Autoradiographs of a 3 days old culture, showing labelled multinucleated myotubes. Note that there are some labelled, flattened mononucleated fibroblastic cells (*Fb*) in the background. × 600.

of well differentiated myofibrils were observed in the cylindrical muscle cells. The 14 days old culture exhibited highly developed cylindrical muscle cells running in different directions (Fig. 17). Most of these fibres were undergoing spontaneous contractions and many were contracting in unison. The studies of thin sections of the 14 days old cultures indicated clearly that a considerable number of cylindrical cells were fully differentiated as muscle fibres (Fig. 18) and contained well organized A- and I-bands and Z-lines. In addition, they contained well organized mitochondria and lipid droplets. When these cultures were continued up to 21 days, they became heavily overgrown with fibroblasts. Myotubes in these cultures did not exhibit any sign of ultrastructural degeneration and did not appear to enlarge further.

DISCUSSION

Our observations suggest that the enzymically liberated myogenic cell population, which gave rise to fully differentiated muscle fibres, was derived from satellite cells: first, the ultrastructure of a population of freshly dissociated cells was similar to that of the *in vivo* satellite cell; second, the dissociated cells, after 24 to 48 hours of





Figs. 12–13. Autoradiographs of 4 days old cultures. Figure 12 shows a large number of labelled spindle-shaped myogenic cells (small arrows) which attained a peak at this stage. Note that some labelled cells are aligned with one another (large arrows) and are probably on the way toward fusion. In the centre of the field a non-labelled myotube (*Mt*) is present. × 500. Figure 13 shows a labelled multinucleated myotube (*Mt*) along with some spindle-shaped myogenic cells (Mc) and flattened fibroblastic cells (Fb). × 500.

culture, differentiated into two different types of cell, one of which contained features of satellite cells while the other possessed fibroblastic features.

We believe that the satellite cells present in the adult muscle fibre serve to provide additional myoblasts for formation of new generations of myotubes or for fusion with pre-existing fibres (Kelly & Zacks, 1969). The progeny of proliferating satellite

