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Electron microscope observations on human fetal striated muscle

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INTRODUCTION

Information concerning the mode of establishment of functional connexions between developing motor nerves and developing muscle in the human fetus is fragmentary. Contact between spinal nerve and myotome is said to occur as early as the 4 mm stage (*c.* 30 days), but the nature of the contact has not been studied in detail (see Frazer, 1940). The nature of the earliest contacts of motor nerves with extrinsic ocular muscles is equally obscure, and as recently as 1976 Landon & Hall referred to the possibility that Schwann cells might be present *in situ* in tissues before the appearance of the axons. In embryos of 5 mm crown–rump length the extrinsic ocular muscles are represented by a single mass of condensed mesenchyme from which individual muscle anlagen become recognizably differentiated by the 10 mm stage (*c.* 5½ weeks of fetal life).

The earliest reflex movement elicitable in the human fetus occurs at *c.* 7½ weeks of fetal life (e.g. Hamilton, Boyd & Mossman, 1962) when some neck muscles contract in response to cutaneous stimulation close to the mouth. In the upper limb careful histological study of the m. biceps brachii (Cuajunco, 1942) suggested that there was no contact between nerve fibres and myoblasts before 10 weeks of development. The further finding that histogenesis was much less advanced in the muscles of the hand conformed with Coghill's (1929) and Hewer's (1934) observations that the structural development of muscle proceeds cephalocaudally. It is not known, however, where the establishment of functional connexions of extrinsic ocular muscles fits into this pattern: the earliest appearance of eye movements is not known and might anyway be obscured to some extent by the fusion of the eyelids until the seventh month of intrauterine life. It is known, however, that the histogenesis and the apparent establishment of motor innervation of extrinsic ocular muscles lag significantly behind the same processes in the intrinsic muscles of the tongue, and in the intercostal muscles and the diaphragm (Hewer, 1934).

Against this background it was decided to investigate the ultrastructure of human fetal extrinsic ocular muscles. In the event, recent advances in the techniques employed to terminate pregnancy have greatly reduced the numbers of fetuses suitable for examination by electron microscopy, so that the present observations have been made on only four specimens of fetal superior rectus oculi muscle.

MATERIALS AND METHODS

Four human fetuses of *c.* 5, 9·2, 12 and 24 cm crown–rump length (approximately 10, 12, 15 and 23 weeks of fetal life respectively) were obtained at hysterotomies.

After removal of the cerebral hemisphere the roof of the orbit was opened and the underlying muscles bathed, *in situ*, in chilled 5 % glutaraldehyde in phosphate buffer at pH 7·3. No contraction of muscles was seen. Some 20–30 minutes later small pieces of superior rectus oculi muscle, from approximately midway between origin and insertion, were dissected out for further fixation (2 hours or more) in buffered glutaraldehyde. After several rinses in a 10 % solution of sucrose, similarly buffered, the tissues were further fixed in buffered 1 % solution of osmium tetroxide for 1 hour. After dehydration and embedding in Araldite, thick sections (*c.* 1 μm) were stained with a mixture of Azur II and methylene blue (Richardson, Jarett & Fink, 1960) and examined by light microscopy. Thin sections were mounted on uncoated grids, stained with uranyl acetate and examined in an A.E.I. EM6B electron microscope.

Serial sections were often obtained, although the exact sequence was not always certain upon the grid. Preservation of tissue was generally good, but mitochondria in more deeply situated muscle cells were sometimes swollen and distorted. In the largest specimen, but not in the others, the plasma and nuclear membranes of some muscle cells had scalloped outlines as a consequence of strong contraction.

RESULTS

5 cm crown–rump length specimen

In this specimen there were a few large nerve bundles. Muscle cells with 'myotube satellite cells' occurred in groups which were often large. The cells contained relatively few myofilaments and were often rich in glycogen. Blood vessels and connective tissues generally were sparse and no motor nerve endings were seen.

Muscle cells could be identified by their content of myofilaments set in characteristic hexagonal arrays. As many as 23 muscle cells were found grouped closely together along with 'myotube satellite cells' or their cytoplasmic processes, but the cells wholly lacked basal laminae and, because of the sparsity of connective tissues, it was often difficult to set limits to a group, or bundle, of muscle cells (Fig. 1). Muscle cells ranged from *c.* 3×5 μm to 7×8 μm when transected through the nuclear region; the nuclei were sometimes centrally, sometimes peripherally placed within the cells, apparently independently of cell size. A Golgi apparatus was often conspicuous close to the nucleus and mitochondria were found in all parts of the cytoplasm except where glycogen was present; lipid inclusions were also occasionally seen. Although rough endoplasmic reticulum was present in small quantities, neither smooth endoplasmic reticulum nor transverse tubules were identifiable at this stage.

The plasma membrane of the muscle cells often showed micropinocytotic vesicles, but 'tongue and groove' (peg and socket) associations between adjacent muscle cells (see later) were very rare and seldom amounted to more than a minor elevation on the surface of one apposed to a minor indentation on the surface of the other. In general a gap of *c.* 20 nm separated adjacent muscle cells, but occasionally this appeared to be obliterated over short distances, and some increased electron density was seen at the point of apparent membrane fusion.

'Myotube satellite cells' were numerous among groups of muscle cells, extending their cytoplasmic processes widely to contact both their fellows and neighbouring muscle cells. 'Myotube satellite cell' cytoplasm was generally of moderate electron density and contained a Golgi apparatus, a few mitochondria and a little granular endoplasmic reticulum; centrioles and a cilium were occasionally seen. A basal

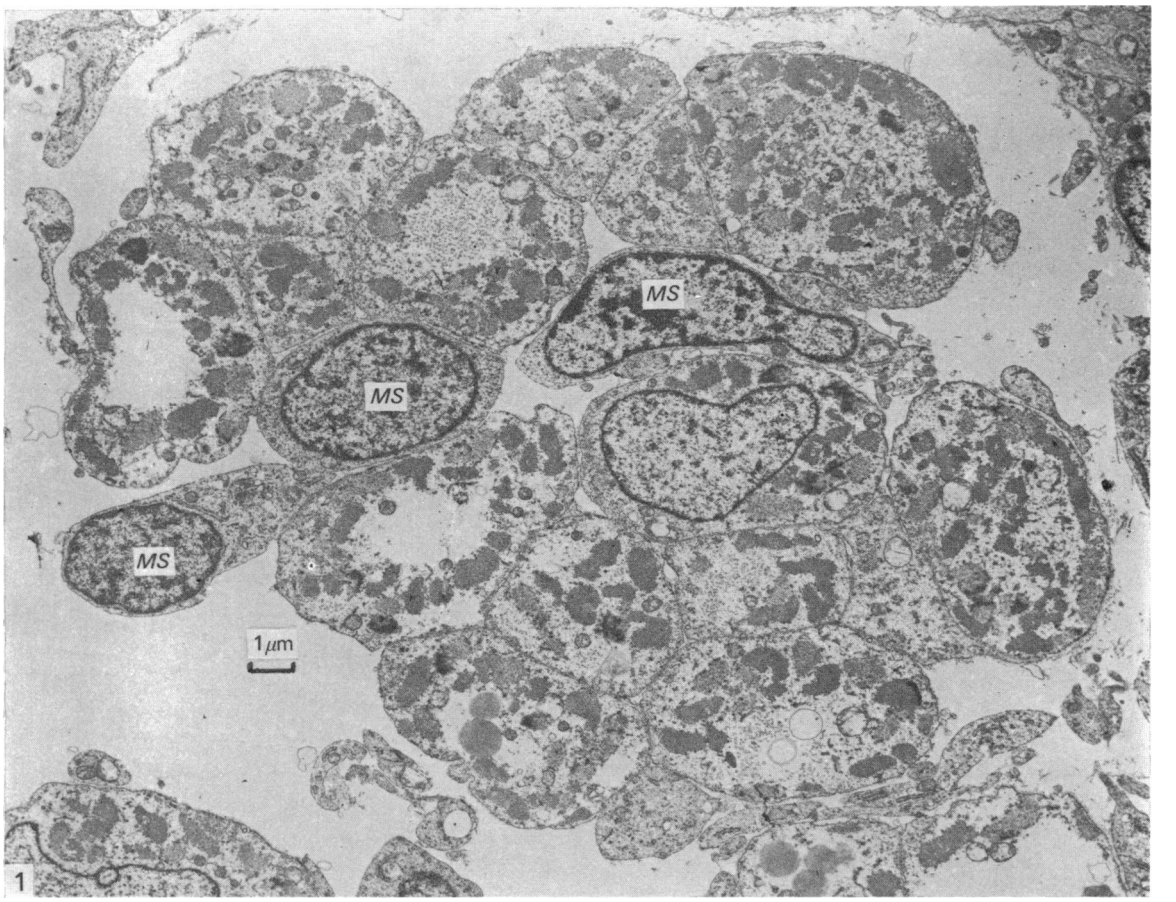

Fig. 1. From superior rectus oculi muscle of 5 cm crown–rump length specimen. A bundle of muscle and 'myotube satellite cells' lies close beside a large nerve bundle. Although the limits of the muscle bundle are ill-defined it contains 14 certain muscle cells (plus one doubtful), three nucleated 'myotube satellite cells' (*MS*) and several small, unidentified cytoplasmic processes. Endomysial collagen is very scanty. × 4800.

lamina was not present in association with these cells. Small cytoplasmic processes assumed to belong to 'myotube satellite cells' were extremely numerous in the muscle cell bundles; many of them were extremely small. They have not been counted in any systematic way, but nuclei have been counted in low power survey micrographs made especially to exclude the tendency, at higher magnifications, to concentrate on especially interesting fields in which, perhaps, nuclei of cells might be unduly prominent. From such survey micrographs there were counted 130 'myotube satellite cell' nuclei associated with 157 muscle cells transected through their nuclei, i.e. the 'myotube satellite cell' nuclei constituted approximately 45 % of the nuclei in muscle bundles.

