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Improved culture of individual muscle fibres with and without spinal cord explants in a collagen gel

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Suspension culture of single adult rat flexor digitorum brevis (FDB) muscle fibres in Vitrogen a purified collagen on tissue culture plastic or glass with mesh ring supports is superior to culture upon other substrates including collagen- laminin- or Vitrogen-coated tissue culture plastic. The Vitrogen gel-fibre mixture which attaches to glass or plastic provides at least 10 times more fibres per dish than does plating fibres on other substrates. Use of Vitrogen gel permits variable plating densities and the production of adequate numbers of cultures for long-term experimental comparisons of acetylcholinesterase (AChE) and rhodamine- α -bungarotoxin (RBTX) distribution on muscle fibres. Use of 40 μ g/ml ovotransferrin (OT) instead of chick embryo extract in the culture medium significantly improves long-term survival. Cultured fibres with or without the addition of ventral spinal cord explants may also be examined with electrophysiological techniques.

Several techniques have been developed for culturing single denervated adult skeletal muscle fibres such as those of the rat flexor digitorum brevis (FDB). Fibres have been plated on collagen (Beckoff and Betz, 1977b), on layers of fibroblasts stabilized by exposure to gamma radiation (Rubin et al., 1979), on beds of 7-day-old chick skin fibroblasts (Gundersen, unpublished), and in fibrin clots (Bischoff, 1975). No data on the number of viable fibres plated or investigation of viability with time were mentioned in these studies, but small numbers of fibres (1–2%) (Bischoff, 1979) were apparently viable for up to 3 weeks. We adapted a technique of Bischoff's for suspending fibres in a Vitrogen gel (Grega and Jay, 1983). Subsequently our method was used by Styra and Axelrod (1984) to examine acetylcholine receptor mobility on limited numbers of cultured adult fibres maintained for 14 days. Again no viability data were reported in this study.

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We report here good survival of muscle fibres cultured alone or with pieces of the ventral halves of spinal cord explants in Vitrogen gels. Adherence to glass surfaces was greatly improved by addition of a Nitex mesh ring. Substitution of ovotransferrin (40 $\mu\text{g}/\text{ml}$) for chick embryo extract also significantly improved survival in the second week of culture. This technique provided long-term stability of large numbers of fibres for up to $2\frac{1}{2}$ weeks. Experimental assessment of acetylcholinesterase (AChE) and acetylcholine receptors (AChRs) with histochemical or fluorescent labels was then possible on fibres maintained in culture for long time periods.

Two FDB muscles were first treated with collagenase by following a procedure similar to that used by Beckoff and Betz (1977a,b), collagenase digested the connective tissue and tendinous insertions of skeletal muscle without affecting membrane and electrical properties or acetylcholine sensitivity (Betz and Sakman, 1973, Beckoff and Betz, 1977a), apparently the basal lamina remained intact (Bischoff, 1979). Single fibres which remained suspended in the dissociation solution were removed and transferred to a nutrient medium (Barald and Berg, 1978) containing 10^{-7} M tetrodotoxin (TTX). Muscle fibres contained in Vitrogen drops were plated on specially prepared 35-mm tissue culture dishes with glass bottoms. Rings of Nitex mesh 4 mm in width were cut to fit the outer edge of the dish bottom and were secured to the glass surface with hot 0.5% (w/v) agarose in $3\times$ distilled water. The agarose solution was allowed to dry overnight and could be stored up to one month before addition of Vitrogen suspensions. Cocultures were made with pieces (approximately 100 μm) of the ventral halves of embryonic spinal cords, explants were initially cultured by our previous method (Grega, 1984), excised, and positioned in target fields of 24-h-old muscle fibres. Approximately 12 explants were excised from a 1- to 10-day-old explant culture and pipetted into a muscle fibre culture from which the nutrient medium was removed. Excess liquid was drained from the culture until only a thin film remained. Explants were positioned by means of a No. 5 Inox forceps in a target field of 1–6 muscle fibres. All remaining medium was removed, and a drop of Vitrogen mixture was applied to each explant or several explants which were in the same vicinity. Cocultures were placed in a humidity chamber which consisted of a lidded glass dish lined with several water-soaked "lint-free" tissues (Kimwipes). Cocultures were incubated for 20 min at 37°C in a 5% CO_2 atmosphere to allow the Vitrogen to gel. Nutrient medium used for muscle cultures (Barald and Berg, 1978) was applied and changed every other day.