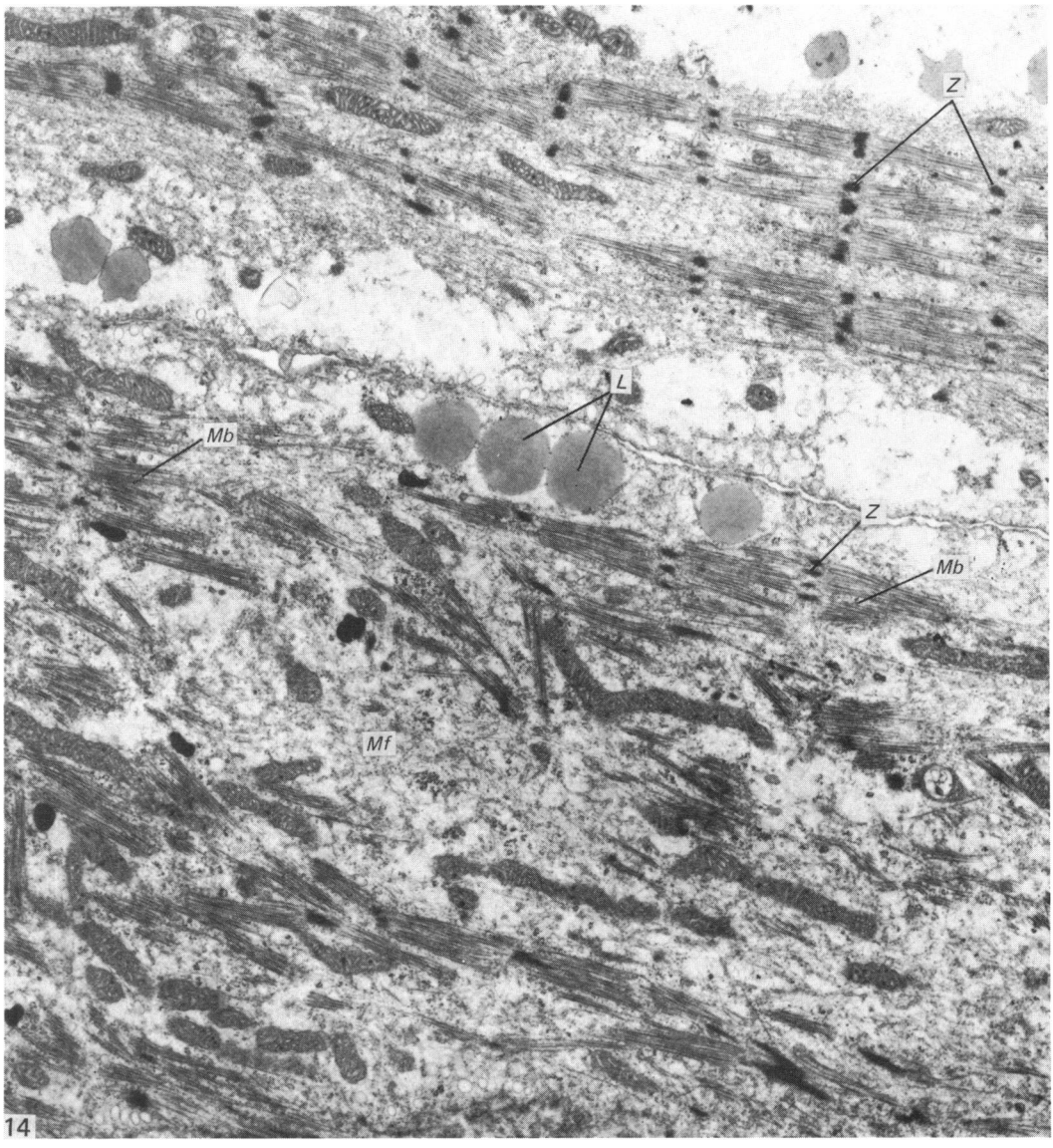
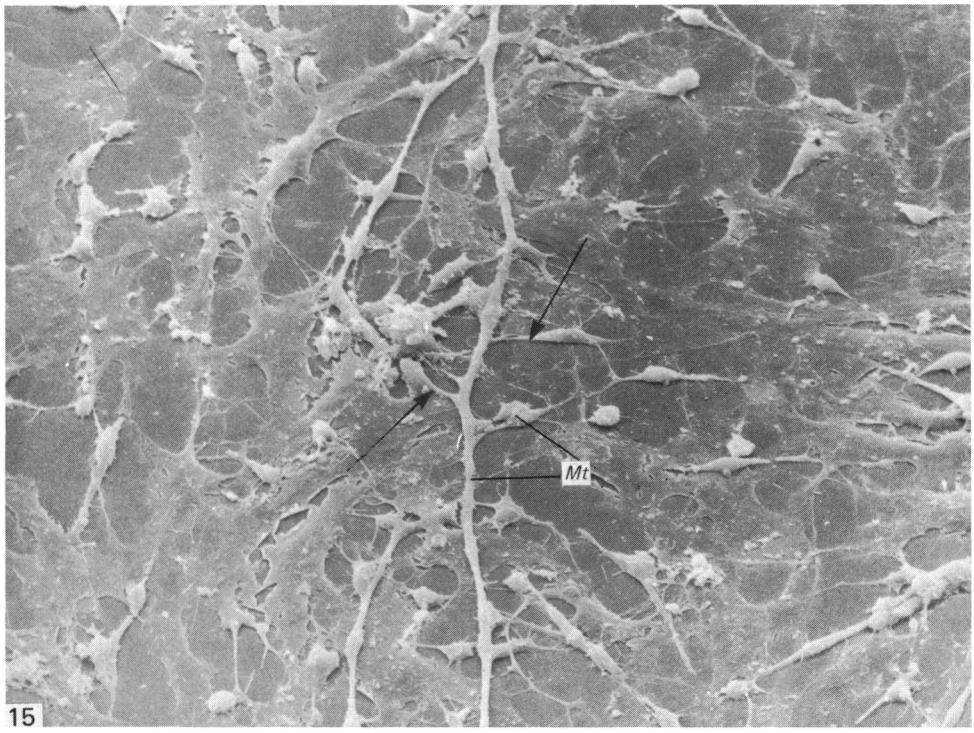


