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De novo neuromuscular junction formation on human muscle fibres cultured in monolayer and innervated by foetal rat spinal cord: ultrastructural and ultrastructural–cytochemical studies

VALERIE ASKANAS, HELEN KWAN, RENATE B. ALVAREZ, W. KING ENGEL, TAKAYOSHI KOBAYASHI, ANDREA MARTINUZZI and EDWARD F. HAWKINS

Neuromuscular Center, University of Southern California School of Medicine, Hospital of the Good Samaritan, 637 South Lucas Avenue, Los Angeles, CA 90017, USA

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Summary

Ultrastructural features of neuromuscular junction formation and transverse tubule development were studied utilizing a newly developed model in which human muscle fibres cultured in monolayer are innervated by foetal rat spinal cord with dorsal root ganglia attached. At early innervation (7–10 days), when distinct 'boutons' are contacting muscle fibres, the contacts of nerve terminals with the muscle fibres are, ultrastructurally, superficial and unorganized, and there is no basal lamina-like material between nerve terminals and muscle fibres. A bouton consists, ultrastructurally, of a cluster of small nerve terminals contacting the muscle fibre. At 2–3 weeks of innervation, shallow 'beds' are formed on the muscle fibre just beneath nerve terminals, and occasionally there are irregular and miniscule fragments of basal lamina-like material in the cleft. There is no Schwann cell apposing the nerve terminal at this stage of innervation. After 4–5 weeks of innervation there is more definite basal lamina material in the cleft and suggestive postsynaptic plasmalemmal densities and invaginations. However, there is no Schwann cell apposing the nerve terminal at this stage. At 6–8 weeks of innervation, deep postsynaptic folds are present, a Schwann cell apposes the nerve terminal, and basal lamina surrounds the entire muscle fibre. At all four stages of innervation examined, ultrastructural cytochemistry of alpha-bungarotoxin binding reveals that nicotinic ACh receptors are located exclusively at the neuromuscular junctions. After 1–2 weeks of innervation, very few lanthanum-positive transverse tubules are observed and only in close proximity to the surface membrane. After 3 weeks of innervation, more lanthanum-positive tubules are present, and they are located deeper within the muscle fibre. Five weeks after innervation, somewhat more elaborated tubules (but no lateral sacs) appear, and honeycomb structures are often present. After 6–7 weeks of innervation the tubular system is very elaborate and lateral sacs are present. Hence, this study describes consecutive stages of the formation of neuromuscular junctions and transverse tubules in innervated cultured human muscle, and provides an important basis to which similar studies related to the diseased human muscle can be compared.

Introduction

In various animal species, *de novo* neuromuscular junction (NMJ) formation has been extensively and systematically studied both during embryonic development *in vivo* and in tissue culture (Hirano, 1967; Kelly & Zacks, 1969; Koenig & Pécot-Dechavassine, 1971; Fischbach, 1972; Shimada & Fischman, 1973; Bennett & Pettigrew, 1974; Kullberg *et al.*, 1977; Fischbach *et al.*, 1979; Frank & Fischbach, 1979; Weldon & Cohen, 1979; Nakajima *et al.*, 1980; Denis, 1981 (review); Denis *et al.*, 1981; Bennett, 1983 (review); Bursztajn, 1984). For similar systematic studies of human NMJ formation, the best model currently available is innervated cultured human muscle derived from muscle biopsies, which permits analysis of NMJ development in both normal and diseased human muscle fibres. This is because human foetal material is practically unavailable for such studies and, even if it were, a spectrum of human muscle disease would not be available as it is with diagnostic muscle biopsies.

We have recently developed a new culture system in which adult human muscle fibres cultured in monolayer from liberated individual myoblasts are innervated by neurons of foetal rat spinal cord. In this

system, nicotinic ACh receptors (AChRs) and AChE accumulate at the newly formed nerve–muscle contacts, and there is a definite trend from multifocal innervation of muscle fibres in early co-culture toward unifocal innervation in longer co-culture (Kobayashi & Askanas, 1985; Kobayashi *et al.*, 1986). In this system, innervated muscle fibres can survive for up to 5–6 months. They contract nearly continuously and the contractions are reversibly stopped by 1 mM d-tubocurarine (Askansas *et al.*, 1985). Innervated in this way, the expression of genes for the muscle-specific isozymes of creatine kinase, glycogen phosphorylase and phosphoglycerate mutase of the cultured human muscle fibre is significantly enhanced, and the degree of enhancement correlates with the duration of innervation (Martinuzzi *et al.*, 1986, 1987). Histoenzymatic maturation of innervated cultured human muscle is also much more advanced than that of the non-innervated controls (Vita *et al.*, 1987). Microelectrode studies of these innervated cultured human muscle fibres have shown increased resting membrane potentials (as compared to aneurally cultured human muscle) and curare-sensitive miniature endplate and endplate potentials (Saito *et al.*, 1986; and in preparation).

Previous important studies by Crain and Peterson had established in what they termed 'organotypic' culture that adult human muscle *in vitro* can be innervated by rodent spinal cord neurons (Crain *et al.*, 1970; Peterson & Crain, 1979). Innervation of adult human muscle in that organ culture system provides a good *in vitro* model of *in situ* regeneration and innervation–reinnervation. However, in that system one is not able to study easily the completely *de novo* formation of neuromuscular junctions, because in organ cultures the original explanted muscle fibres are damaged but their basal lamina survives. Therefore, *de novo* formation of basal lamina and basal lamina specialization due to innervation are impossible to evaluate in the organ culture system. Moreover, in organ culture new myofibres form within the original basal lamina of the explanted muscle fibres; they can then sometimes be innervated at the original synaptic sites on the basal lamina (Ecob, 1984) as is known usually to occur during muscle regeneration *in vivo* (Marshall *et al.*, 1977; Sanes *et al.*, 1978; Burden *et al.*, 1979). Thus, our system is different from the Crain–Peterson system.

In the present paper we describe the major ultrastructural steps of NMJ development in the monolayer-cultured, innervated human muscle, and demonstrate that they are very similar to those shown by others in animal muscle *in vivo* (Dennis, 1981 (review); Bennett, 1983 (review); Matthews-Bellinger & Salpeter, 1983).

For studying details of development, this innervated human muscle culture system offers certain advantages over cultured animal muscle. For example, even aneurally cultured embryonic animal muscle and cell lines have well-developed basal lamina (reviewed by Sanes, 1986) and their AChRs are in numerous aggregates, whereas adult human muscle aneurally cultured in monolayer has a remarkably unspecialized muscle surface membrane complex, characterized by: (i) lack of basal lamina (Askanas & Engel, 1979), (ii) AChRs evenly and weakly distributed over the entire muscle plasmalemma (Askanas *et al.*, 1977), and (iii) negative AChE staining (Kobayashi & Askanas, 1985). Therefore, the events associated with NMJ formation, from short-term through long-term innervation, can be precisely studied in the innervated human muscle culture system. Human muscle cultured aneurally in monolayer for as long as 2–2.5 months is not well cross-striated and does not have well-developed transverse (T)-tubules (Askanas & Engel, 1979). Hence, the influence of innervation on general morphologic maturation of cultured human muscle fibres can also be carefully analysed in this system, since it allows parallel comparative studies between aneural and innervated cultured human muscle for up to 2–2.5 months (after which time aneurally cultured human muscle fibres progressively degenerate), as well as the subsequent maturity of the muscle fibres innervated for up to 6 months in culture.

All of these culture innervation studies are applicable to both normal human muscle, as demonstrated in this report, and genetically abnormal human muscle.