The nerve bundles seen in this specimen were mainly large (e.g. over 370 axons counted in one bundle) and of rather simple construction, with Schwann cells forming little more than an outer investing layer. Cytoplasmic processes of the Schwann cells did not penetrate among the axons, nor were basal laminae present on the outer aspects of the Schwann cells. The Schwann cell cytoplasm contained a little granular

endoplasmic reticulum, mitochondria and a Golgi apparatus close to the nucleus; centrioles and a cilium were occasionally seen. Axons in the nerve bundles ranged in size from *c.* 0·2 to 2·0 μm in diameter and, in general, were of healthy appearance containing occasional mitochondria set among neurofilaments and neurotubules. Occasionally what appeared to be axon material was seen bounded by an incomplete axonal membrane, or with its components grossly disorganized. Such axon remnants were often set among healthy and well preserved neighbours, so that it is more plausible to attribute their degenerate appearance to ante-mortem changes *in vivo* than to locally inadequate fixation.

In addition to the large nerve bundles a few small ones of *c.* 50 axons were also present, and these were sometimes very close to muscle cells, although nothing resembling a neuromuscular contact was seen.

Neither small nor large bundles were contained within distinct perineurial sheaths, although scattered cells did form a discontinuous investment and perhaps represented the precursors of such a sheath. These cells wholly lacked basal lamina, and their cytoplasmic organelles, Golgi apparatus, mitochondria, granular endoplasmic reticulum, centrioles and occasional cilium, all set in a moderately electron-dense cytoplasm, were similar to those seen elsewhere in 'myotube satellite cells' and Schwann cells.

Collagen fibrils were extremely sparse in this specimen, a very few being seen between the large bundles of muscle fibres and in association with blood vessels and nerve bundles. Blood vessels were themselves rare, of small calibre, and generally empty of blood cells. Except over short distances, basal lamina was absent from the surface of the endothelial cells, and also from the surfaces of closely associated cells on the outer aspect of the vessels – cells which, but for the lack of basal lamina, would doubtless be classified as pericytes.

9·2 *cm crown–rump length specimen*

In this specimen nerve bundles were numerous, often quite small, and widely distributed. Muscle cells occurred in groups of up to 5 or 6, usually associated with 'myotube satellite cells' (Fig. 2); they also occurred in pairs, and singly. Motor end plates were frequently seen, but sensory nerve endings were not recognized. Blood vessels and connective tissues were much more conspicuous than in the smaller specimen and basal laminae were present on muscle cells as well as on the cells of blood vessels; in relation to Schwann cells and 'myotube satellite cells' the presence of basal laminae is uncertain.

Nerve bundles were present in widely differing sizes with axon numbers varying from 4 or 5 to 110 and more. Some of the larger bundles were contained within perineurial sheaths (the cells of which lacked basal laminae) but so too were some

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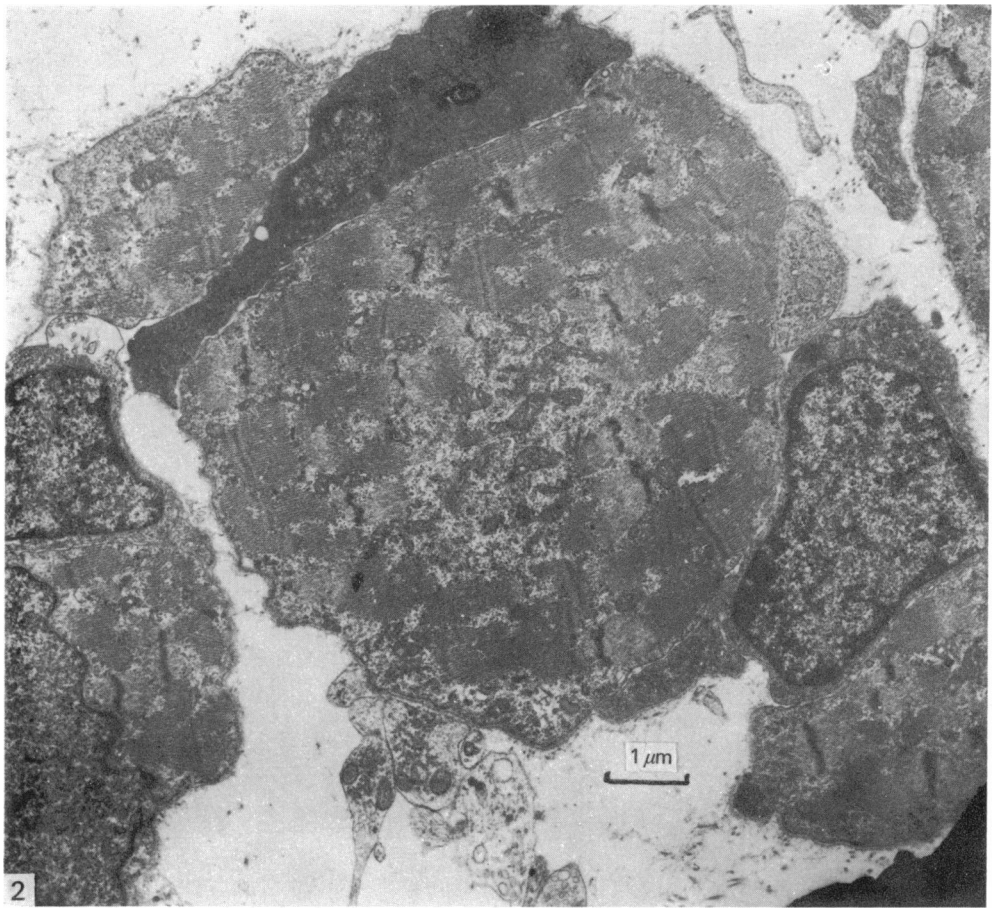
Fig. 2. From superior rectus oculi muscle of 9·2 cm crown–rump length specimen. A muscle cell, of *c.* 8 μm diameter, contacts moderately and very dense 'satellite' cells (lower right and upper left, respectively) with which it shares basal lamina investment. It also makes contact with axonal profiles rich in electron-lucent vesicles; here the plasma membrane is of increased density. × 10000.

Fig. 3. From superior rectus oculi muscle of 9·2 cm crown–rump length specimen. A muscle cell, obliquely sectioned, is closely associated with three electron-dense cells. One of these cells (upper left) contains myofilaments; the other two contain much granular endoplasmic reticulum and many mitochondria. × 10000.



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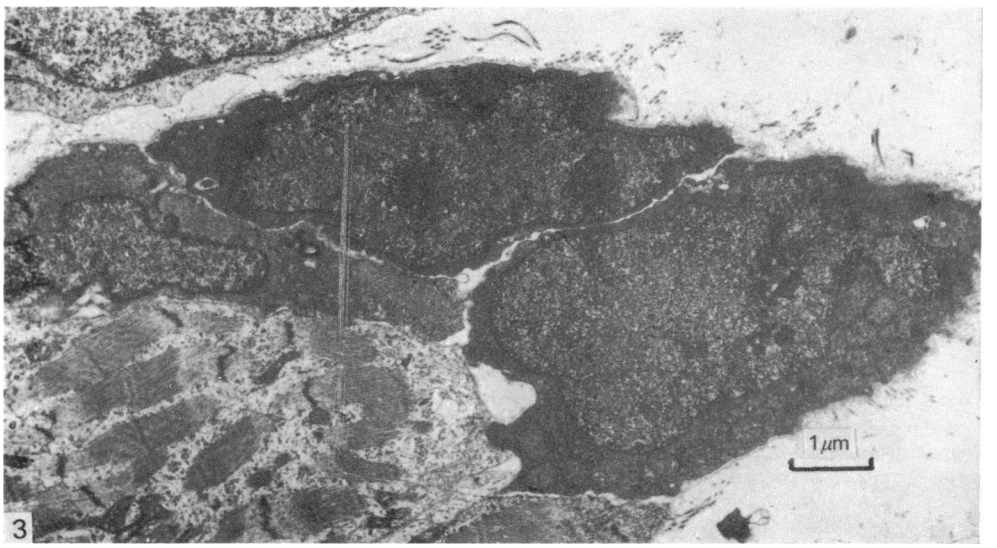

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comparatively tiny bundles; mere size of nerve bundle did not, apparently, determine the presence or absence of a sheath.

Schwann cells of these nerve bundles were sometimes of very complex form and were (except where close to their terminations upon muscle cells) invested by basal laminae. In addition to a Golgi apparatus, granular endoplasmic reticulum, centrioles and cilium (as seen also in the younger specimen), dense bodies of comma or dumb-bell shape, and small lipid inclusions, were frequently present.

Axons ranged in size from *c.* 0·2 to 2·0 μm in diameter, the great bulk of them sharing a communal investment by Schwann cell cytoplasmic processes; a very small number were singly invested by Schwann cell processes. As in the younger specimen, occasional axons appeared degenerate among their healthy, well-fixed neighbours.

The muscle cells of this specimen, although of similar calibre to those in the younger specimen, seemed generally to be more densely packed with myofilaments, so that glycogen, when present, occupied only a small part of the cross sectional area of a cell. Nuclei were sometimes centrally and sometimes peripherally located, apparently independently of cell diameter. Cells containing only a few myofilaments were extremely rare. Some muscle cells were of extreme electron density (Fig. 3) so that only very close inspection revealed the presence of myofilaments. In the more 'normal' (and vastly more numerous) muscle cells the same cytoplasmic organelles were present as in the younger specimen, as well as a little sarcoplasmic reticulum. Nothing resembling triads was seen. Although the bulk of the specimen was sectioned transversely, some fibres were sectioned obliquely at the periphery of the block, allowing the certain identification of M bands in the myofibrils (Fig. 2).

Neuromuscular contacts occurred frequently, as many as eight axonal terminals (some richly supplied with electron-lucent vesicles) making contact with a single muscle cell, with only its basal lamina intervening (Fig. 4); axonal terminals occasionally contained mitochondria. Other axonal profiles, close to but not in contact with muscle cells, frequently contained many electron-lucent vesicles. Schwann cell cytoplasm was almost always closely associated with axonal terminals contacting muscle to give a more or less complete investment. Schwann cell nuclei were frequently present in the immediate neighbourhood of such contacts.

