Expression of asymmetric forms of acetylcholinesterase during myogenesis *in vitro*

J. P. TOUTANT

*Laboratoire de Physiologie animale, I.N.R.A., 9. place Viala, 34060 Montpellier Cedex, France.*

**Summary.** Chick muscle cells differentiating *in vitro* in the absence of nerve cells produce asymmetric forms of acetylcholinesterase (AChE) only if they originate from muscles which accumulate these forms *in ovo* (i.e. after embryonic day 5). The presence of nerve cells does not induce the synthesis of A forms in cultures of 5 day-old myoblasts and does not increase their proportion in cultures of 7 day-old myoblasts. Thus, the capacity to synthesize (or assemble) the complex polymeric forms of AChE does not reflect a direct neural influence but might rather be considered as an intrinsic property of the « late » categories of myoblasts that sequentially occur during the differentiation of leg muscles.

We studied the synthesis of ChE molecular forms in the mouse muscle C₂ cell line. From these experiments we suggest that the synthesis of A forms (or their assembly) can take place as soon as the cells are withdrawn from the cell cycle, but does not require cell fusion by itself.

These observations are related to other recent studies that challenge the validity of A forms as topographical/physiological markers of neuromuscular interactions.

**Introduction.**

Acetylcholinesterase (AChE, EC 3117) of vertebrate skeletal muscle can exist in six molecular forms: three globular forms G₁, G₂ and G₄ corresponding to monomer, dimer and tetramer of catalytic subunits, and collagen-tailed, asymmetric forms, in which 1, 2 or 3 tetramers of catalytic subunits are associated to a collagene component that confers their specific hydrodynamic properties to the molecules. These forms are referred to as A₄, A₈ and A₁₂ (Bon *et al.*, 1979; Massoulié and Bon, 1982; Massoulié and Toutant, 1987).

A forms were found concentrated at rat neuromuscular junctions (Hall and Kelly, 1971; Hall, 1973). They appeared at the moment of innervation in developing muscles (Vigny *et al.*, 1976; Koenig and Vigny, 1978), disappeared after denervation and reappeared with reinnervation (Vigny *et al.*, 1976). It was therefore considered that these forms represented topographical markers and physiological correlates of nerve-muscle interactions (Vigny *et al.*, 1976).

In cell cultures it was demonstrated that rat myoblasts removed *in utero* at embryonic day 14 (i.e. before the innervation of muscle) did not produce A forms.
They acquired this capacity if they were cocultured with nerve cells originating from the spinal cord (Koenig and Vigny, 1978). It was therefore suggested that the synthesis of A forms was induced by a direct neural influence (review in Toutant and Massoulié, 1987).

We tested this hypothesis in the case of chick muscle cells differentiating in ovo and in vitro (Toutant et al., 1983). Some of the results reported in this paper do not fit with earlier observations in rat and challenge the idea of a neural determination of the expression of AChE asymmetric forms. Other experimental data are also reported concerning the possible relationship between early events of myogenesis (withdrawal from cell cycle, cell fusion) and the synthesis of A forms.

Material and Methods.

Cell cultures. — A complete description of chick muscle cultures is given in Toutant et al. (1983). Muscle cells were grown in Dulbecco’s minimum Eagle medium (DMEM) containing 5 % of fetal calf serum (FCS).

Cultures of C2 cells were obtained from C. Pinset at the Pasteur Institute. Medium conditions were manipulated in order to obtain C2 cells in three different stages of differentiation, as described previously in the case of L6 cells (Pinset and Whalen, 1984, 1985):

Type 1 cultures: cells were cultured for 4 days in Ham’s F12 medium supplemented with fetal calf serum (20 %) and 10⁻⁶ M dexamethasone: in this medium, the C2 cells proliferate.

Type 2 cultures: cells were cultured for 4 days in DMEM + insulin (10 µg/ml) + transferrin without serum, in the presence of 0.2 mM Ca²⁺. In this medium the cells cease their divisions but do not fuse into myotubes.

Type 3 cultures: cells were cultured for 4 or 7 days in a medium similar to type 2 but the concentration of Ca²⁺ was increased to 2 mM. In these conditions, the C2 cells fuse into multinucleated myotubes.