Material and methods

Establishing muscle–spinal cord co-cultures

From diagnostic muscle biopsies of eight patients considered free of intrinsic muscle disease after all diagnostic studies were performed, human muscle cultures were established according to our modified explant–re-explanation technique (Askanas & Engel, 1975a).

Cultures were initiated from 1 mm^3 muscle explants, five of which were placed in each 35-mm Petri dish (Falcon) coated with a gelatin–human plasma mixture. After an abundant growth of cells had emerged from the explants, the explants were removed and re-explanted into collagen-coated Petri dishes. After another abundant growth of cells had emerged from the re-explanted explants, those explants were removed and discarded, and the muscle cells were allowed to continue to grow in monolayer (see Fig. 1a). The muscle was cultured in F_{14} medium (Gibco) (Vogel *et al.*, 1982), modified to contain 600 mg dl^{-1} glucose, 10% foetal bovine serum (FBS), 50 ng ml^{-1} fibroblast growth factor (FGF), 10 ng ml^{-1} epidermal growth factor (EGF) and 10 µg ml^{-1} insulin (Askanas & Gallez-Hawkins, 1985). FBS was purchased from Hy-Clone, FGF and EGF from Collaborative Research, and insulin from Sigma.

Cultures were fed twice a week, and were examined every day or every other day by phase-contrast inverted microscopy. About 10–15 days after myoblast fusion, explants of whole transverse slices of 13- to 14-day-old rat embryo spinal cord with dorsal root ganglia attached were placed on the muscle monolayer cultures, four fragments per Petri dish (see Fig. 1b). In this study, the muscle–spinal cord co-cultures were maintained for up to 8 weeks in the same medium as above, but without FGF and EGF.

Transmission electron microscopy

Thirty-five cultures (nine cultures after 7–10 days of co-culture, ten cultures after 2–3 weeks of co-culture, eight cultures after 4–5 weeks of co-culture and eight cultures after 6–8 weeks of co-culture) were studied by transmission electron microscopy. The whole culture dish was fixed in 1.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 30 min. After fixation, cultures were postfixed in 1% OsO_4, dehydrated and embedded (Askanas & Engel, 1975b). After embedding, the areas of nerve–muscle contacts designated for ultrastructural studies were identified in phase contrast microscopy, marked, and core-drilled out (Askanas & Engel, 1975b). Drilled-out cores containing the exact areas of interest were glued in Epon blanks and processed for thin sectioning (Gorycki & Askanas, 1977). To study NMJ formation, 40 to 50 muscle fibres in each culture age were cut in serial sections along their transverse or longitudinal axis. For study of general ultrastructural morphology, muscle fibres were cut longitudinally parallel to the bottom of an embedded disc (i.e. parallel to the bottom of the original culture dish).

Ultrastructural studies of binding of horseradish peroxidase-labelled alpha-bungarotoxin

Alpha-bungarotoxin was labelled with horseradish peroxidase (HRP) according to the method of Nakane & Kawaoi (1974) with minor modifications (A. G. Engel, personal communication). Briefly, 5 mg HRP (Sigma, type VI) were dissolved in 1 ml freshly prepared sodium bicarbonate, pH 8.1, at room temperature. Fluorodinitrobenzene (0.1 ml of a 1% solution in absolute ethanol) was then added and the solution gently mixed for 1 h. Next, 1 ml of 0.016 M ethylene glycol in distilled water was added, and stirring continued for a further 1 h at room temperature. The solution was then dialysed against three 1-litre changes of 0.01 M sodium carbonate buffer, pH 9.5 at 4° C, prior to the addition of 1 mg of alpha-bungarotoxin (Miami Serpentarium). After mixing for a further 3 h at room temperature, 0.5 mg sodium borohydride in 0.1 ml distilled water was added and the solution left at 4° C overnight. The following day the solution was dialysed against two 1-litre changes of phosphate-buffered saline (PBS), pH 7.2 at 4° C, and was then applied to the top of a pre-calibrated, 80 × 1.5 cm Sephadex G-100 column. Elution was accomplished with PBS at a flow rate of 15 ml^{-1}. Fractions of 1 ml were collected and absorbance at 280 and 403 nm measured. Fractions corresponding to the first peak were pooled and concentrated fivefold by ultrafiltration (Diaflo YM10 membranes, Amicon) prior to use.

For ultrastructural cytochemistry, living cultures were incubated for 2 h at 37° C in a tissue culture incubator in HRP–alpha-bungarotoxin diluted from 1:1 to 1:5 with culture medium containing 2 mg ml^{-1} bovine serum albumin. After incubation in HRP–alpha-bungarotoxin, cultures were repeatedly washed for 1 h to remove unbound conjugate. Subsequently, they were fixed in 1.25% glutaraldehyde and 2% paraformaldehyde for 30 min. After fixation, cultures were washed three times in sodium phosphate buffer followed by three washes in 0.5 M Tris buffer, pH 7.6. They were then incubated in 3, 3-diaminobenzidine (DAB; Sigma) (5 mg per ml of 0.05 M Tris buffer, pH 7.6) and 0.01% H_2O_2 for 45 min. After being incubated in DAB, cultures were washed and processed for electron microscopy as above. With this procedure, positive staining at the nerve–muscle contacts was only occasionally visible by bright-field, light microscopy. When staining was evident, NMJs were marked and core-drilled out as described above. Otherwise, nerve–muscle contacts were localized under phase contrast microscopy, marked and core-drilled out. For serial sectioning, the cores containing the selected areas were mounted in plastic blanks after having been oriented according to their designation for longitudinal or transverse sectioning.

To evaluate the specificity of the reaction, control cultures were preincubated in unconjugated alpha-bungarotoxin for 1 h at 37° C, followed by incubation in HRP–alpha-bungarotoxin, and processed for electron microscopy as above.

Ultrastructural cytochemistry of lanthanum staining

For ultrastructural visualization of T-tubules by lanthanum staining, cultures were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde on 0.1 M sodium cacodylate buffer at pH 7.2. After fixation, cultures were incubated for 2 h by vibratory agitation in a mixture of 2% lanthanum nitrate and 0.5% OsO_4 in 0.2 M S-collidine buffer, according to a modification of the method of Revel & Karnovsky (1967). After being incubated, cultures were processed for electron microscopy as above.

Results

Observation of living cultures

By 2–3 days after the start of the co-culture of rat spinal cord with the human muscle, long neurites had emerged from the spinal cord explants and bouton-like structures were present at the nerve–muscle contacts (Fig. 1c,d). After 7–10 days of co-culture, occasional and asynchronous contractions of a few individual muscle fibres were visible in close proximity to the spinal cord explant.

By 14–16 days after co-culture, the number of contracting muscle fibres had increased and the majority of the contracting fibres had become entirely cross-striated (Fig. 1e). Morphologically they were quite different from non-innervated muscle fibres of the same age (Fig. 1f). This time course of the appearance of muscle fibre contractions was consistently

present in innervated cultured muscle fibres from over 70 different muscle biopsies (each biopsy established in 20 to 40 Petri dishes) cultured to date. Contractions were monitored with a videotape recording system attached to a Zeiss inverted microscopy (Askanas *et al.*, 1985). One week later (21–23 days after co-culture), large areas of virtually continuously contracting muscle fibres became morphologically distinct from the areas of non-contracting (non-innervated) muscle fibres in the same culture dish. Densely packed contracting muscle fibres, generally parallel to each, were present predominantly in proximity to the ventral part of the spinal cord explants (Fig. 1g), and they could be maintained in such a state for up to 5–6 months. Muscle fibres around the dorsal part of the spinal cord explants progressively degenerated after 6–8 weeks of co-culture.