The muscle cell at a neuromuscular contact usually exhibited increased density and thickness of its plasma membrane, and occasionally small indentations of the surface were seen. Frequently the muscle cell also showed a local concentration of mitochondria or, at least, a local absence of myofilaments.

'Myotube satellite cells' were numerous in this specimen, sharing basal laminae with associated muscle cells, and provided with extensive cytoplasmic processes: these processes often extended through the investing basal lamina to contact fibro-

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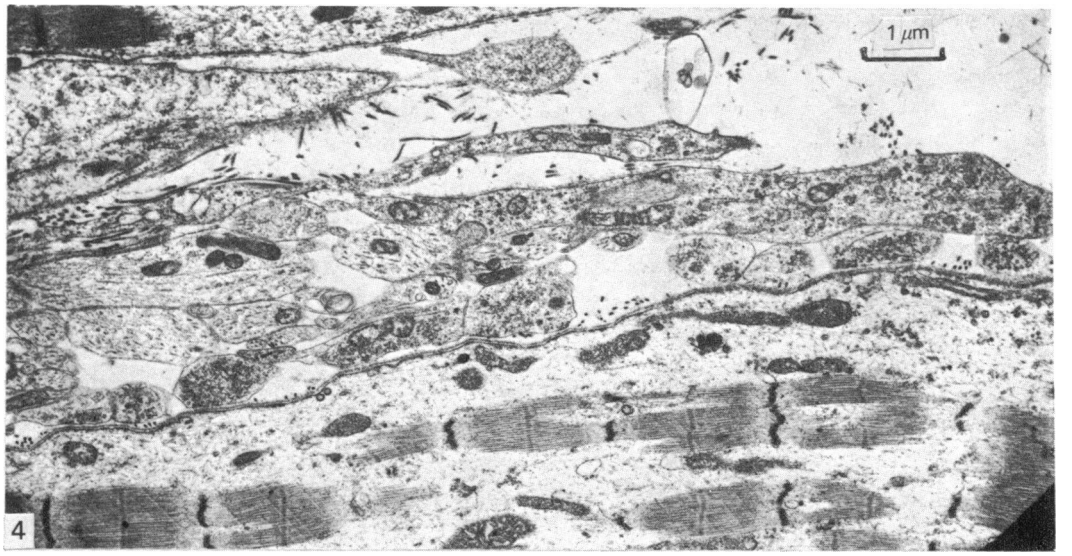
Fig. 4. From superior rectus oculi muscle of 9·2 cm crown–rump length specimen. A muscle cell, cut longitudinally, shows M banded myofibrils set in a rather pale cytoplasm containing mainly elongated mitochondria. Some 20 μm of the plasma membrane is of increased electron density and thickness and confronts seven axonal profiles, each of which is rich in electron-lucent vesicles. × 10000.

Fig. 5. From superior rectus oculi muscle of 12 cm crown–rump length specimen. Two immature muscle cells (*IM*) containing only few myofilaments extend cytoplasmic processes into invaginations of the surfaces of two other more mature muscle cells (*MM*). A continuous basal lamina runs across the outer surface of all four muscle cells. × 15000.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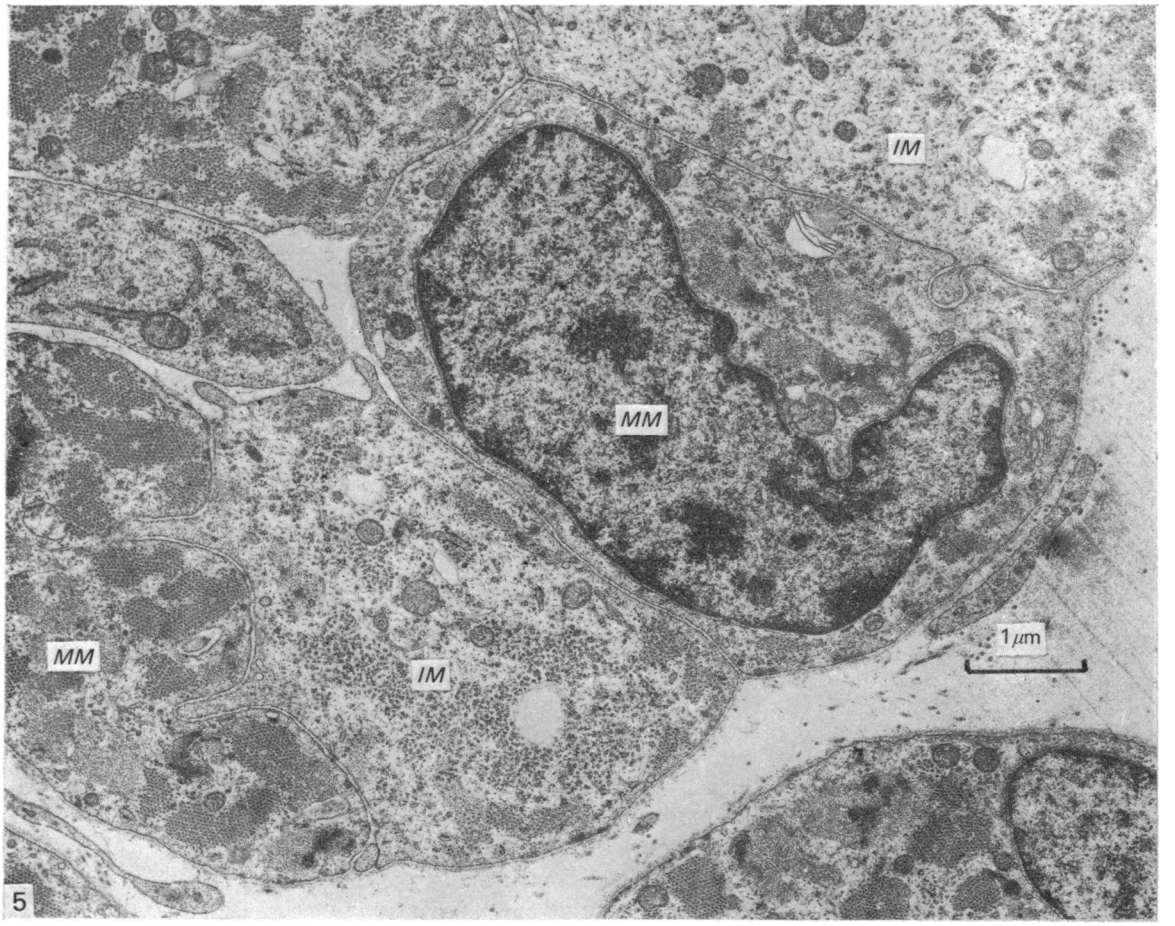
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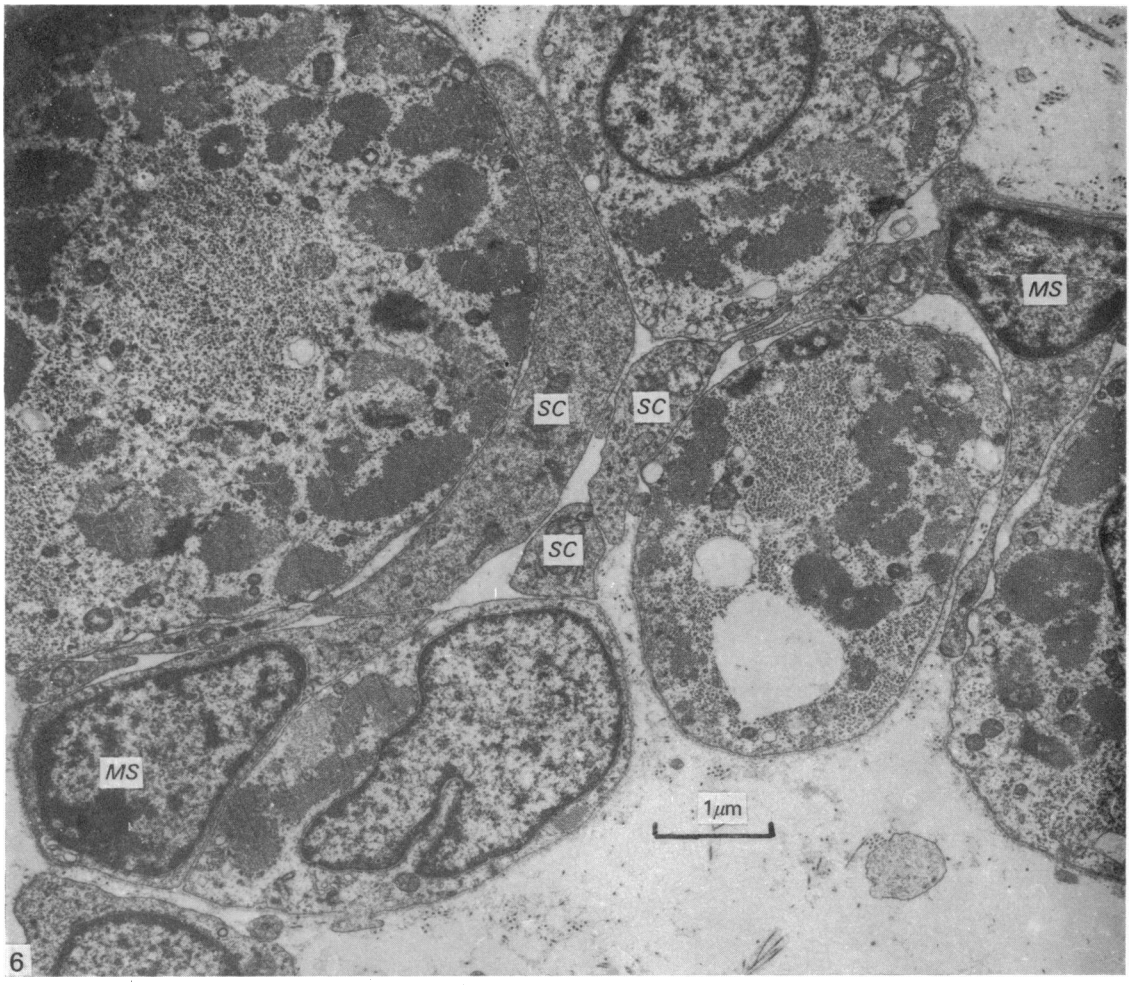


Fig. 6. From superior rectus oculi muscle of 12 cm crown–rump length specimen. A bundle of muscle cells is permeated by 'myotube satellite cells' (*MS*) or their processes (*SC*). The greater number of these lie within the basal lamina investing the muscle bundle. ×15000.

blast-like cells in the endomysial connective tissue, as well as contacting similar cells within the muscle bundle. Some 'myotube satellite cells' were of moderate electron density and contained a Golgi apparatus, granular endoplasmic reticulum, centrioles and cilium as in the younger specimen; others were of very extreme electron density (Fig. 2) and were identified by their form and by their basal lamina shared with muscle cells. As in the 5 cm crown–rump length specimen, counts of muscle and 'myotube satellite' nuclei have been made from low magnification electron micrographs; there were 101 satellite cell nuclei in fields containing 134 muscle cell nuclei, i.e. the 'myotube satellite cells' constituted approximately 43 % of all the nuclei present in the muscle bundles.