Tissue homogenization, centrifugation and AChE assay. — These routine operations were performed as indicated in Toutant et al. (1983) and Toutant (1986). Centrifugation parameters are indicated in figure legends.

Collagenase treatment. — The homogenates of cell cultures were subjected to collagenase digestion according to Toutant et al. (1985). 3 000 units of collagenase (Advance Biofacts Co., form III) were solubilized in 500 µl of 1 M Tris-HCl, pH 8.0 and 50 mM CaCl₂. Incubations (300 µl extract + 100 µl of collagenase solution) were performed at 20 °C for 1 h or 2 h at 37 °C.

Results.

Synthesis of AChE A forms in chick muscle cultures.

The expression of A forms by chick muscle cells in vitro has been source of conflicting reports (see review in Toutant and Massoulié, 1987). It was demonstrated however that the use of fetal calf serum was a prerequisite for the
synthesis of A forms in 11 day-old myoblasts cultures (Bulger et al., 1982; Toutant et al., 1983) and that horse serum interferes with the analysis of molecular forms in gradient centrifugation (Toutant et al., 1983; Vallette et al., 1986).

We compared the proportions of AChE molecular forms produced in cultures seeded with myoblasts removed at embryonic days (ED) 5, 7, 9 and 11. Figure 1 shows that in culture of ED5 myotubes synthesized only the globular forms G1, G2 and G4. At ED7, 9 and 11, the A12 form (20S) was also detected in addition to G2 and G4 forms, and represented 3% (ED7), 9% (ED9) and 10% (ED11) of the total activity after six days in culture.

![Diagram showing AChE activity](image)

FIG. 1. — Molecular forms of AChE synthesized by chick muscle cells in culture. Myoblasts used to initiate the cultures were removed from chick embryos at different ages: A: 5 day-old myoblasts, 6 days in culture. AChE activity: 0.23 OD/min/ml. No A form. G1 form accounts for 30% of total activity. B: 7 day-old myoblasts, 6 days in culture. Activity: 0.28 OD/min/ml. A12 form: 3%; G1 form: 23%; C: 9 day-old myoblasts, 6 days in culture. Activity: 4.9 OD/min/ml. A12 form: 9%; G1: 15%; D: 11 day-old myoblasts, 6 days in culture. Activity: 2.4 OD/min/ml. A12: 10%; G1: 8%.

All cultures were seeded at a density of 2.10⁶ cells/dish (Ø = 10 cm)
Sedimentation analyses were performed in 5-20% sucrose gradients (1 M NaCl, 1% Triton × 100) centrifuged for 19 h at 36 000 rpm at 4 °C in a Beckman SW41 rotor. 165: E. coli β-galactosidase; 6.1S: E. coli alkaline phosphatase.
The A forms of AChE appeared in leg muscles after ED5\textit{ in ovo} (Toutant et al., 1983) in apparent correlation with the establishment of the first neuromuscular junctions (Landmesser and Morris, 1975). Therefore it seems that «late» myoblasts (ED7, 9 and 11) possess the information necessary to express the \( A_{12} \) form. We tested the influence of cocultured nerve cells on «late» and «early» (ED5) myoblasts.

\textit{Effect of cocultured nerve cells on A form synthesis in muscle cultures.}

Figures 2 and 3 show the effect of cocultured nerve cells on the molecular forms of AChE produced by 7 day-old (2) or 5 day-old chick myoblasts (3).

![Graph showing molecular forms of AChE](image)

FIG. 2. \textit{— AChE molecular forms synthesized in 7 day-old myoblasts cultures.} A: Aneural muscle culture: \( 3.10^6 \) muscle cells cultured for 6 days. B: Coculture of \( 3.10^6 \) myoblasts and \( 3.10^6 \) nerve cells from spinal cord of 7 day-old chick embryo for 6 days. Total AChE activities were 0.36 OD/min/dish in A; 1.1 OD/min/dish in B. Centrifugation was performed as in figure 1. The 20S regions of each profile are redrawn with an expanded scale (\( \times 10 \)).