By 6–7 weeks of co-culture, nerve trunks, often containing myelinated nerve fibres, were prominent. They were emerging from spinal cord explants and branching on the clustered, parallel, contracting muscle fibres (Fig. 1h).

Transmission electron microscopy

At 7–10 days after innervation (days of 'innervation' are arbitrarily defined as the number of days after beginning the co-culture) nerve–muscle contacts, in the form of 'boutons' were very superficial and unorganized (Fig. 2a). Clusters of various-sized nerve terminals usually composed of three or four (sometimes more) terminals, were contacting the muscle plasmalemma. Many clear vesicles and some dense-core vesicles were present in the nerve terminals. There was no basal lamina-like material between the nerve terminals and the muscle fibre (nor elsewhere along the fibre), no thickening and no invaginations (folds) of the postsynaptic muscle plasmalemma beneath the terminal.

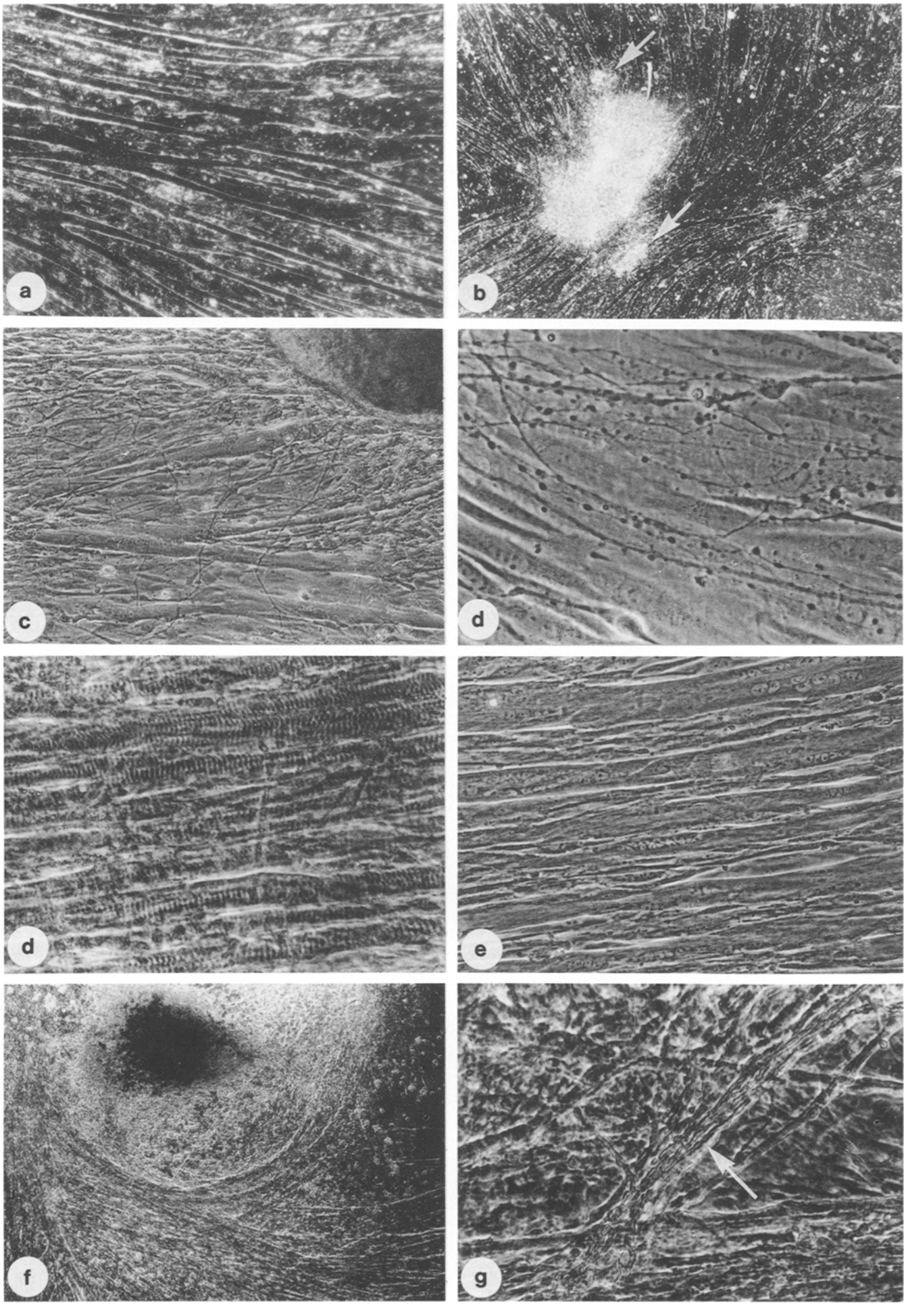
At 2–3 weeks after innervation, nerve–muscle contacts continued to be quite unorganized, even though the muscle fibre itself was often very well cross-striated and contracting. Nerve terminals contained many vesicles, and shallow 'beds' consisting of a large indentation of the membrane (presumably to accommodate the nerve terminal) were formed on the muscle fibres just beneath the terminals (Fig. 2b). Occasional irregular and miniscule fragments of basal lamina-like material were present in the nerve terminal–muscle–plasmalemmal cleft. There was no Schwann cell apposing the nerve terminal (Fig. 2b).

After 4–5 weeks of innervation, there was definite basal lamina-like material in the nerve terminal–muscle cleft (Fig. 2c–e), and suggestive postsynaptic plasmalemmal densities and invaginations. Most commonly, Schwann cells were still not apposing nerve terminals at this stage of innervation, although occasionally a Schwann cell was present in proximity to a terminal, partially covering a terminal (Fig. 2d), or apposing a muscle fibre independently from a terminal (Fig. 2e). Plasmalemmal densities of muscle cells were often present beneath a Schwann cell, and basal lamina-like material was also occasionally present between the muscle plasmalemma and an adjacent Schwann cell (Fig, 2d, e). Desmosome-like junctions were also occasionally observed at the contact of a Schwann cell and a muscle cell (Fig. 2d). Basal lamina was not present extrajunctionally along the muscle fibre at this stage of innervation.

In both 2- to 3-week-old and 4- to 5-week-old co-cultures, endocytotic vesicles were present subjacent to the postsynaptic muscle plasmalemma, immediately beneath the nerve terminal.

At 6–8 weeks of innervation, distinct postsynaptic folds were present. There was definite basal lamina in the nerve–muscle cleft, and a Schwann cell typically apposed the nerve terminal (Fig. 3b, c). Immediately beneath the terminal, the muscle fibre cytoplasm was rich in rough endoplasmic reticulum ribosomal clusters, free ribosomes, and mitochondria (Fig. 3a, c). Occasional darkly osmiophilic, lyosome-like structures were present in this region (Fig. 3a, c). The tips of postsynaptic folds were thicker and more darkly stained than the bottom of the folds (Fig. 3b, arrows). One muscle fibre usually had two or three terminals in very close proximity to each other, while the rest of the fibre remained junction-free. Nerve terminals contained many clear and some dense-core synaptic vesicles, and usually some glycogen granules (Fig. 3b, c). Active zone-like regions were