In order to compare the effects of the presence of nerve upon the distribution of muscle fibre AChE and RBTX binding sites over time it became necessary to establish a culture system which would yield sufficient numbers of viable fibres for investigation. Culture methods other than suspending fibres in Vitrogen did not provide sufficient numbers of viable fibres (see Table 1). Plastic tissue culture dishes were coated with one of the following: Vitrogen, collagen (Barald and Berg, 1978), chick fibroblasts (Temin, 1960), chick fibroblasts over a layer of collagen, chick fibroblast-derived extracellular matrix, and laminin (1.1 $\mu\text{g}/\text{ml}$, 0.25 ml/dish). Fibres, which were suspended in 1 ml of nutrient medium, were plated on these substrates and were allowed to settle overnight. Medium containing suspended fibres was removed from each dish, and fresh medium was applied. Phase contrast criteria

TABLE 1

PLATING EFFICIENCY OF VIABLE FDB MUSCLE FIBRES ON VARIOUS SUBSTRATES

Values are means + S E M

Substrates *	Percent fibre adherence
Fibres <i>suspended in</i> Vitrogen	99.8 ± 0.20
Fibres <i>plated on</i> Vitrogen	4.6 ± 0.77
Fibres plated on collagen	4.3 ± 1.15
Fibres plated on chick fibroblasts	10.2 ± 1.14
Fibres plated on chick fibroblasts grown on collagen	7.9 ± 2.53
Fibres plated on chick fibroblast-derived extracellular matrix **	5.1 ± 1.12
Fibres plated on laminin (1.1 µg/ml-0.25ml/dish)	5.0 ± 1.11

* Initial plating density was 150-200 fibres/35 mm culture dish n = 3 for each substrate tested

** Fibroblasts were plated and then removed by lysing them in 3 × distilled water

(the presence of definite striations and bulging peripheral nuclei as observed by Beckoff and Betz, 1977b) were used to compare the number of viable fibres suspended in Vitrogen gels to the number of viable fibres which adhered to the other substrates after 24 h (see Table 1) Plating fibres in Vitrogen resulted in a 10-fold increase in plating efficiency, providing an adequate sample size (8-12 cultures/2 FDB muscles with 20-400 fibres/dish) Viability studies were carried out on cultures with a glass surface and mesh support in which 0.1% trypan blue was used as a viability indicator At the 96 h time point, of 172-390 fibres plated per dish, 38.5% ± 1.95 (S E M, n = 13) of the initial population were viable In muscle fibre-spinal cord explant cocultures, which contained 173-382 fibres at the time of explant addition, 39.2% ± 2.49 (S E M, n = 31) of the fibres were viable at 96 h Endplate AChE and RBTX binding persisted throughout the period examined Viability was confirmed by electrophysiological intracellular recordings of -50 to -70 mV resting potentials (Jay et al, unpublished), these results were in agreement with those of Beckoff and Betz (1977a) Neither viability nor the distribution pattern of AChE and RBTX binding sites was affected by plating density or coculture conditions