Fig. 14. Transmission electron micrograph of two differentiating muscle cells of a 5 days old culture. The lower cell exhibits patches of differentiating myofibrils (*Mb*), meshes of myofilaments (*Mf*) and lipid droplets (*L*) in the sarcoplasm. Note the differentiation of the dense Z-band materials (*Z*) in rows. The Z-band has not yet differentiated as the continuous band present in a well-differentiated muscle cell.

cells were also found to be fused with the developing muscle fibre in young rats (30 g body weight) (Moss & Leblond, 1971). Recently, Bischoff (1975), using dissociated adult muscle cell suspension, and Konigsberg *et al.* (1975), using segments of mature muscle fibre, reported that a reserve population of mononucleated cells contained between the sarcolemma and basal lamina of the mature fibres possessed myogenic potential. These cells were identical with the muscle satellite cells.

The only alternative explanation of the origin of the myogenic cells is that they originate by the formation of a new plasmalemma around a myonucleus and its



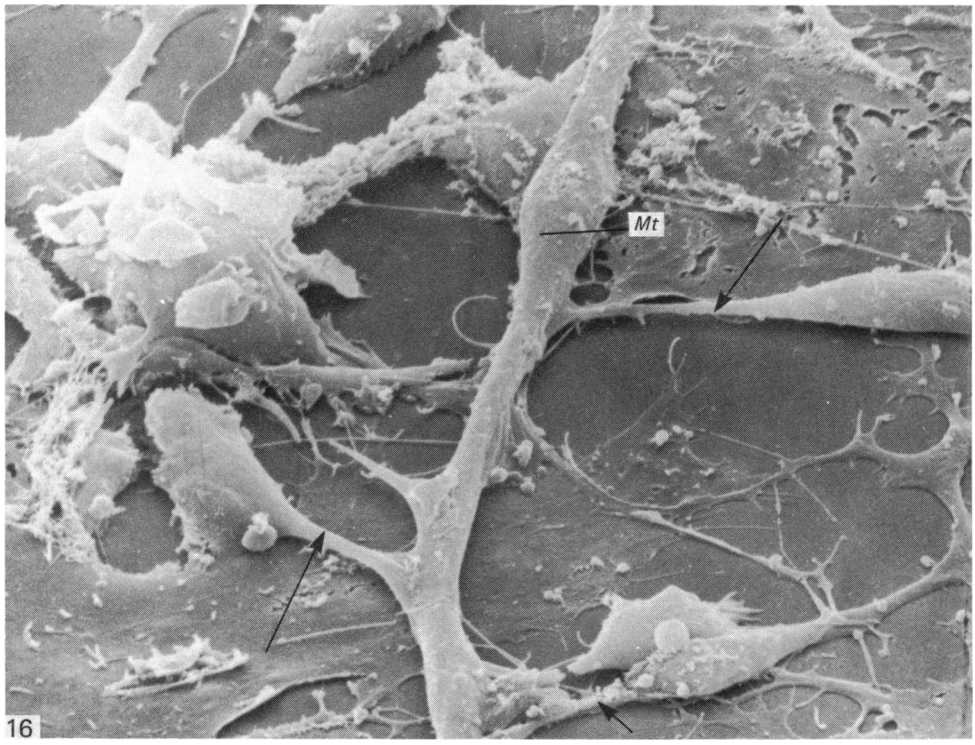


Fig. 15. Scanning electron micrograph of a newly formed myotube (*Mt*) of a 6 days old culture. Some of the myoblasts from both sides of the field are fused with myotube at right angles (arrows). These myoblasts on the sides may give rise to a branching appearance of the mature myotube observed occasionally in the late culture. × 310.

Fig. 16. Higher power scanning electron micrograph of a portion of Fig. 15, showing particularly the participation of the myoblasts at the sides, in the fusion process (arrows). Very commonly the myoblasts align in linear fashion with one another during fusion. × 1500.