In the experiment of figure 2, the total AChE activity in the coculture was 1.1 OD/min/ml, and the activities of the separate control cultures were 0.36 OD/min/ml for the muscle cells and 0.6 OD/min/ml for the nerve cells. The activity of the coculture was therefore almost entirely accounted for by the sum
of the two activities. Figure 2 shows that the proportion of A forms was similar in the coculture and in the aneural muscle cell cultures (3% in each case). A similar result was obtained by Kato et al. (1980) using ciliary ganglion cells in coculture with chick muscle cells.

In the experiment of figure 3, we noted that the total AChE activity (1.05 OD/min/ml) was higher than expected from the values of nerve or muscle cells cultured alone, seeded at the same density as in the coculture (0.34 and 0.23 OD/min/ml respectively). The specific activity of AChE was also increased.
in the coculture (0.41 OD/min/mg protein versus 0.3 and 0.1 OD/min/mg protein in nerve and muscle cell cultures, respectively). In spite of this positive effect of the coculture on the overall AChE activity, we did not detect any A form in this case.

These experiments suggest that the expression of A forms does not require a direct neural influence but is rather a property of the « late » categories of leg myoblasts (see White et al., 1975).

In the next section we examine whether the production of A forms is restricted to multinucleate muscle cells or if these forms may be produced by mononucleate premuscle cells, prior to innervation.

Effect of cell fusion on the expression of A forms.
For these experiments, we used the C2 cell line derived from adult mouse muscle (Yaffe and Saxel, 1977).

The culture conditions used by C. Pinset are indicated in the section Materials and Methods and in the legend of figure 4. We used three types of cultures:

Type 1: medium rich in mitogenic factors in which the cells proliferate;
Type 2: medium without these factors but in which the concentration of calcium is reduced to 0.2 mM: the cells cease to proliferate but do not fuse (no myotube was observed in these conditions);
Type 3: medium of type 2 but the concentration of extracellular calcium is raised to 2 mM: the cells fuse into myotubes.

We analyzed the molecular forms of AChE produced in the three types of cultures (fig. 4A, B and C). In type 1 cultures, proliferating myoblasts synthesized only G1 (3S) and G4 (10.5S) forms of AChE. In type 2 and 3 cultures, A forms were produced (A12: 16.4S, A8: 13S) in addition to G1 and G4 forms. The proportions of A forms were however different in type 2 and 3 cultures after 3 days: they accounted for 8 % of the total activity in type 2 (mononucleated cells) and 18 % in type 3 (multinucleated cells). The proportion of A forms increased in type 3 cultures with further differentiation (see fig. 4, D). Figure 5 shows that the A forms produced in type 2 cultures were collagenase-sensitive. After 1 h of collagenase treatment at 20 °C, we noted that the sedimentation coefficients of A12 form (17.4S vs 16.4S in the control sample) and A8 form (14.2S vs 13S) were increased as a result of the partial digestion of the collagen tail and that the amount of G4 form was also increased. When the collagenase treatment was performed at 37 °C for 2 h, we observed a total disappearance of A forms in parallel with an increase of the G4 forms (not shown).

These experiments show that the expression of A forms of AChE in muscle cell does not require cell fusion.

Discussion.

Clonal analysis of chick myogenesis has shown that distinct classes of myoblasts (defined by their medium requirements and clonal morphology) sequentially occur during leg development (White et al., 1975). The phenotypes
of these cells are maintained through subclonal passages (Rutz and Hauschka, 1982). Early and late classes of myoblasts differ by the type of myosin light chains synthesized (Toutant et al., 1984; Mouly et al., 1987). The present results indicate that the production of asymmetric forms of AChE by muscle cells in vitro does not depend on the presence of nerve cells but may be considered as a property of «late» classes of myoblasts. «Early» myoblasts do not synthesize A forms. Thus, AChE A forms cannot be used as a marker of neuromuscular interactions.