In order to increase the viability with time in culture an effort was made to improve the nutrient medium Although addition of ascorbic acid to the nutrient medium did not increase viability, medium in which 40 µg/ml of ovotransferrin (OT) (Ii et al, 1982) was used in place of 5% chicken embryo extract (C E E) (Barald and Berg, 1978) significantly increased viability during the second week of culture In one such experiment dishes containing between 190 and 357 fibres were maintained in either C E E or OT medium In the second week, 15 × as many fibres survived in cultures with OT medium compared to cultures with C E E medium (up to 20% survival) The substitution of OT for C E E provided a better-defined medium in which to assess the effects of nerve tissue on denervated muscle fibres, since no constituents derived from nervous tissue were present

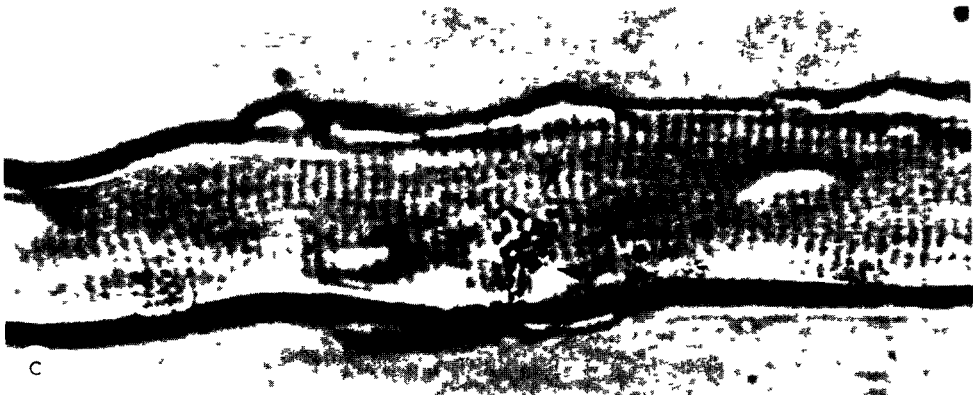
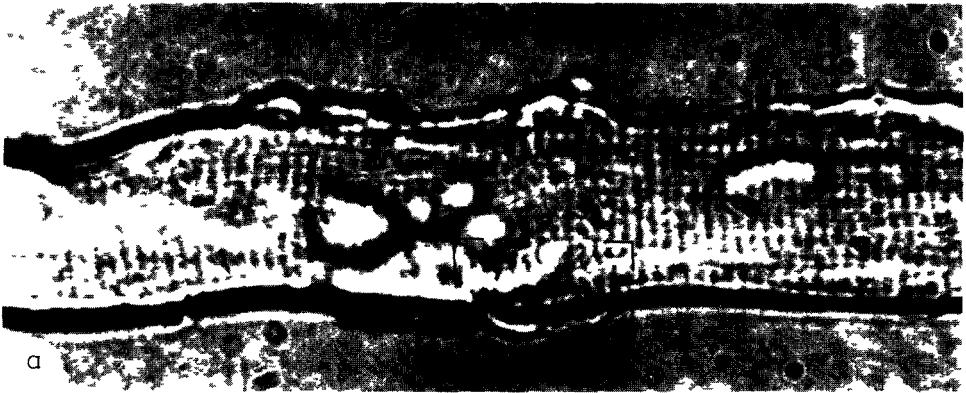


Fig 1 a the fibre in Fig 1a-c was photographed in a culture which did not contain explants. Fibre viability was initially determined in such cultures by staining with 0.1% trypan blue. Viable fibres exclude the dye. Cultures were fixed for 30 min in an L-15 based solution (Moody-Corbett and Cohen 1981). RBTX binding sites were fluorescently labelled and AChE was revealed histochemically. Each viable fibre was examined for both RBTX binding sites and AChE. The effect of the presence of nerve on the distribution of the above-mentioned parameters was then investigated in cocultures. Prominent cross-striations and peripheral location of nuclei are apparent in this phase contrast micrograph of a viable fibre from a 48-h-old culture. a the endplate region is boxed. b fluorescence micrograph of the fibre in (a). After fixation this culture was incubated in 1×10^{-8} M RBTX to locate putative acetylcholine receptors. Note the fluorescently labelled area which is delimited by the arrow tips and marks the postsynaptic