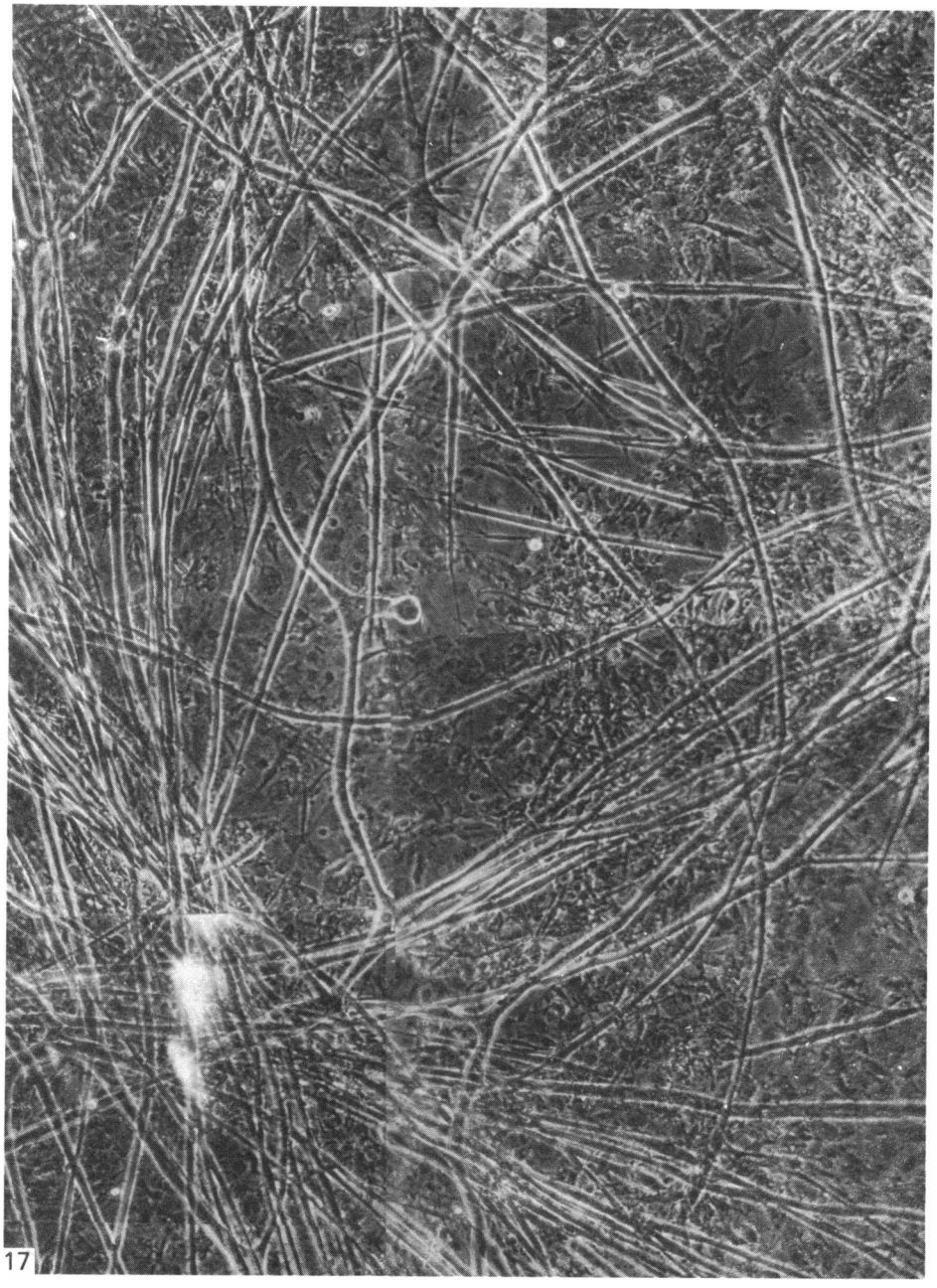


Fig. 17. A composite phase contrast micrograph of à 14 days old culture, showing the well differentiated muscle cells in the field. The cylindrical muscle cells extend in various directions. In the background flattened fibroblastic cells are visible. × 190.