Fig. 1. Dark-field, low-power micrographs (a, b) and phase contrast micrographs of living cultured human muscle (c–h). (a) Human muscle, aneurally cultured in monolayer, photographed 1 day before co-culture with foetal rat spinal cord. Note the abundance of muscle fibres. × 140. (b) Micrograph obtained 1 day after foetal rat spinal cord with dorsal root ganglia (arrows) was placed on top of the monolayer cultured human muscle. × 75. (c) At 3 days after co-culture with foetal rat spinal cord, long neurites are emerging from the spinal cord explant. × 480. (d) Many bouton-like configurations are present at nerve–muscle contacts, 3 days after co-culture. × 1200. (e) Human muscle fibres, well cross-striated and contacted by neurites, 16 days after co-culture with rat spinal cord. × 1200. (f) Aneurally cultured human muscle fibres, 1 month old, are immature and not cross-striated. × 600. (g) At 23 days after co-culture, a large area of densely packed muscle fibres in parallel to each other, in proximity to one part of spinal cord explant, were continuously contracting. × 75. (h) At 7 weeks after co-culture, a myelinated 'nerve trunk' (arrow) that was emerging from the spinal cord explant (not shown), is seen branching among muscle fibres that are densely packed and parallel to each other. × 400.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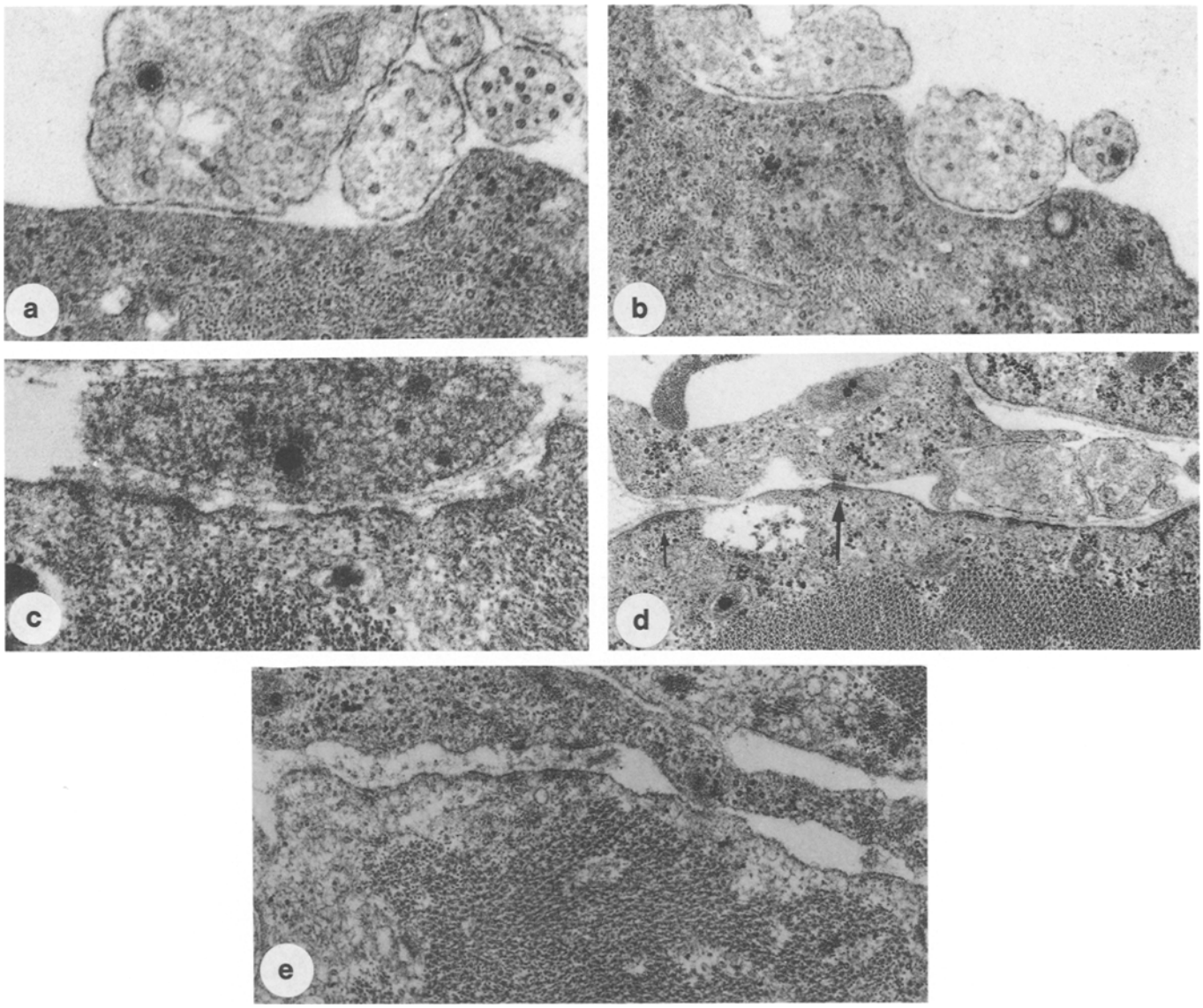
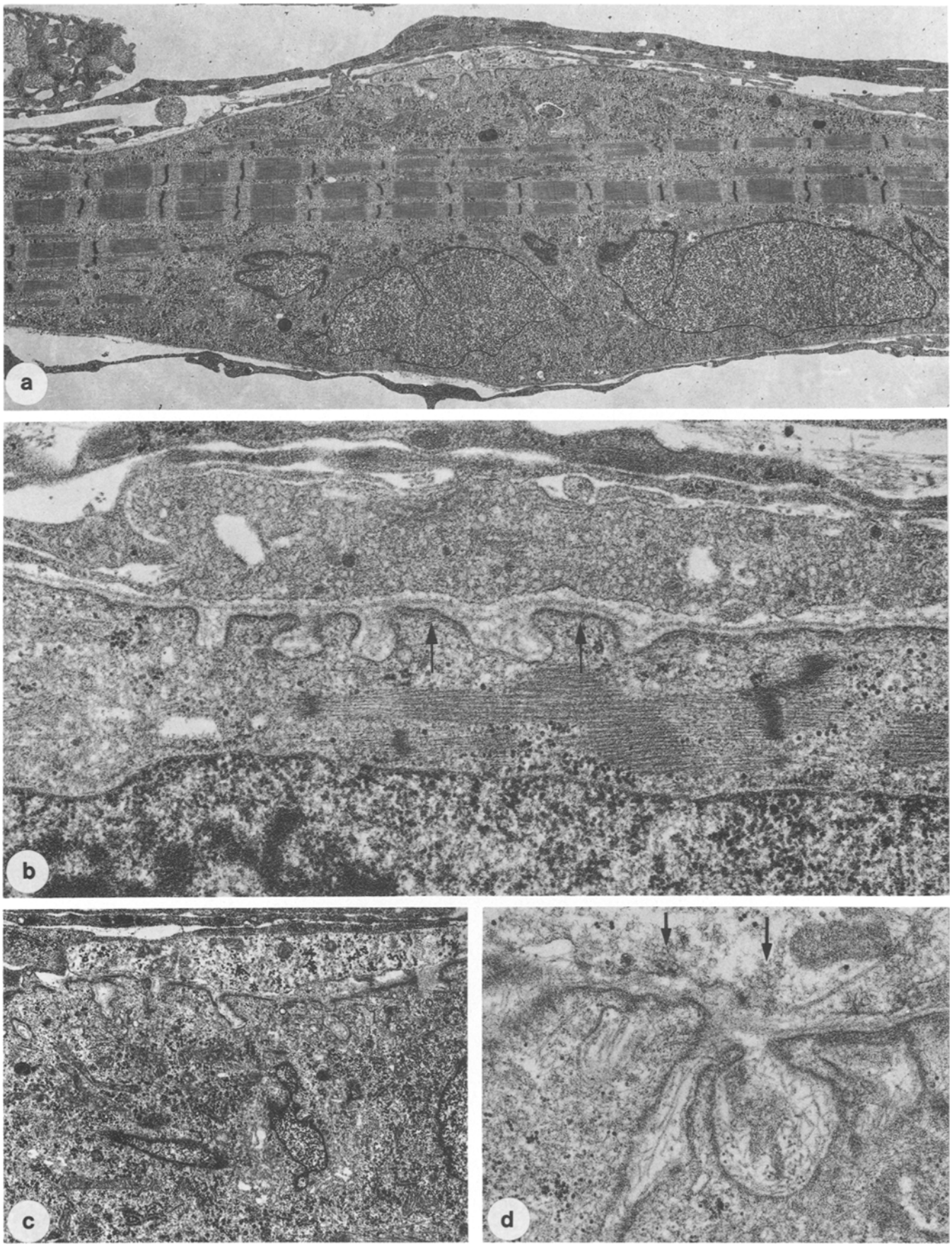