Collagen fibrils were very numerous in the endomysium of this specimen and were perhaps most conspicuous in association with the nerve bundles. There was no indication of any local concentration of collagen fibrils close to the fibroblasts and their processes in the endomysium.

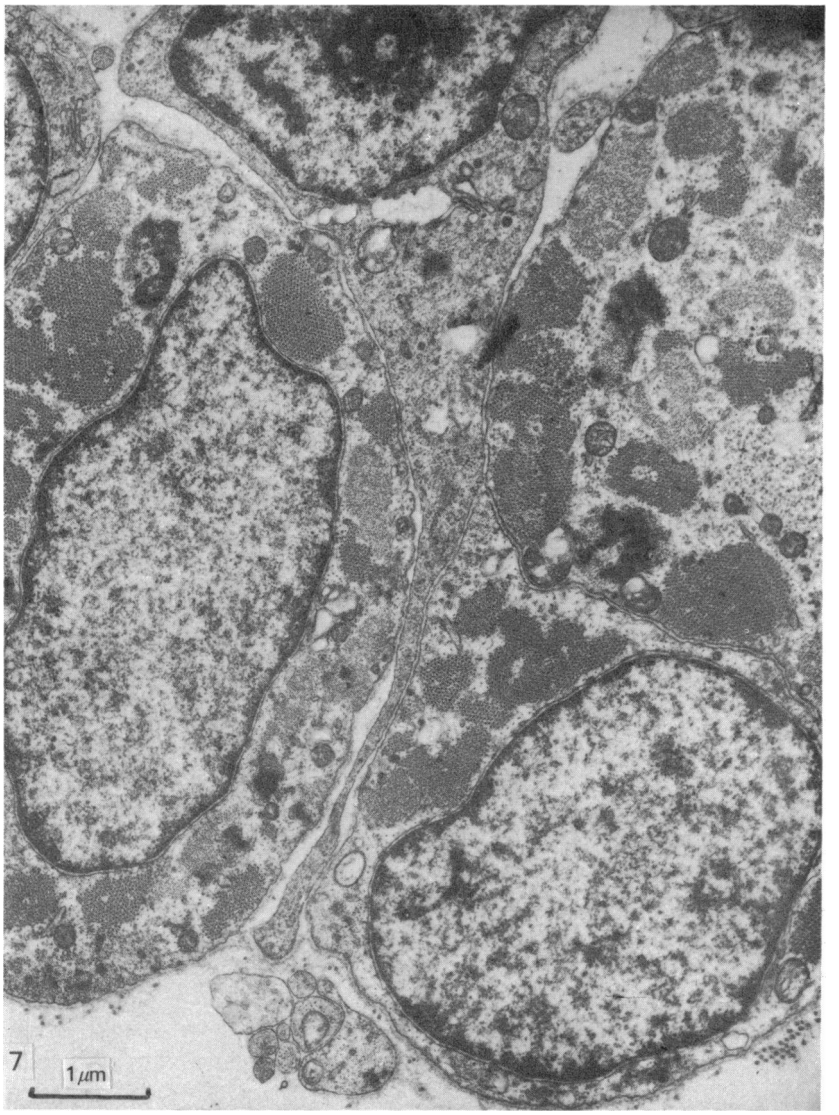


Fig. 7. From superior rectus oculi muscle of 12 cm crown–rump length specimen. A slender elongated cytoplasmic process of a 'myotube satellite cell' extends between two closely associated muscle cells towards a tiny Schwann cell/axon complex. The basal lamina common to the two muscle cells intervenes. ×15000.

12 *cm crown–rump length specimen*

In this specimen muscle cells occurred singly or, invested by a shared basal lamina, in pairs or in small groups (up to four cells). In any of these situations they were frequently associated with 'myotube satellite cells' which shared the basal lamina investment. The muscle bundles were set in an endomysium much richer in collagen fibrils than in the younger specimens, and containing numerous blood vessels, together with large and small nerve bundles. Many neuromuscular contacts were present.

Cytoplasmic organelles in the muscle cells were similar to those in the smaller specimens but the range of cell diameters was greater, extending from 3×5 to $8 \times 9\,\mu$m through the nuclear region of the cell. The myofilament content of the cells was by no means uniform and on this criterion two rather different sorts of muscle cell were frequently seen, lying side by side, and sharing investment by a continuous basal lamina. Commonly a cell rich in myofilaments was grooved, or socketed to receive tongues, or pegs, of cytoplasm from an adjacent cell very conspicuously less endowed with myofilaments (Fig. 5). Desmosome-like thickenings of the adjacent plasma membranes sometimes occurred close to the 'tongue and groove', as did some undercoating of the plasma membranes. In muscle cells well endowed with myofilaments other organelles appeared to occupy, in random fashion, such spaces as remained; the fibrils sometimes so filled the cell as to constitute a 'Felderstruktur'. In cells equipped with tongues (or pegs) of cytoplasm, myofilaments were often extremely scanty (although their characteristic array in cross section allowed certain identification) so that other organelles were very widely, as well as apparently randomly, scattered through the cytoplasm. Smooth endoplasmic reticulum and transversely oriented tubules were very occasionally seen in both kinds of muscle cell, but triads were never identified.

More complex forms of association between well- and ill-developed muscle cells were seen also. A cell moderately endowed with myofilaments extended tongues of cytoplasm into sockets of a better endowed neighbour while at the same time providing sockets to receive pegs from another ill-endowed neighbour. Occasionally an ill-endowed cell extended tongues of cytoplasm into sockets on two well-endowed neighbouring cells, or a well-endowed cell provided sockets to receive processes from two ill-endowed neighbours. The linear extent of the tongues and grooves in the long axis of the muscle cells is not known, but examination of near serial sections showed changes in their outlines without, necessarily, much change in the overall profile of the muscle cell.

'Myotube satellite cells' seemed to occur in two distinct forms in this specimen. One form occurred singly in association only with a muscle cell (or cells), and was contained wholly within a common basal lamina. The cytoplasm was scanty and sparsely provided with organelles set in the narrow perinuclear part or within short cytoplasmic processes – organelles consisting of a few mitochondria and, rarely, a little granular endoplasmic reticulum. The other form of 'myotube satellite cell' often occurred in small groups, again in intimate association with muscle cells. The cytoplasm was of much greater extent and often prolonged into processes extending over and between adjacent muscle cells to contact processes and cell bodies of similar cells (Figs. 6, 7). Some cytoplasmic processes were frequently seen perforating and extending beyond the investing basal lamina to traverse the endomysial spaces (Figs. 9–12) and there effect further contacts with similar processes, and with Schwann cells of small nerve bundles. In cells of this kind, a Golgi apparatus, granular endoplasmic reticulum and mitochondria were often conspicuous; and a centriole, or cilium, or both were not infrequently seen (Fig. 8). The electron-dense inclusion, dumb-bell or comma-shaped, of the kind frequently seen in Schwann cells, also occurred in this form of 'myotube satellite cell'. This type of 'myotube satellite cell', then, appeared to form a network within and between the muscle cell bundles, and forming contacts with Schwann cells. As in the smaller specimens, counts of muscle cell and 'myotube satellite cell' nuclei have been made from low magnification survey electron micrographs: there were 52 'myotube satellite cell'

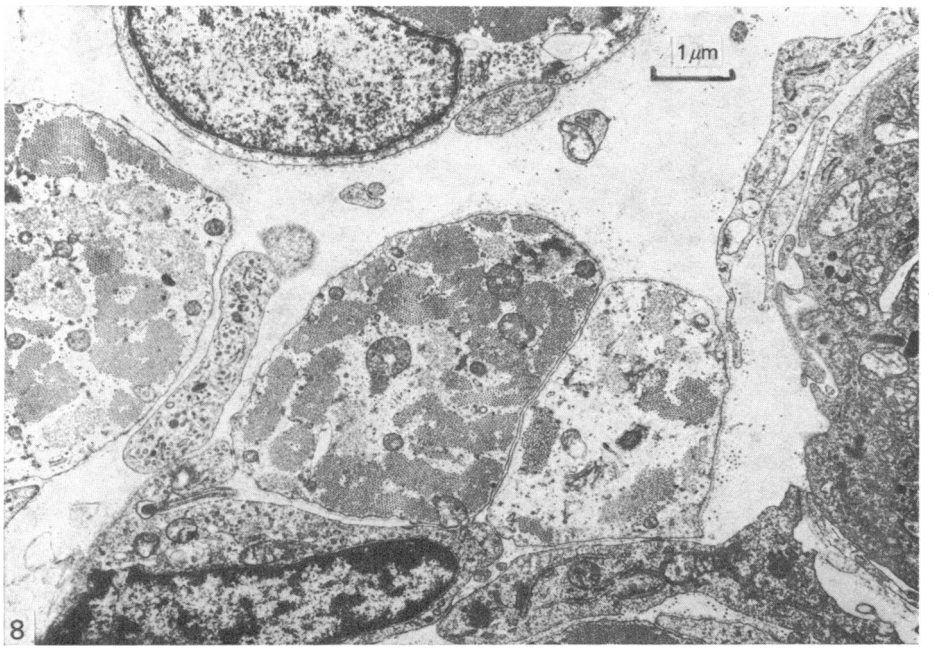


Fig. 8. From superior rectus oculi of 12 cm crown–rump length specimen. Two adjacent muscle cells share investment by a basal lamina; but investment is incomplete so that additional contacts are made with adjacent 'myotube satellite cells', which also contact each other. One 'myotube satellite cell' is equipped with a cilium. ×10000.