adjacent sarcoplasm (Hay, 1959; Reznik, 1969; Hess & Rosner, 1970), in response to trauma involved in isolating and culturing the cells. If this were true, in the present study the process would have to occur within a relatively short time. Since the time required for enzymic dissociation of myogenic cells is approximately 1 hour, it seems unlikely that these cells originated by a process of fibre fragmentation or budding

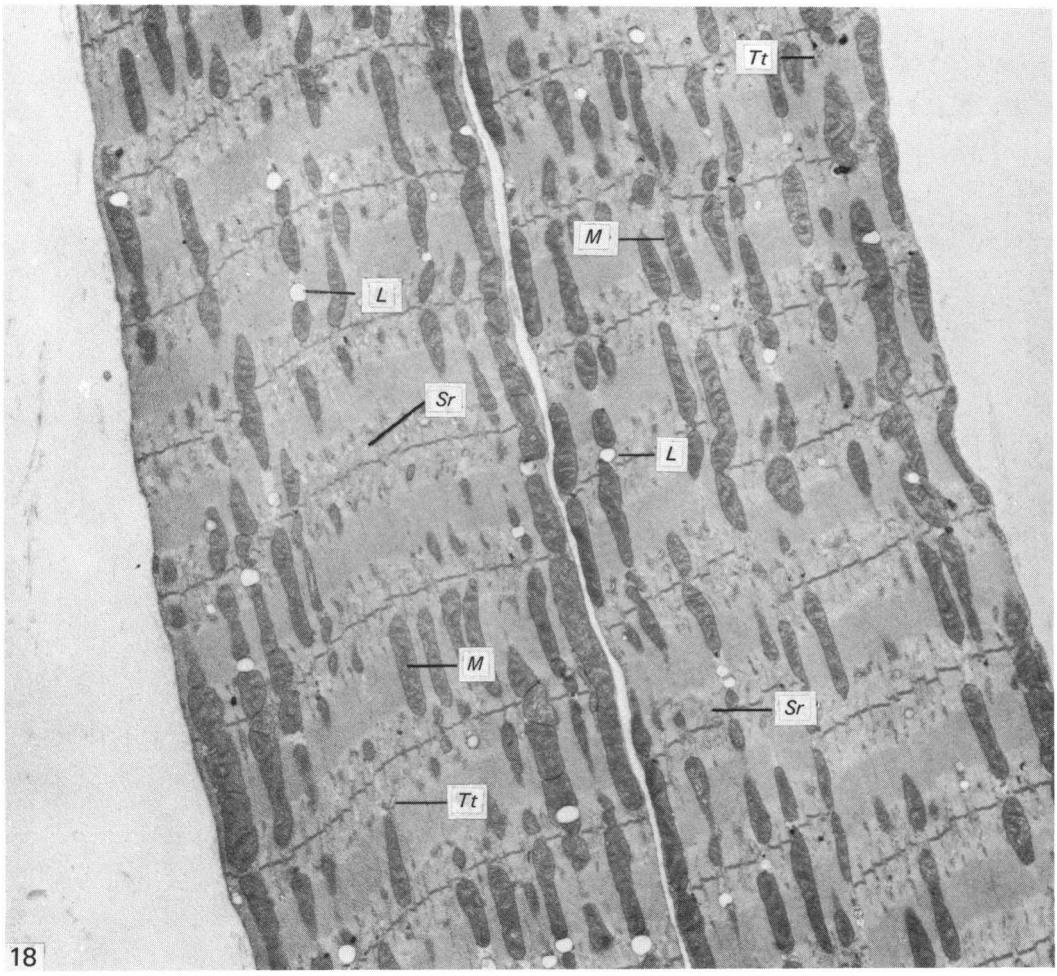


Fig. 18. Transmission electron micrograph of two fully differentiated muscle cells of a 14 days old culture, showing well organized A-, I- and Z-bands. L, Lipid droplet; M, mitochondria; Sr, sarcoplasmic reticulum; Tt, transverse tubules.

which was reported to occur 10–12 hours after compression injury (Teravainen, 1970) and 2 days after denervation (Lee, 1965).