Fig. 2. Transmission electron microscopy of early nerve–muscle contacts. (a) At 10 days after co-culture, several nerve terminals (one containing several clear and one dense-core vesicle) are apposing unspecialized postsynaptic muscle membrane. × 29 700. (b) At 21 days after co-culture, shallow 'beds' are formed on the muscle fibre just beneath nerve terminals. × 37 350. (c) At 30 days after co-culture, basal lamina-like material in the cleft, early postsynaptic invagination, and postsynaptic plasmalemmal densities are present. The nerve terminal contains many clear and a few dense-core synaptic vesicles. × 53 460. (d) At 30 days after co-culture, postsynaptic density is present beneath a Schwann cell (thin arrow) which partially covers the nerve terminal. Desmosome-like junction (thick arrow) is also present between Schwann and muscle cell membranes. × 23 370. (e) Basal lamina-like material is present between the muscle plasmalemma and an adjacent Schwann cell, 30 days after co-culture. × 33 210.

Fig. 3. Transmission electron microscopy of organized neuromuscular junctions. (a, b) Nerve–muscle contacts 6 weeks after co-culture. (a) Distinct postsynaptic folds, cross-striations and basal lamina extending around the muscle fibre are present. Cross-section of a part of a nerve trunk is seen in the upper left. × 5 670. (b) Nerve–muscle contact 8 weeks after co-culture. Similar postsynaptic organization as in (a) and (c), but plasmalemma at the tips of postsynaptic folds (arrows) is thicker and more darkly stained than at the bottom of the folds. A Schwann cell is apposing the nerve terminal. Many clear and several dense-core vesicles are present in the nerve terminal. × 35 280. (c) Definite basal lamina in the cleft and distinct postsynaptic folds are present. Rough endoplasmic reticulum ribosomal rosettes, free ribbons, mitochondria and lysosome-like structures are present. A Schwann cell is apposing the nerve terminal. Clear vesicles, glycogen granules and mitochondria are present in the nerve terminal. × 8910. (d) Active zone-like regions (arrows) in one of the nerve terminals. × 36 500.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evident in some nerve terminals (Fig. 3d, arrows). At this stage of innervation basal lamina surrounded the entire muscle fibre, even when the NMJ had a less mature appearance (Fig. 4a). Large nerve trunks, which were emerging from the spinal cord explants, examined just before branching among the muscle fibres (Fig. 1h), contained bundles of axons, most of which were ensheathed by Schwann cells and some of which were well myelinated (Fig. 4b).

Ultrastructural localization of HRP–alpha-bungarotoxin binding

In virtually all stages of innervation, HRP–alpha-bungarotoxin was localized only at the nerve–muscle contacts. In the 28 fibres containing nerve–muscle contacts that were examined, only one muscle fibre had weak staining along the entire extrajunctional plasmalemma (as occurs in non-innervated cultured human muscle (Askanas *et al.*, 1977), in addition to the much stronger staining of the junctional plasmalemma. Early nerve terminal–muscle fibre contacts always had a simple, linear pattern of staining (Fig. 5a), whereas more mature NMJs had staining related to the developing postsynaptic folds (Fig. 5b, c).

Often, several neighbouring innervated muscle fibres had a 'step-like' pattern of their junctional complexes, evident on serial sections of adjacent fibres.

In addition to the staining of the muscle plasmalemma immediately beneath a nerve terminal, HRP–alpha-bungarotoxin staining was often present on the muscle plasmalemma beneath an immediately