This conclusion is further supported by other experimental evidence obtained in vivo. Embryonic muscles in which the innervation is impaired by the early
destruction of nerve cells do accumulate A forms (rat: Harris, 1981; chick: Toutant et al., 1983; duck: Sohal and Wrenn, 1984). In adult muscle, A forms do not always appear dependent on innervation either: these forms disappear, for example, from the innervated chick anterior latissimus dorsi muscle in aged animals (Lyles and Barnard, 1980). They are actively neosynthesized in the slow-twitch part of the rabbit semimembranous muscle after denervation (Bacou et al., 1982) as well as in certain slow-twitch muscles in other mammals (Lai et al., 1986).

Thus the temporal correlation between the expression of A forms and the establishment of innervation does not mean that the two phenomena are causally related: we suggest that it is largely coincidental.

The second purpose of this study was to investigate the synthesis of AChE molecular forms during early stages of muscle differentiation. Using quail myoblasts infected by a thermosensitive mutant of Rous sarcoma virus (Fiszman and Fuchs, 1975), we have previously reported that proliferating cells (cultured at 35 °C) synthesized only globular forms, whereas myotubes originating from the same cells (at 41 °C) accumulated A12 and G forms (Toutant et al., 1983). Similarly, murine C2 cells have been shown to produce A forms when fused into myotubes (Inestrosa et al., 1983). We show here that fusion is not a necessary condition: the C2 cells may assemble the A forms, even at the mononucleate state
in culture conditions in which cells are withdrawn from the cell cycle. In these
conditions, C\textsubscript{2} mononucleated cells were also shown to accumulate muscle
specific contractile proteins (C. Pinset, personal communication). This observa-
tion in vitro is supported by the recent result of Vallette et al. (1987) who reported
that A forms of AChE exist in dermomyotomes of 3 day-old quail embryos in
which all premuscle cells are still mononucleated.

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Résumé. Expression des formes asymétriques de l’acétylcholinestérase au cours de la
myogenèse in vitro.

Chez le poulet, les cellules musculaires différenciées en cultures aneurales ne
synthétisent des formes asymétriques d’acétylcholinestérase que si les myoblastes dont
tes cellules sont prélevées des muscles embryonnaires accumulant cette forme
in ovo (c’est-à-dire après le 5\textsuperscript{e} jour de développement embryonnaire). La présence de

cellules nerveuses n’induit pas la synthèse des formes A dans les cultures initiées à partir
de myoblastes prélevés au 5\textsuperscript{e} jour in ovo, ni n’augmente leur proportion dans les cultures
dérivant de myoblastes de 7 jours. La synthèse (ou l’assemblage?) des formes A d’AChE
apparaît donc comme une propriété des catégories «tardives» de myoblastes et non
comme le reflet d’une interaction nerf-muscle.

L’étude de cultures de myoblastes de la lignée cellulaire C\textsubscript{2} de souris suggère que la
synthèse (ou l’assemblage) des formes complexes nécessite la fin des divisions cellulaires
mais non la fusion : des cellules musculaires mononucléées sont donc capables, dans
certaines conditions de culture, de synthétiser les formes asymétriques d’AChE.

References

BACOU F., VIGNERON P., MASSOULIÉ J., 1982. Acetylcholinesterase forms in fast and slow

BON S., VIGNY M., MASSOULIÉ J., 1979. Asymmetric and globular forms of acetylcholinesterase

BULGER J. E., RANDALL W. R., NIEBERG P. S., PATTerson G. T., McNAMEE M. G., WILSON
B. W., 1982. Regulation of acetylcholinesterase forms in quail and chicken muscle cultures.

FISZMAN M., FUCHS P., 1975. Temperature sensitive expression of differentiation in transformed

HALL Z. W., 1973. Multiple forms of acetylcholinesterase and their distribution in endplate and

HALL Z. W., KELLY R. B., 1971. Enzymatic detachment of endplate acetylcholinesterase from

HARRIS A. J., 1981. Embryonic growth and innervation of rat skeletal muscles. II. Neural regulation

INESTROSA N. C., MILLER J. B., SILBERSTEIN L., ZISKIND-CONHAIM L., HALL Z. W.,
1983. Development and regulation of 16S acetylcholinesterase and acetylcholine receptors