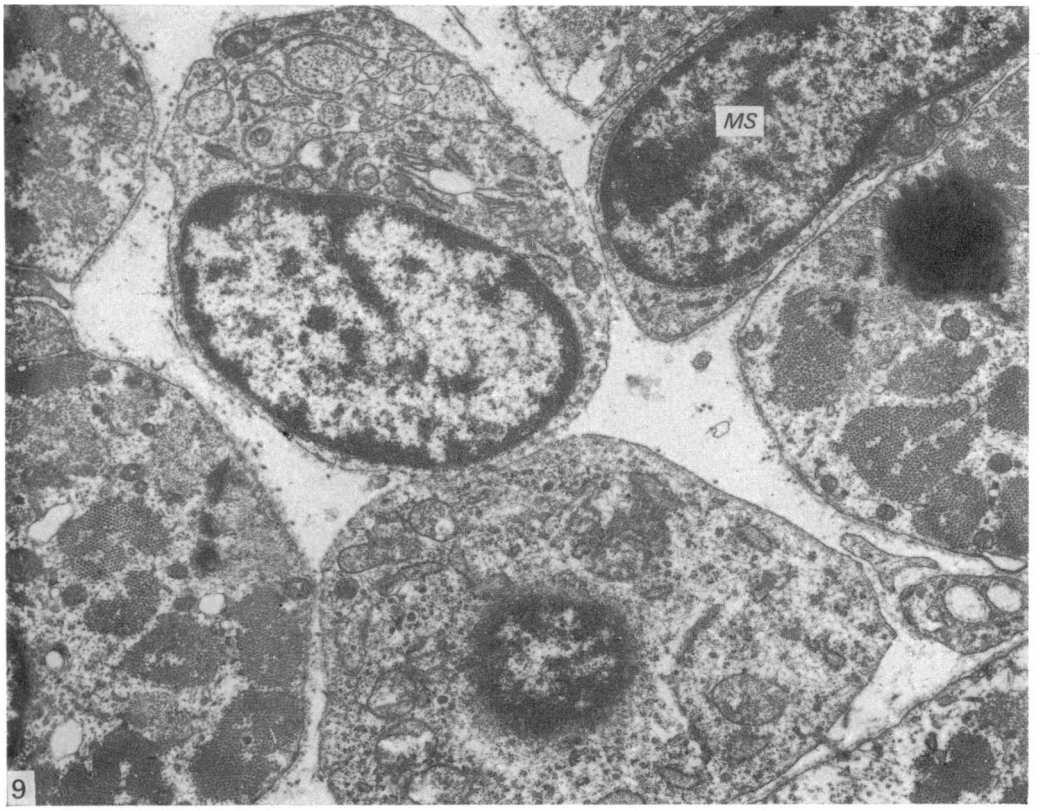
nuclei in fields containing the nuclei of 77 muscle cells, i.e. 'satellite' cells constituted 40 % of the nuclei within the muscle bundles.

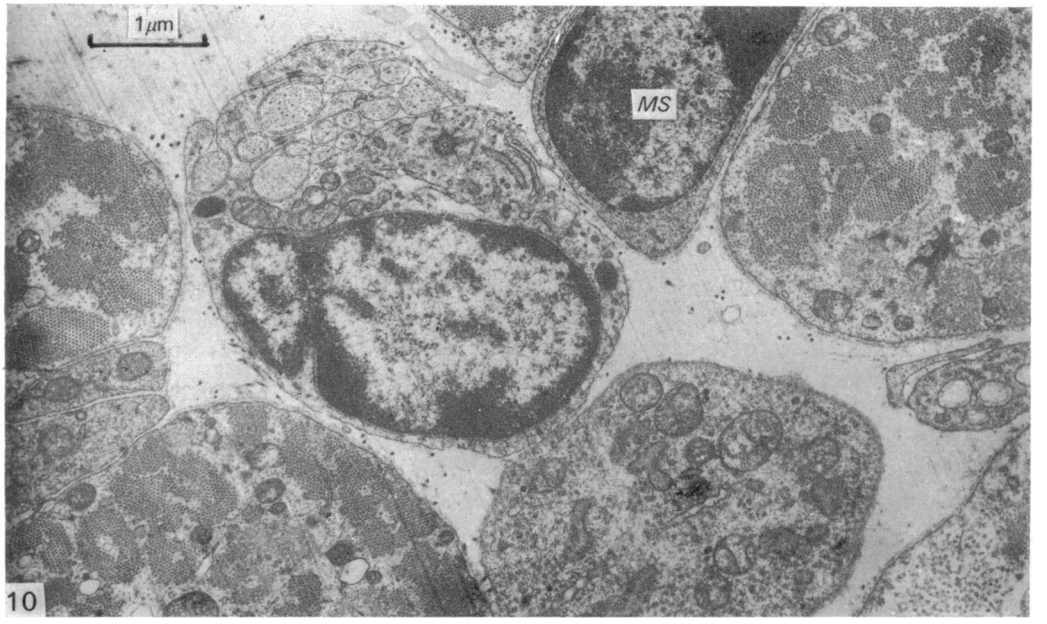
Running through the muscle were nerve bundles of all sizes, from one axon invested by a tiny Schwann cell process to as many as 324 axons associated with Schwann cells to produce ten Schwann cell/axon complexes. The larger nerve bundles were commonly invested by perineurial sheath formed by the attenuated cytoplasmic processes of fibroblast-like cells arranged in one or two layers. The Schwann cells were invested by conspicuous and continuous basal laminae. The smaller nerve bundles contained Schwann cells in which basal lamina was sometimes complete, sometimes incomplete, and sometimes wholly absent.

Schwann cells were sometimes of very bizarre form (when seen in cross section) as they extended around and between the associated axons; sometimes merely shallowly indented. In most cases, however, the Schwann cell cytoplasm looked very active, with many free ribosomes as well as a Golgi apparatus, granular endoplasmic reticulum, dumb-bell or comma-shaped dense bodies, centrioles and a cilium.

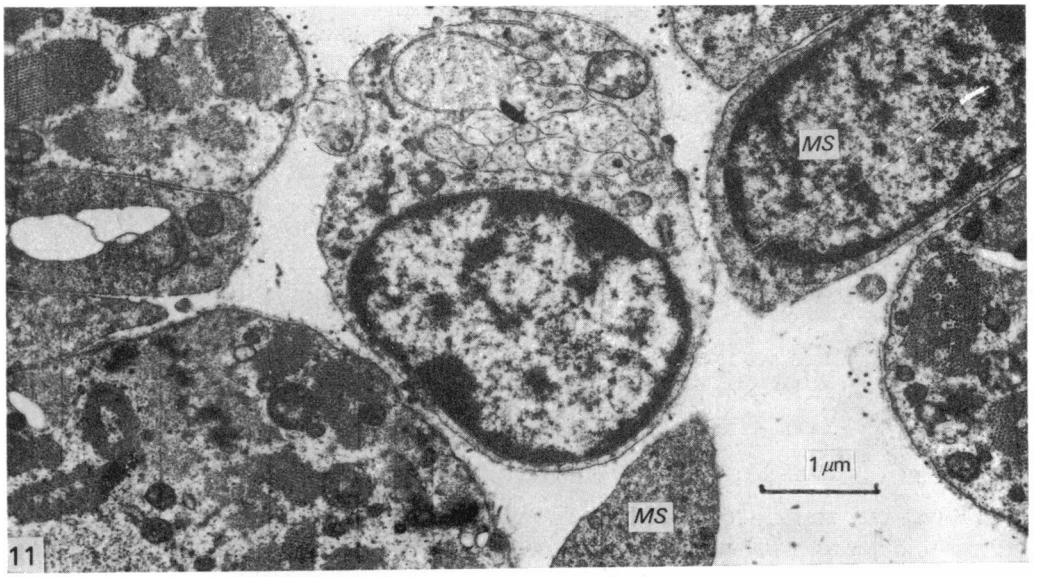
Axons ranged in size from 0·1 to 2·0 μm in diameter, and although some were singly invaginated by Schwann cell processes, none was seen with an elongated mesaxon indicating the beginning of myelin formation. Axons for the most part appeared well preserved, with neurotubules, neurofilaments and occasional mitochondria; some few, however, appeared empty of the usual organelles, or were bounded by an incomplete axonal membrane and were, perhaps, undergoing degeneration. Some Schwann cell/axon complexes contained tiny, rounded electron-dense profiles of unknown nature.

Contacts between axons and muscle cells were seen frequently in this specimen.





Figs. 9–12. From superior rectus oculi muscle of 12 cm crown–rump length specimen. In these near serial sections, a Schwann cell, associated with axons, lies among muscle and 'myotube satellite cells'. The nucleated 'myotube satellite cell' (*MS*) at upper right in Fig. 9 is seen to extend a cytoplasmic process toward another muscle bundle making extensive contact with the Schwann cell *en passant*. ×15000.