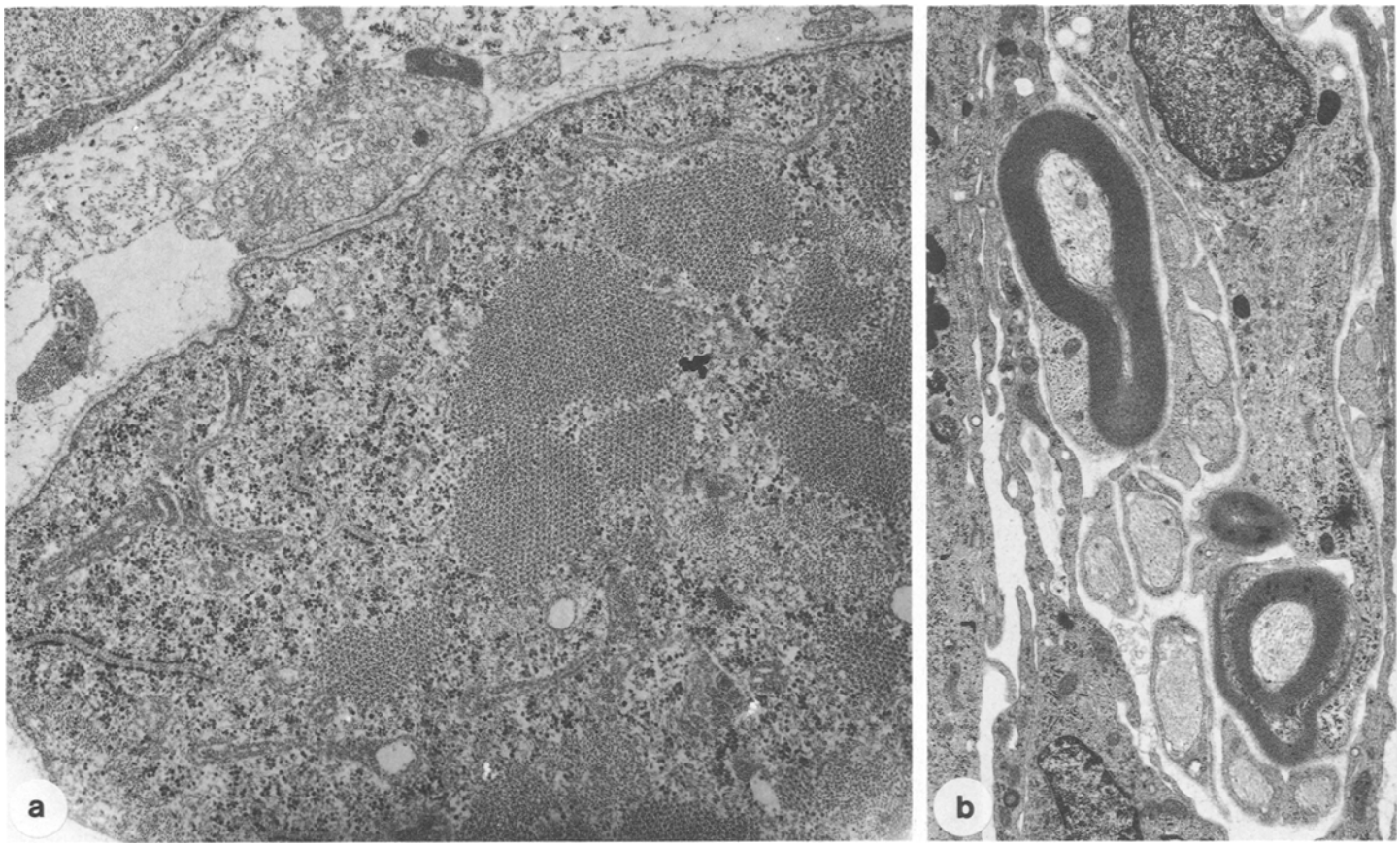
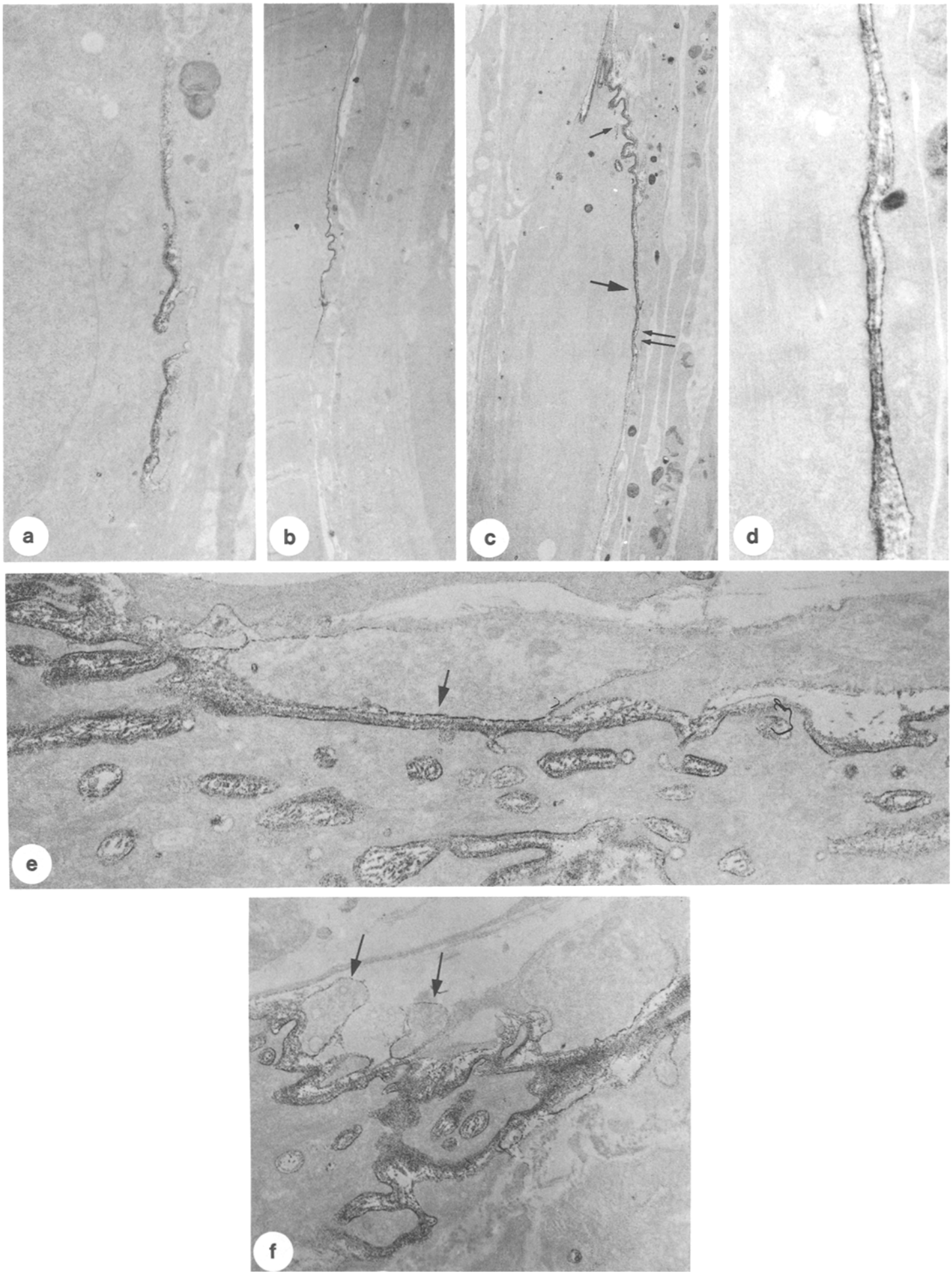


Fig. 4. (a) Cross-section of a muscle fibre innervated for 7 weeks. It is completely surrounded by a well-developed basal lamina. × 30 910. (b) Electron micrograph of a cross-section of a nerve trunk similar to one seen in Fig. 1h. All the axons are ensheathed by Schwann cells and two are well myelinated. × 8480.

Fig. 5. Ultrastructural cytochemistry of HRP–alpha-bungarotoxin at the nerve–muscle contacts. (a) Linear distribution of HRP–alpha-bungarotoxin at early nerve muscle contacts. × 10 935. (b) The reaction product is confined to the organized nerve–muscle contact. × 4590. (c) The reaction product is present on well-developed postsynaptic folds (thin arrow) and on the muscle membrane beneath the Schwann cell (thick arrow) and on the Schwann cell plasmalemma (double arrows) immediately apposing the muscle plasmalemma. × 5670. (d) Higher power of a subsequent section showing pre- and post-'synaptic' staining at the same Schwann cell muscle junction as in (c). × 26 460. (e, f) The reaction product is present on the postsynaptic muscle plasmalemma and on presynaptic plasmalemma of nerve terminals (arrow in 'e'), even in nerve terminal regions (arrows in f) well away from the junctions. (e) × 14 700; (f) × 21 060.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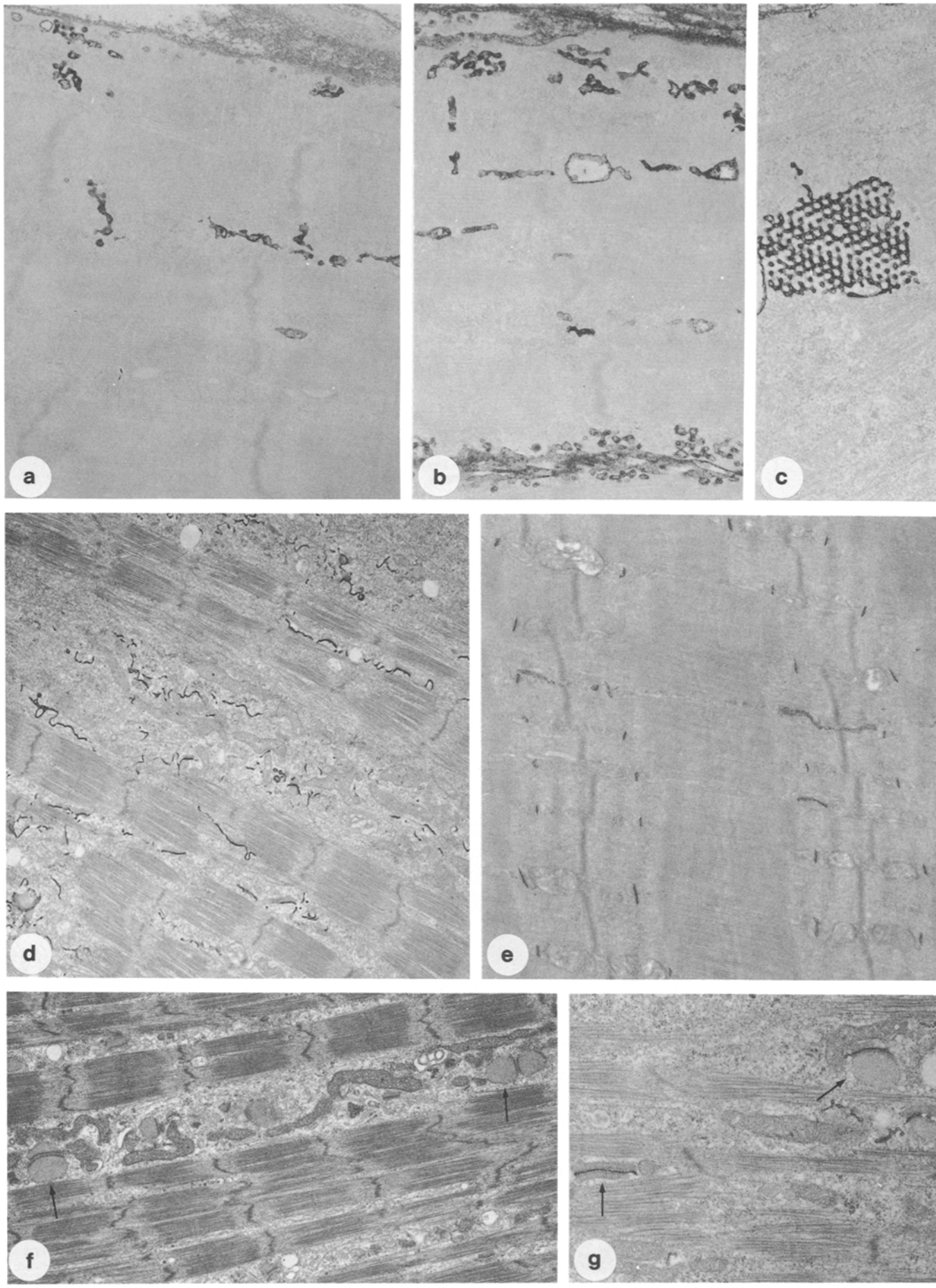
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adjacent Schwann cell (Fig. 5c, d). The HRP–alpha-bungarotoxin reaction product was also occasionally seen on the plasmalemma of Schwann cells, especially where their plasmalemma immediately apposed muscle plasmalemma (Fig. 5c, d). The HRP–alpha-bungarotoxin reaction product was also often present on the presynaptic plasmalemma of a nerve terminal immediately apposing the muscle postsynaptic plasmalemma (Fig. 5e, f), and occasionally on the plasmalemma entirely surrounding the nerve terminal (Fig. 5f). Pretreatment with unlabelled alpha-bungarotoxin resulted in absence of HRP–alpha-bungarotoxin reaction product in all of these regions.