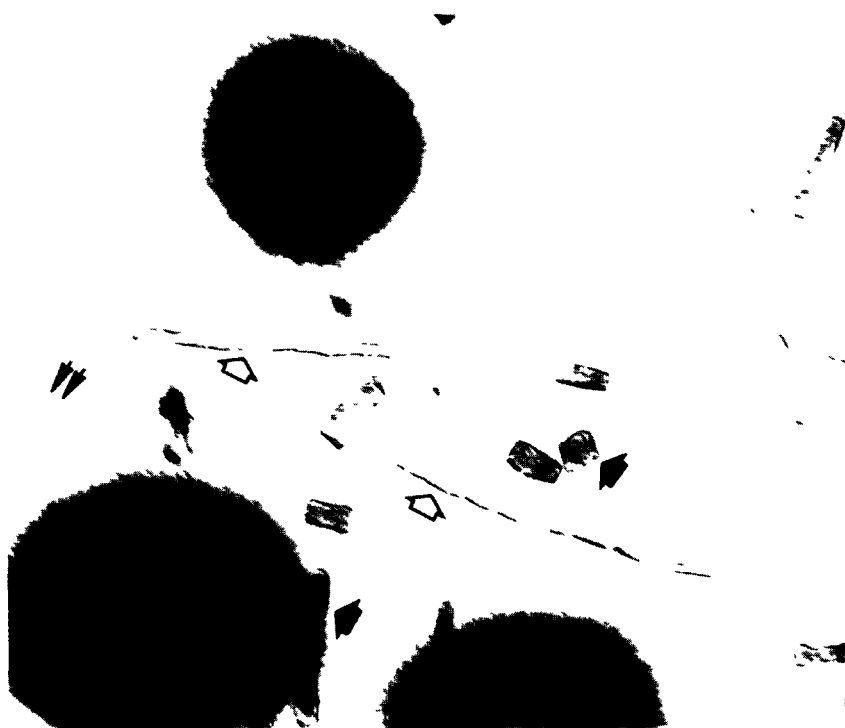


Fig 2 Pieces of ventral halves of embryonic spinal cord were placed in target fields of muscle fibres which had been previously cultured for 24 h This phase contrast micrograph of a 72-h-old coculture was stained with 0.1% trypan blue Both nonviable fibres and fragments (filled arrows) take up the dye viable fibres (hollow arrows) exclude it Neurites (double arrows) extend from the entire circumference of the explants and appear to contact viable muscle fibres randomly

We are currently using this *in vitro* system of muscle fibres \pm nerve to investigate the role of the muscle basal lamina in reinnervation and neurite behaviour with respect to individual muscle fibres The distribution of 2 basal lamina components (AChE and laminin), and 1 plasma membrane component, putative acetylcholine receptors, revealed by RBTX binding sites, is being investigated in muscle cultures and cocultures Fig 1a–c illustrates RBTX binding sites and AChE at the original synaptic site in muscle cultures A typical coculture is seen in Fig 2 Intracellular electrophysiological recording from muscle in cocultures revealed irregularly appearing miniature endplate potentials (MEPPs) of 1–3 mV with a duration of 60 ms 24 h after the addition of explants (Jay et al, unpublished), indicating that the fibres examined were innervated Whether all nerve–muscle contact sites are functional is currently under investigation The culture procedure for individual denervated

region of the neuromuscular junction c phase contrast micrograph of the fibre in (a) and (b) After incubation with RBTX this culture was treated with a modified Karnovsky stain (Moody-Corbett and Cohen 1981) to locate AChE The region which contains AChE is enclosed by arrow tips and is colocalized with endplate RBTX binding sites in (b) a b and c 625 \times

muscle fibres should prove useful for future studies of nerve–muscle interactions and degeneration and/or regeneration of various muscle components

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