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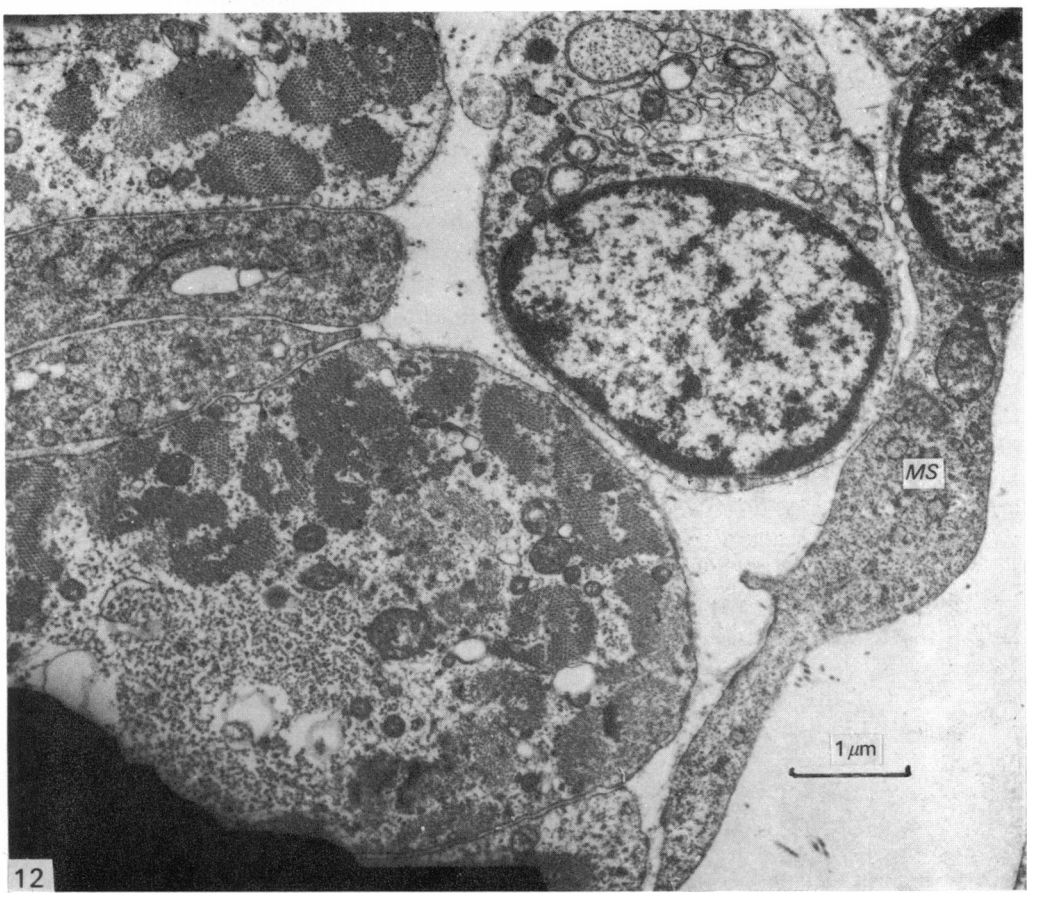

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Basal lamina of the muscle was always continuous across the junctional region, where the underlying muscle plasma membrane was usually thickened and of increased electron density. Folding of the membrane with duplication of the basal lamina was seen, although it recurred only rarely. Axonal terminals (and their preterminal stalks, close to muscle) usually contained an accumulation of vesicles, mostly electron-lucent, but occasionally there were larger ones with an electron-dense core. These terminals were usually small (of *c.* 0·4 μm diameter), but some larger ones sometimes contained one or two small mitochondria. Schwann cell cytoplasm usually invested axonal terminals on all those surfaces not applied to the muscle cell; it was, in its turn, invested by basal lamina, continuous on to the muscle cell surface generally, and into the interval between muscle cell and axonal terminal.

Axonal terminals sometimes confronted a local accumulation of mitochondria within a muscle cell whose plasma membrane showed thickening, increased electron density, and (very occasionally) indentation. Often the muscle confronting an axonal terminal showed no more than a local paucity of myofilaments; often it did not show any features of note.

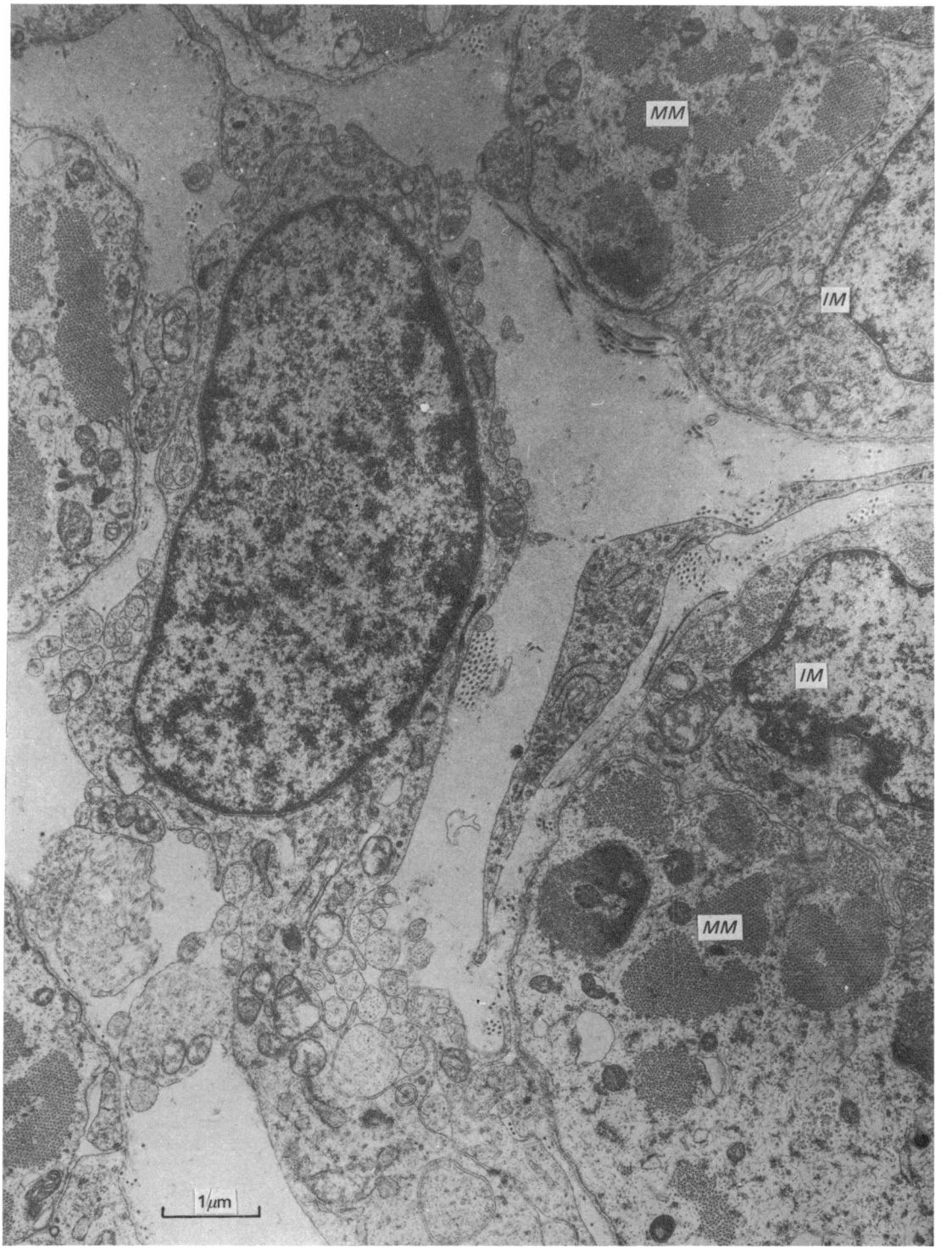
Where well- and ill-endowed muscle cells occurred in 'tongued and grooved' pairs it was not uncommon to see axonal terminals in contact with the well-endowed muscle cell, but not in contact with the ill-endowed neighbour. In Figure 13 a Schwann cell/axon complex lies between seven muscle cells: a well-endowed single fibre and three pairs of muscle cells, two of the pairs being formed by tongued and grooved cells. All four of the well-endowed muscle cells make contacts with axonal terminals; none of the ill-endowed cells does so, at least in the plane of this section.

24 cm crown–rump length specimen

In this specimen the preservation of cellular organelles was generally good except where some muscle cells had contracted markedly so that plasma membrane and nucleus were very crinkled and sarcomeres were of *c.* 1 μm in length. Most muscle cells, however, had maintained perfectly smooth outlines; and a sarcomere length of *c.* 2·0 μm (with M band clearly visible) was seen in some fibres cut longitudinally.

In many respects the tissues in this specimen resembled those seen in the 12 cm crown–rump length specimen except for a marked difference in the form of the 'myotube satellite cells', which now were almost all rather simple cells, elongated alongside the associated muscle cell, and equipped with scanty perinuclear cytoplasm poor in organelles and lying wholly within the basal lamina which they shared with the muscle cell. In some cells the cytoplasm was of moderate electron density, in others of extreme electron density, but no other differences were seen. Cytoplasmic branches were rare, so that the cells did not form a network within the muscle bundles nor (being wholly confined within basal laminae) between adjacent bundles in association with other 'myotube satellites' and with Schwann cells. Counts made in low magnification survey electron micrographs showed the nuclei of 85 'myotube satellite cells' in fields containing the nuclei of 177 muscle cells, i.e. 'satellite' nuclei constituted *c.* 32 % of all the nuclei in muscle bundles.

Fig. 13. From superior rectus oculi muscle of 12 cm crown–rump length specimen. A Schwann cell associated with axons lies among muscle bundles. At upper and lower right are pairs of muscle cells, one in each pair more mature (*MM*) and one immature (*IM*); sockets and pegs are visible. Axonal profiles contact the two more mature muscle cells (and two other muscle cells at upper and lower left), but do not contact the two immature muscle cells. × 15000.


MM
IM
IM
MM
1 μm

Muscle cells in this as in the other specimens contained only a little sarcoplasmic reticulum distributed in random fashion. Tubules were occasionally seen, but not triads, and the only evidence of greater maturity was the almost universal peripheral location of the muscle nuclei; only one muscle nucleus was seen centrally located within the cell. All muscle cells were invested by basal lamina, and while many occurred singly (often associated with a 'myotube satellite cell'), others occurred in pairs, or in groups of three or four. The largest fibres seen were *c.* 10 μm in diameter.

Nerve bundles were conspicuous in this specimen, set in a well developed endomysium of collagen and fibroblasts. The larger bundles were sometimes contained within a perineurial sheath formed by flattened cells wholly lacking basal lamina, but containing endoplasmic reticulum close to the nucleus. Schwann cells were rich in cytoplasmic organelles, including a Golgi apparatus, granular endoplasmic reticulum, dense bodies of dumb-bell or comma-shape as well as centrioles and a cilium. All Schwann cells seen were invested by basal lamina.