Development of T-tubules demonstrated by lanthanum staining

In cultured human muscle innervated for 1–2 weeks, very few lanthanum-positive tubules were observed, and only in close proximity to the surface of the muscle fibre. After 3 weeks of innervation, more lanthanum-positive tubules were present, and they were located deeper within the muscle fibre (Fig. 6a). Four to five weeks after innervation, the T-tubule system was somewhat more elaborate (Fig. 6b). It often formed honeycomb-like structures (Fig. 6c). The plasmalemma of the muscle fibre and subplasmalemmal caveolae were also positively stained (Fig. 6a, b). Usually, lateral sacs could not be observed at this stage. After 6–7 weeks of innervation the T-tubule system was very elaborate (Fig. 6d), and lateral sacs were present (Fig. 6f, g). Even as late as 8 weeks after innervation, however, the pattern of organization of the T-tubules still had not achieved the pattern of adult human muscle fibres *in vivo*, wherein the T-tubules are (i) nearly all transverse rather than longitudinal and (ii) located regularly at the level of the I-band near the A–I junctions (Fig. 6e).

Discussion

NMJ formation

This study provides the first description of consecutive steps involved in NMJ development in innervated cultured human muscle fibres. It demonstrates that rather mature junctions can appear on human muscle cultured in monolayer and innervated by foetal rat spinal cord. We have divided this innervation into four developmental stages, which strikingly resemble developmental stages of NMJ formation in mice (from embryonic day 16 until 1 month postnatally), as detailed by Matthews-Bellinger & Salpeter (1983) and in rats as described by Kelly & Zacks (1969).

At stage I (1–2 weeks of innervation), clusters of small nerve terminals contacted the muscle plasmalemma, but there were no consistent signs of muscle postsynaptic plasmalemmal specialization. There was no Schwann cell apposing the nerve terminal. At stage II (2–3 weeks of innervation), shallow beds were formed postsynaptically to accommodate nerve terminals, and small bits of basal lamina were present between the presynaptic and postsynaptic plasmalemma. At stage III (4–5 weeks of innervation), the postsynaptic plasmalemma had developed densities and invaginations, there was definite basal lamina-like material in the nerve–muscle cleft, and subplasmalemmal cytoplasmic densities were prominent. Subsynaptic vesicles were also prominent in the muscle fibre. At stage IV (6–8 weeks of innervation), clearly developed postsynaptic folds were present. A Schwann cell always apposed the nerve terminal in the well-developed NMJs. Active zone-like structures of the nerve terminals, which are considered to be the regions where transmitter release occurs (Couteaux & Pécot-Dechavassine, 1970; Dreyer *et al.*, 1973), were occasionally observed in the region of the nerve terminal immediately apposing the postsynaptic membrane.

Not all the NMJs at a given stage of innervation had the same degree of development. Even at 8 weeks of innervation there were many NMJs that did not appear to have well-developed postsynaptic folds. However, basal lamina was always present in synaptic clefts of those junctions and was usually surrounding the entire muscle fibre. Even though all junctions with well-developed postsynaptic folds seemed to have a Schwann cell apposing the nerve terminal, not all junctions in which a Schwann cell apposed the nerve terminal had deep postsynaptic folds. Even though NMJs had various degrees of differentiation, HRP–alpha-bungarotoxin was virtually always localized exclusively at the junctional area, even in the very early stages of differentiation. The reaction product was present mainly at the postsynaptic muscle plasmalemma; however, presynaptic neuronal plasmalemma and Schwann cell plasmalemma also often contained a certain amount of

Fig. 6. Ultrastructural cytochemistry of lanthanum staining (a–e, g) and regular transmission electron microscopy of T-tubules (f). (a) At 3 weeks after innervation, a few T-tubules are present. × 17 940. (b, c) At 5 weeks after innervation more prominent T-tubules are present in (b). × 21 560; a prominent honeycomb-like structure is present in (c) × 21 060). In (a) and (b) muscle plasmalemma and caveolae are also stained. (d) Elaborate T-tubules 7 weeks after innervation. × 8000. (e) Organized T-tubules in normal adult human muscle biopsy. × 17 940. (f, g) Well-developed lateral sacs (arrows), 7 weeks after innervation. (f) × 9000; (g) × 14 820.

HRP–alpha-bungarotoxin reaction product. In the past, some investigators have considered positive HRP–alpha-bungarotoxin staining of the presynaptic neuronal plasmalemma a real phenomenon (Bender *et al.*, 1976; Lentz *et al.*, 1977), whereas others considered it to be an artifact (Engel *et al.*, 1977; Jones & Salpeter, 1983). How the staining of presynaptic membranes in our cultures compares to that of adult NMJs is not known.