Although the process of fibre fragmentation or budding cannot be ruled out on the basis of time, we have seen no evidence to support it. On the other hand, satellite cells are readily demonstrable in the freshly dissociated cell suspension and subsequently in the culture, thus supporting the hypothesis that they are the source of the myogenic cells. It is now fairly well established that the frequency of satellite cells declines with maturity (Kelly & Zacks, 1969; Muir, 1970; Ishikawa, 1966; Allbrook, Han & Hellmuth, 1971). In the adult rat approximately 1 % of all nuclei within the basement lamina belongs to satellite cells (Ontell, 1974). Proliferation of this small population of myogenic cells in response to injury, and subsequent fusion of their progeny, would sufficiently account for the restoration of the normal complement of myofibres in the injured area within a few days.

The present study has demonstrated that 80 % of the satellite cells are capable of

incorporation of [^{3}H]-thymidine into their nuclei between 24 and 96 hours of culture. This indicates the capability of these cells for DNA synthesis and presumably cell proliferation. Although all these labelled cells did not participate in the formation of myotubes, nevertheless more than half of them did so and formed myotubes which were fully differentiated into muscle fibres. The rationale for the involvement of fewer myogenic cells in the formation of the myotubes is that the culture conditions did not mimic fully the *in vivo* condition which would favour the higher rate of proliferation and differentiation of satellite cells into fully formed muscle fibres.

The present study has demonstrated the sequences of differentiation of the released myogenic and non-myogenic mononucleated cells at different periods of culture. Furthermore, this study focuses light on DNA synthesis and proliferative behaviour of the enzymically liberated mononucleated cells in culture. The finding of similar ultrastructures in *in vivo* satellite cells and in a population of freshly dissociated mononucleated cells during their subsequent culture strongly suggests that the satellite cells are a reserve population of myogenic cells. We believe that these myogenic cells would serve as a source of myonuclei during muscle regeneration in addition to providing a source of myonuclei during normal muscle growth (Moss & Leblond, 1971).

SUMMARY

An injury to adult mammalian skeletal muscle is followed by regeneration, which involves a process believed to be similar to the differentiation of muscle fibres in the embryo. The origin of these differentiating myogenic cells is conjectural. The aim of the present study was to examine the source of myogenic cells and the process of myogenesis in adult skeletal muscle. Mononucleated cells were released from adult rat leg muscle mince after incubation with 0·1 % pronase for 50–60 minutes at 37 °C. The ultrastructural studies revealed that the freshly dissociated mononucleated cells consisted of at least two populations of cells: myogenic satellite cells and non-myogenic fibroblastic cells. These cells were plated in growth media at various densities in cell culture dishes and incubated for 3 weeks in a balanced air atmosphere at 37 °C. The culture was routinely examined with a phase contrast microscope for evidence of myogenic activities of the plated cells. At selected time intervals, the cell cultures were processed for autoradiography and scanning and transmission electron microscopy (SEM and TEM). Attachment of cells to the dish began soon after plating, with flattening of some non-muscle cells. The round- to spindle-shaped cells, indicative of myoblasts, began to appear within 24 hours. DNA synthesis and cell proliferation were observed in myogenic and non-myogenic cells within 24 hours of culture. SEM revealed that at 72 hours some myoblasts aligned and fused with one another, forming myotubes. Quantitation of autoradiographs indicated that the maximum number of labelled myotubes were present in the 3 days old culture, and thereafter, the labelled myotubes decreased in number and were absent in the 7 days old culture. Within 5–7 days the myotubes became larger and showed contractility. TEM of 6 to 21 day cultures revealed that the myotubes contained well differentiated myofibrils, T-tubules and sarcoplasmic reticulum. It was evident from our studies that the mononucleated cells, having satellite cell morphology, were capable of differentiating into fully formed muscle fibres. This study lends support to the satellite cell hypothesis for regeneration of the skeletal muscle.

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