In the larger nerve bundles, of several Schwann cell/axon complexes, as many as 30 axons were invested communally: such axons ranged from 0·1 to 2·5 μm in diameter. Many axons, of *c.* 0·3–2·5 μm diameter were invested singly, although no sign of myelin formation was ever seen: and some axons appeared to be wholly free of Schwann cell investment, being covered only by basal lamina.

Axonal contacts with muscle cells were quite frequently seen, but were not obviously more differentiated than those seen in the 12 cm crown–rump length specimen.

Blood vessels were numerous in the endomysium, the majority being very small and empty of blood cells. In some vessels the wall consisted of endothelium and basal lamina only, but in others pericytes and smooth muscle cells were identified. Very rarely, tiny nerve bundles were seen close to the thicker walled blood vessels, but no axonal contact with a smooth muscle cell was ever seen.

DISCUSSION

The development of mammalian skeletal muscle has been described in some detail by Kelly & Zacks (1969*a*, rat intercostal muscle) and by Church (1969, fruit bat web) where the events occurring are similar in nature and sequence (with some exceptions) to those reported here in developing human extrinsic ocular muscle. The time scale of events, however, is very different; events which occupy a few days in the rat, or a few weeks in the fruit bat, appear to be spread over many weeks in human development (see also Ishikawa, 1966), as might be expected in view of the very different gestation periods in these species.

In the rat, the last 5 days before birth appear to suffice for development to proceed from the first appearance of myotubes (as members of large groups of cells, many of which are undifferentiated) to the stage when they (and several later generations of myotube) almost always had peripherally placed nuclei, were packed with myofibrils and were individually ensheathed by basal lamina. Almost all, besides, made contact with axonal terminals, although not all of these were of the mature motor end plate form (Kelly & Zacks, 1969*b*). At the eighteenth day of gestation (and apparently at the twentieth also) complex interdigitations of the contiguous plasma membranes of more and less mature muscle cells were present (Kelly & Zacks, 1969*a*; Fig. 8), an arrangement identical with those seen in the rectus oculi muscle of the 12 cm crown–rump length specimen of the present report, the age of which has been estimated to be *c.* 15 weeks. Kelly & Zacks (1969*a*) were of the opinion

that all the undifferentiated cells present in their specimens either differentiated to form later generations of myotube (having been myoblasts prior to this) or, if present in the muscle at the time of birth, persisted as satellite cells of the muscle. As described in their papers, these cells are of less active appearance than some of those present in developing human rectus oculi muscle, although they are capable of mitosis and extend long cytoplasmic processes between adjacent muscle cells. Figures 9 and 10 of Kelly & Zacks' paper also show that they may contain local concentrations of mitochondria and distended cisternae of granular endoplasmic reticulum.

In the web of the fruit bat, Church (1969) was able to recognize myotubes at 3½ weeks' gestation, but basal laminae did not become recognizable before 6 weeks, when it covered the outer aspect only of aggregated muscle cells. It did not cover what were thought to be early satellite cells until the eighth week, by which time it also covered individual muscle cells. Basal lamina was not seen on any cells of a 5 cm crown–rump length specimen (*c.* 10–11 weeks) of human rectus oculi muscle, although it was present, apparently, at the ends of the gastrocnemius muscle of a 4 cm crown–rump length human fetus studied by Ishikawa (1966).

The breakdown of muscle cell aggregates in the fruit bat extended over a lengthy period, so that cells occurred in groups of 2–6 at 8 weeks; as singletons (50 %) and in groups of 2–3 (50 %) at 15½ weeks; and as singletons only at 19 weeks. This process is far more rapid in the rat, as noted above, but much retarded in developing human muscle, where large groups of muscle cells were present in the 5 cm crown–rump length specimen and (while some singletons were seen in the 12 cm crown–rump length specimen) many groups of 3–4 cells were present even in the 24 cm crown–rump length specimen of some 26 weeks' developmental age. Ishikawa (1966) found groups of 5–10 muscle cells in his specimen of 22 cm crown–rump length (*c.* 25 weeks).

The migration of muscle cell nuclei from a central to a peripheral location was found to be almost complete at birth in the rat. It had begun in 12 weeks (estimated) specimens of the fruit bat, but it is uncertain when the process is completed. In the human rectus oculi muscle, it had begun in the smallest specimen (of 5 cm crown–rump length) and was apparently almost complete in the largest (of 24 cm crown–rump length), conforming very largely with events recorded by Ishikawa in developing human gastrocnemius muscle.

A dual nature of human extrinsic ocular muscle has been emphasized by Lockett (1968), and discussed by Breinin (1971) among others, but such features as size (cross sectional diameter) and myofilament content (whether of 'Fibrillenstruktur' or 'Felderstruktur') in incompletely developed muscle cells can hardly be related to adult structure. Small diameter muscle cells still may grow, and filament (and fibril) density may change, as glycogen content becomes reduced or sarcoplasmic reticulum content becomes increased. Features of adult muscle fine structure which enable one to categorize them as 'twitch' or 'slow' are not present in fetal muscle: sarcoplasmic reticulum is sparse and apparently randomly distributed, while transverse tubules have not been identified with any certainty. (It is well known, of course, that sarcoplasmic reticulum and transverse tubules develop relatively late, as Schiaffino & Margreth (1969) and Muir (1974) have shown.) It is certain, however, that many fibres show M bands in their myofibrils, suggesting that fast fibres are present (Page, 1965; Miller, 1967), although there is no evidence of their single innervation by which their identification might have been made assured (Cheng-Minoda *et al.* 1968).

It is well established that there is early 'neuromuscular' contact in the human embryo; ventral spinal nerve roots are recognizable at the 4 mm stage and very soon thereafter make contact with the medial aspect of myotomes (see, for example, Frazer, 1940; Hamilton *et al.* 1962). There is, however, no detailed knowledge of the nature of these contacts, nor of the cells making them. The widely held view that axons, because they have been shown to grow out from the central nervous system, are the pioneering elements in developing nerve, was recently questioned by Gamble (1974), and the alternative view that migrating Schwann cells might perform this role is no more than a return to views expressed before (and, indeed, after) Harrison's (1907) classical *in vitro* experiments. These conflicting views have been extensively discussed by Hughes (1960).

The early 'neuromuscular' contact, then, may involve Schwann cells rather than axons, and undifferentiated mesodermal elements rather than muscle, since it is said to occur at the early appearance of the myotomes. Such contacts can be of little immediate use to the neuromuscular system since neither conducting nor contractile elements are present, but it is easy to envisage a valuable role later when ingrowing axons arrive and require guidance to suitable terminals. Guidance, of course, presupposes an earlier presence of Schwann cells among the developing muscle cell elements, and although there is no direct evidence of this, there is evidence which is compatible with such an idea. In the human fetal muscle described here there is a population of cells intimately associated with the muscle cells and described as 'myotube satellites'. Some of these cells are present at all the stages examined and are generally of undistinguished appearance, with little cytoplasm, and what there is but little equipped with cytoplasmic organelles. These are, perhaps, true satellites. There is, however, another variety of 'myotube satellite cell', very different from the first in that its cytoplasm is rich in organelles. Some of these, perhaps, are myoblasts but many, with their fellows, form an extensive network within and without the basal lamina of associated muscle cells. These frequently contain centrioles and are equipped with a cilium; they contact undoubted Schwann cells and their numbers are greatest (relatively) in the youngest specimen. In all these characteristics, save those of extending beyond the basal lamina and contacting undoubted Schwann cells, these last cells conform precisely with Ishikawa's (1966) description of certain cells in developing human gastrocnemius muscle. In many respects they resemble some of the undifferentiated cells described and figured by Kelly & Zacks (1969*a*; see their Figs. 2, 6, 9). Their appearance and situation in the developing rectus oculi muscle is such that, but for an absence of directly associated axons, they would be identified confidently as Schwann cells. Very similar cells were described as 'satellites' by Church (1969) in the developing muscle of the fruit bat, and his Figure 15 shows that here, too, they may extend cytoplasmic processes beyond the confines of the basal lamina of associated muscle cells. It is proposed, then, that some of the 'myotube satellite cells' found in developing muscle (as, for example, in Figs. 6, 7 and 8) are Schwann cells introduced into the muscle anlage at the time of the earliest neuromuscular contact, which, as the mesodermal elements involved in that contact mature through myoblast to myotube to myocyte, form bridges over which later ingrowing axons may extend to form true neuromuscular contacts. Since such contacts must form with later as well as with earlier maturing generations of myocytes, it is apparent that a continuing process of innervation is required, and that as new muscle cells develop, new gaps between them and existing Schwann cell/axon complexes will develop needing to be bridged.

If this interpretation of the role of 'myotube satellite cells' be correct, then the decline in the number of their 'active' forms becomes comprehensible; they have been used up in forming additional bridges between nerve bundles and muscle cells. The persistence of 'satellite' cells of inactive form would represent no more than the continued presence of true satellites of the kind demonstrated by Mauro (1961) in adult striated muscle.

The relationship between axon terminals and muscle cells shown in Figure 13, where axons do contact well-developed muscle cells and fail to contact ill-developed adjacent muscle cells would, by itself, suggest that no innervation occurs until a certain stage of muscle differentiation had been achieved. The situation in developing rat muscle has been thought to be different. Kelly & Zacks (1969*b*) have shown axonal processes close to closely contiguous surfaces of well- and ill-developed muscle cells (see their Figs. 7 and 8) in a specimen of 18 days' gestation. However, in these situations, although the axonal components look much alike, the development of the post-synaptic element is more advanced in the better developed muscle cell; its plasma membrane is of increased thickness and density, whereas the less developed muscle cell shows no specialization of its plasma membrane. Closer inspection of these figures, however, may throw some doubt on the original interpretation. The axonal process lying close to the undifferentiated cell in Figure 8 (and interpreted as being involved in a neuromuscular contact with that cell) seems more probably to be one of some six or seven axonal processes which, in the near-serial section shown in Figure 7, are now closely adjacent to the specialized plasma membrane of the more developed cell. The precocious innervation of the undifferentiated cell is not proven by these figures. While the earliest stages of neuromuscular contact in the rat may not, then, antedate the first appearance of myofilaments, it does seem likely that the almost explosive rapidity of events in the development of muscle in the rat telescopes a sequence of changes which, in human development, is spread out over several weeks or longer, so that their manifestations may occasionally be seen in isolation.