In mice from embryonic day 16 until 4 weeks postnatally, the steps of NMJ development are very similar to the steps occurring during the 8 weeks of NMJ development in our human, innervated, cultured muscle fibres, execpt that in our preparation muscle plasmalemmal alpha-bungarotoxin was associated virtually exclusively with the junctional area, whereas in early embryonic (16-day) mouse muscle, alpha-bungarotoxin binding extended well beyond the region of nerve–muscle contact. At all postnatal ages of the mouse, alpha-bungarotoxin binding was present exclusively at the nerve–muscle contacts. In neither our human cultured muscle innervated for 8 weeks nor in 4-week postnatal mouse muscle did the organization of postsynaptic folds resemble those in an adult mammalian NMJ in regard to their number and depth (Matthews-Bellinger & Salpeter, 1983).

Various aspects of neuromuscular synapse formation in monolayer cultures have been studied in embryonic animal muscle and muscle cell lines. A detailed electron microscopic description of consecutive steps of such NMJ formation was reported by Nakajima *et al.* (1980) in embryonic rat muscle cultured in monolayer and innervated for 14 days by microexplants of foetal rat spinal cord. There are several differences between the findings in their embryonic rat muscle culture system and our human muscle culture system: (i) the degree of postsynaptic membrane specialization achieved during the 14 days of innervation in the rat muscle cultures resembled the degree of specialization achieved in our cultures during 6–8 weeks of innervation; (ii) dorsal root ganglia and Schwann cells were not present in their culture system, but they seemed necessary for the development of well-organized NMJs in our system; and (iii) postsynaptic plasmalemmal densities were less extensive in their cultured rat muscle fibres than in our cultured human muscle fibres. Another important difference is that in the rat cultures, AChRs did not accumulate at the majority of nerve–muscle contacts as judged by physiologic techniques (Kidokoro *et al.*, 1975; Kidokoro, 1980), but they did in our human muscle cultures as shown by alpha-bungarotoxin binding. The existence of several differences in NMJ development between cultured innervated embryonic rat and human muscle is not surprising, and it underscores the importance of performing such studies with *normal human* muscle, because this is the reference against which one must compare *diseased human* muscle innervated in culture.

Formation of T-tubules

Sarcoplasmic reticulum and T-tubules are functionally related membrane systems that play important roles in exciting contractile activity of muscle fibres. The T-tubules are invaginations of the surface plasmalemma and their lumens connect directly with the extracellular space. In adult mammalian twitch muscle fibres the T-tubule network is at the level of the A–I junctions, and there are usually two sarcoplasmic reticulum segments per T-tubule (for review see Franzini-Armstrong, 1986).

Formation of T-tubules during development *in vivo* and *in vitro* has been studied in animal muscle. In contrast to well-organized T-tubules and sarcoplasmic reticulum systems in adult muscle, muscle developing *in vivo* and *in vitro* contains T-tubules and sarcoplasmic reticulum components in a disorganized fashion (Ezerman & Ishikawa, 1967; Shimada *et al.*, 1967; Edge, 1970; Kelly, 1971, 1980; Luff & Atwood, 1971; Schiaffino *et al.*, 1977; reviewed by Franzini-Armstrong, 1986). In aneurally cultured chick and rat embryonic muscle, T-tubules develop as caveolar invaginations, appearing either as long, irregular tubes or as elaborate networks of honeycomb structures (Ezerman & Ishikawa, 1967; Schiaffino *et al.*, 1977).

Since the T-tubule system in aneurally cultured embryonic animal muscle fibres never reaches the regular form present in adult innervated muscle fibres, it has been suggested that either innervation and/or actvity may be required for complete T-tubule development (Franzini-Armstrong, 1986). In aneurally cultured human muscle, T-tubules are developed either minimally or not at all (Askanas & Engel, 1979). By contrast, in our innervated cultured human muscle fibres the developmental steps were evident of T-tubule formation from single tubules to an elaborate tubular network, along with distinct lateral sacs. This resembled T-tubule development in aneurally cultured embryonic chick and rat muscle (Ezerman & Ishikawa, 1967; Schiaffino *et al.*, 1977). However, even after 8 weeks of innervation of our cultured human muscle fibres, the pattern of T-tubule organization was still not as organized as that in adult human muscle fibres *in vivo*.

General comments

According to the above, it can be concluded that innervation of human muscle fibres cultured in monolayer influences their morphologic maturation. In contrast to cultured animal embryonic muscle or cell lines, which do not require innervation for the development of contractile activity, basal lamina well-defined cross-striations and T-tubules (Konigsberg, 1963; Ezerman & Ishikawa, 1967; Shimada *et al.*,

1967; Schiaffino *et al.*, 1977; Beach *et al.*, 1982; Silberstein *et al.*, 1982), human muscle aneurally cultured in monolayer does not spontaneously contract, does not have basal lamina, is not well cross-striated and has virtually no T-tubular system. Innervated cultured human muscle fibres, besides receiving 'neural factors' influencing their maturation, also contract nearly continuously. In animal systems, muscle activity has been considered important for the development of basal lamina (Sanes & Lawrence, 1983) and T-tubules (Franzini-Armstrong, 1986), and for organization of the postsynaptic membrane (Lømo, 1984). Therefore, it becomes important to delineate which features of morphological differentiation of innervated cultured human muscle are activity-dependent and which depend purely on 'neural trophic factors'. Since contraction of innervated cultured human muscle can be inhibited by d-tubocurarine (Askanas *et al.*, 1985), tetrodotoxin and alpha-bungarotoxin (V. Askanas, unpublished), consecutive steps of NMJ formation and other aspects of morphologic maturation of human muscle can now be studied over extended periods of time in cultured innervated but paralysed muscle fibres vis-à-vis actively contracting fibres.

Other uses of this co-culture system include the following. (i) Putative hormonal modulation of NMJ formation can be evaluated in this highly controlled environment, especially employing our new serum-free medium for culturing adult human muscle (Askanas *et al.*, 1985). (ii) The other (neuronal) end of this spinal cord, human muscle co-culture system can be studied. Explants of spinal cord survive in this human muscle co-culture system up to 6 months, in contrast to only 30–35 days when that spinal cord is cultured without muscle (Schmidt-Achert *et al.*, 1984). Thus, the influence of various factors, including muscle activity, on the motor neurons can be studied.

Perhaps the most important potential benefit of this culture system, which cannot be replaced by animal models, is that it enables one to study: (i) the influence of innervation on cultured human diseased muscle; (ii) NMJ formation in diseased human muscle in which muscle membrane dysreception has been postulated (Engel, 1979); and (iii) the influence of circulating factors and/or circulating cells (e.g. lymphocytes) on their own autologous, rather-mature, innervated cultured muscle (for example, the influence of autologous myasthenia gravis patient serum on NMJ formation, or of autologous lymphocytes on polymyositis muscle, and of autologous periodic paralysis serum obtained during an attack).

Although aneural cultures of human muscle have provided important insights into the pathogeneses of several neuromuscular disorders (Askanas, 1984; Witkowski, 1986a, b), there are certain inherited neuromuscular diseases, such as Duchenne muscular dystrophy, myotonic atrophy (dystrophy), myophosphorylase deficiency and muscle carnitine deficiency, in which defects have not been manifested in aneurally cultured, non-contracting muscle (Roelofs *et al.*, 1972; Meienhofer *et al.*, 1977; DiMauro *et al.*, 1978; Avigan *et al.*, 1983; Tahmoush *et al.*, 1983; Miranda & Mongini, 1984). It is quite conceivable that expression of the presumed genetic protein abnormality in muscle fibres of those disorders may require more advanced maturation of the muscle fibres, perhaps achievable with long-term innervation in culture. After the basic genetic defect is discovered (e.g. a missing protein) and if the defect is specific for human muscle cells, cultured pathologic human muscle will allow detailed analysis of the various pathogenic steps, and possibly treatment of the muscle in culture before a putative treatment is applied to the actual patient.

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