Kelly & Zacks (1969*a*) described specialized regions of contact between myotubes, and between myotube and less differentiated cells, which they interpreted as close and tight junctions, and which they suggested may be involved in electrical couplings as well as in the adherence of one cell to another. They considered it likely that the contraction of the one cell would result, through the coupling, in the contraction of the other, and that this second cell's contraction would be necessary for the maintenance of points of coupling and adherence alike. Such contacts were apparently most numerous in the 16 days' gestation rats, and while some were still seen at 18 and 20 days other, more complex, contacts of a 'peg and socket' kind were also found. The point was made again, and emphasized, that the contraction of one of these cells must now be accompanied by contraction of the other or else dislocation of pegs from sockets must occur. There is, however, no evidence that interconnected cells contract at all. It is true that thoracic movements have been observed at day 18 in the rat fetus (East, 1931), but this is not to say that coupled myotubes in intercostal muscles are necessarily involved; it appears that some myotubes at day 18 are not coupled with, nor adherent to, less developed myofilament-containing cells. If it be assumed, however, that myofilament-containing muscle cells can, and, from time to time, do contract, it seems reasonable to envisage that their doing so might deform adherent cells without other damage being done. The same considerations presumably apply to the various contacts between muscle cells, and between muscle

and 'myotube satellite cells', which have been described in the human fetal superior rectus oculi muscle. Kelly & Zack's (1969*b*) proposition, that the specialized contacts occurring between adjacent muscle cells, requiring that the contraction of one should lead to contraction of its neighbour(s), and their observation that closely adjacent muscle cells frequently receive axonal terminals from the same tiny Schwann cell/axon complex, were taken by them to indicate 'mutual innervation' (*sic*) and so, perhaps, to be an early manifestation of the development of a motor unit. This idea, however, would require that all the muscle cells of the unit should lie very closely adjacent in their earlier stages of development and it is difficult to envisage a sideways migration of elongated cells in later stages, the more especially so since the cells are presumably more or less firmly anchored at both ends; yet it is commonly assumed that the muscle cells of a motor unit are widely scattered throughout a muscle (e.g. Muir, 1974).

In fact there is no evidence that the axonal processes in a Schwann cell/axon bundle complex lying in contact with adjacent muscle cells stem from the same motor neuron, while there is some quite weighty evidence to suggest that they do not. Redfern (1970) showed that, at birth, end plate potentials can be evoked in the same muscle fibre of rat diaphragm by the stimulation of more than one nerve fibre, and he concluded that an initial polyneuronal innervation was followed by the loss of all but one neuromuscular junction. These observations were later confirmed histologically by Bennett & Pettigrew (1974). Prestige (1976) has remarked that the time course of cervical ventral root fibre loss (Fraher, 1974) did not match the loss of polyneuronal innervation, and suggested that a decrease in pre-terminal branching might provide an explanation. Korneliussen & Jansen (1976), however, have noted an apparent absence of any sign of degenerating intra-muscular axons or axon terminals in skeletal muscle of newborn rats, and have suggested that redundant axonal terminals are eliminated by retraction of the parent axon. A possible explanation of these various findings is attempted in the drawings of Figure 14.

The extreme electron density of some 'myotube satellite cells' and of some few muscle cells in the rectus muscle of the 9·2 cm crown–rump length specimen, is not at present explicable. It does not appear to be related to shrinkage or contraction of the cells concerned, and the seemingly good preservation of immediately adjacent cells tends to rule out the likelihood of poor fixation being to blame.

The present results do not bear very directly upon the functional states of developing extrinsic ocular muscle. The presence of axonal terminals, often containing vesicles, upon the surface of muscle cells rich in myofilaments would seem to imply the possibility of some nervous control of muscular contraction. Even in the largest specimen, however, there is no more than a hint of the elaboration of a sub-neural apparatus, while within the muscle cell itself sarcoplasmic reticulum is scanty and transverse tubules apparently as yet unformed. One may suppose that, should nervous activity induce muscle contraction, then it would be slow and followed by a slow relaxation. Whether such movements of the eyeball occur behind the fused eyelids of the 24 cm fetus is not recorded. It seems certain, at least, that the extrinsic ocular muscles lag behind the muscles of the tongue and those involved in respiration, as Hewer pointed out many years ago (1934).

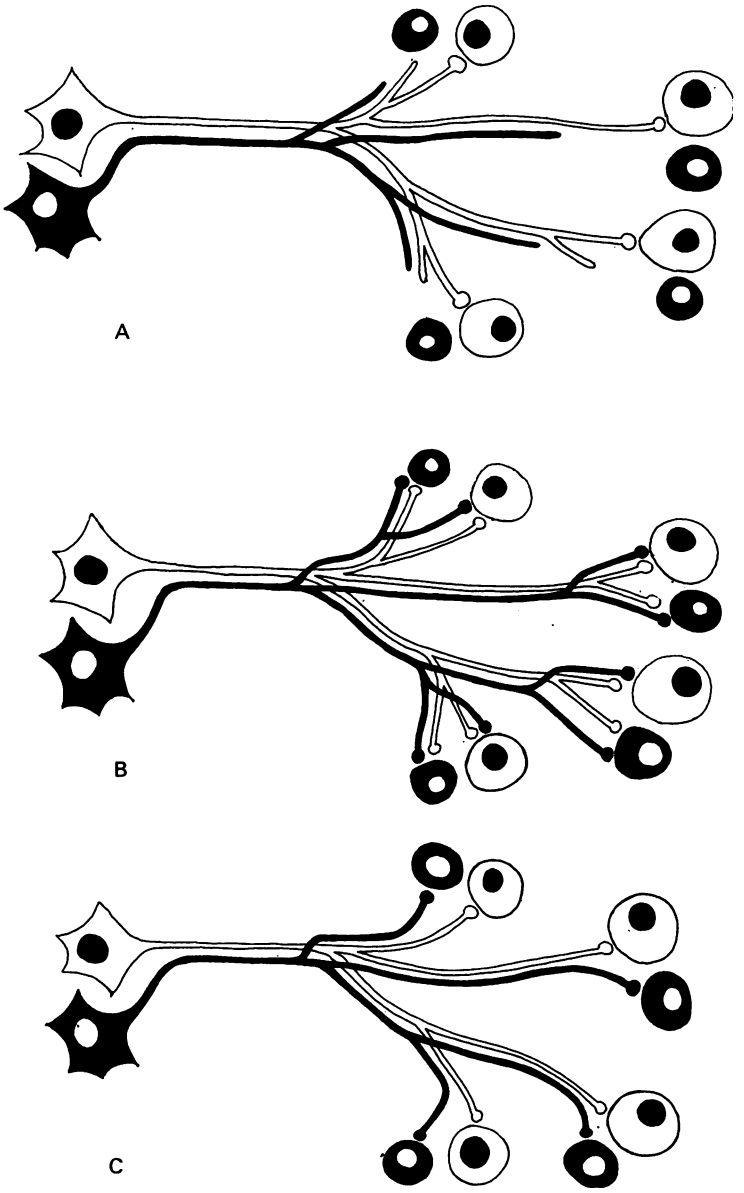


Fig. 14. Diagrams to illustrate suggested sequence of motor axon development. (A) To represent earliest pattern of innervation. A motor neuron extends axonal terminals to each of four relatively mature (larger) muscle cells, with branches towards two other less mature (smaller) muscle cells. Another motor neuron extends branches, but none reaches a muscle cell. (B) To represent a later pattern of innervation (polyneuronal). A motor neuron still contacts the four mature muscle cells with branches now contacting the four less mature associated muscle cells. The other motor neuron has also extended axonal processes to contact all eight muscle cells. (C) To represent final pattern of innervation. The two motor neurons have each lost terminal axonal branches (possibly by their retraction, as suggested by Korneliussen & Jansen, 1976) so that each muscle cell is innervated by only one neuron. In effect two motor units are shown.

SUMMARY

The superior rectus oculi muscle from human fetuses of 5, 9·2, 12 and 24 cm crown–rump length (of ages estimated to be 10, 12, 15 and 23 weeks respectively) have been examined by electron microscopy.

'Myotube satellite cells' closely associated with myotubes and myocytes were present in all specimens, but their relative numbers declined with advancing age. Some were small with scanty cytoplasm containing few organelles. Others were rich in organelles, including Golgi apparatus, granular endoplasmic reticulum, comma and dumb-bell shaped dense bodies and centriole or basal body: these cells were numerous in the three smaller specimens but almost absent from the largest.

Seemingly active 'myotube satellite cells' often extended cytoplasmic processes beyond their confining basal laminae into the endomysial space to contact free-lying cells of similar appearance, as well as axon-associated Schwann cells, often to form an extensive network. These 'myotube satellite cells' resembled Schwann cells in all respects save association with axons, and it is suggested that they are, indeed, Schwann cells so disposed as to promote axonal growth towards differentiating, but as yet uninnervated, myocytes.

Neuromuscular contacts were increasingly numerous with advancing age, usually where several or many axonal terminals contacted a relatively mature (myofilament-rich) muscle cell. Immature myotubes seldom made contact with axonal terminals, even when a closely adjacent (and 'coupled') mature muscle cell did so. A sequence of axonal growth and retraction has been proposed which reconciles accounts of early but temporary polyneuronal innervation with commonly accepted ideas regarding the scattered distribution of the muscle components of motor units.

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ADDENDUM

Martinez, McNeer, Hay & Watson (*Acta neuropathologica*, 1977, **38**, 87–93) have reported myelination of axons within extrinsic ocular muscle at 18 weeks, which we did not find at 23 weeks. They also reported multiple convolutions of the sarcolemmal membrane at 20–24 weeks, which we did not see.