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

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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 differentation of leg muscles.

We studied the synthesis of ChE molecular forms in the mouse muscle C_2 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these experiments we suggest that the synthesis of A forms (or their assembly) can take place as soon as the cells are withdrawn from the cell cycle, but does not require cell fusion by itself.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 G_1, G_2 and G_4 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 collagenic component that confers their specific hydrodynamic properties to the molecules. These forms are referred to as A_4, A_8 and A_{12} (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 C_2 cells were obtained from C. Pinset at the Pasteur Institute. Medium conditions were manipulated in order to obtain C_2 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 F_{12} medium supplemented with fetal calf serum (20 %) and 10^{-6} M dexamethasone : in this medium, the C_2 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 Ca^{2+}. 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 Ca^{2+} was increased to 2 mM. In these conditions, the C_2 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 Biofactures Co., form III) were solubilized in 500 µl of 1 M Tris-HCl, pH 8.0 and 50 mM $CaCl_2$. 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 G_1, G_2 and G_4. At ED7, 9 and 11, the A_{12} form (20S) was also detected in addition to G_2 and G_4 forms, and represented 3 % (ED7), 9 % (ED9) and 10 % (ED11) of the total activity after six days in culture.

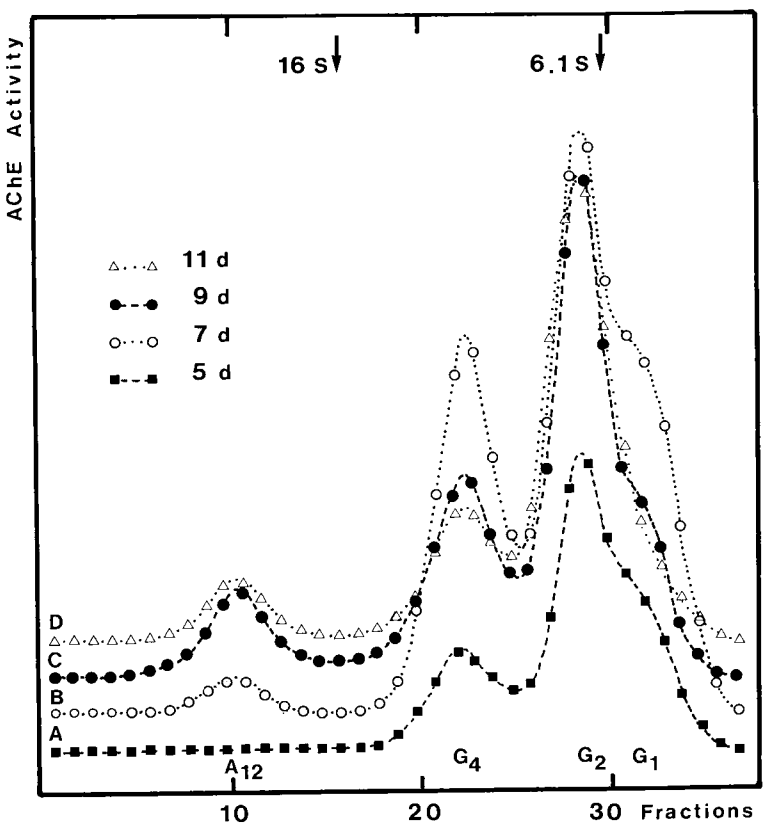

FIG. 1. — *Molecular forms of AChE synthesized by chick muscle cells in culture.* Myoblasts used to initiate the cultures were removed from chick embryos at different ages : A : 5 day-old myoblasts, 6 days in culture. AChE activity : 0.23 OD/min/ml. No A form, G_1 form accounts for 30 % of total activity. B : 7 day-old myoblasts, 6 days in culture. Activity : 0.28 OD/min/ml. A_{12} form : 3 % ; G_1 form : 23 % ; C : 9 day-old myoblasts, 6 days in culture. Activity : 4.9 OD/min/ml. A_{12} form : 9 % ; G_1 : 15 % ; D : 11 day-old myoblasts, 6 days in culture. Activity : 2.4 OD/min/ml. A_{12} : 10 % ; G_1 8 %.

All cultures were seeded at a density of 2.10^6 cells/dish (∅ = 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. 16S : *E. coli* β-galactosidase ; 6.1S : *E. coli* alkaline phosphatase.

The A forms of AChE appeared in leg muscles after ED5 *in ovo* (Toutant *et al.*, 1983) in apparent correlation with the establishment of the first neuromuscular junctions (Landmesser and Morris, 1975). Therefore if 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.

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).

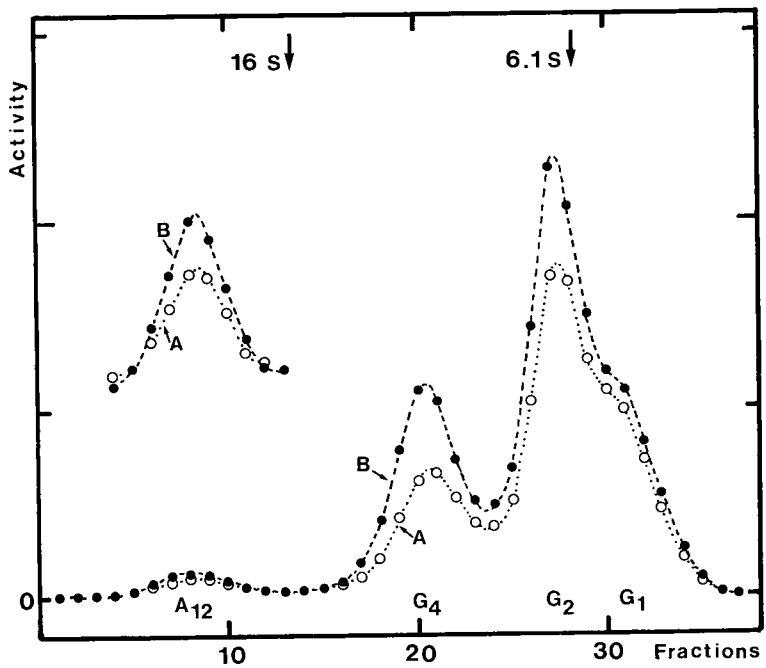

FIG. 2. — *AChE molecular forms synthesized in 7 day-old myoblasts cultures.* A : Aneural muscle culture : 3.10^6 muscle cells cultured for 6 days. B : Coculture of 3.10^6 myoblasts and 3.10^6 nerve cells from spinal cord of 7 day-old chick embryo for 6 days.
Total AChE activities were 0.36 OD/min/dish 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 (× 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

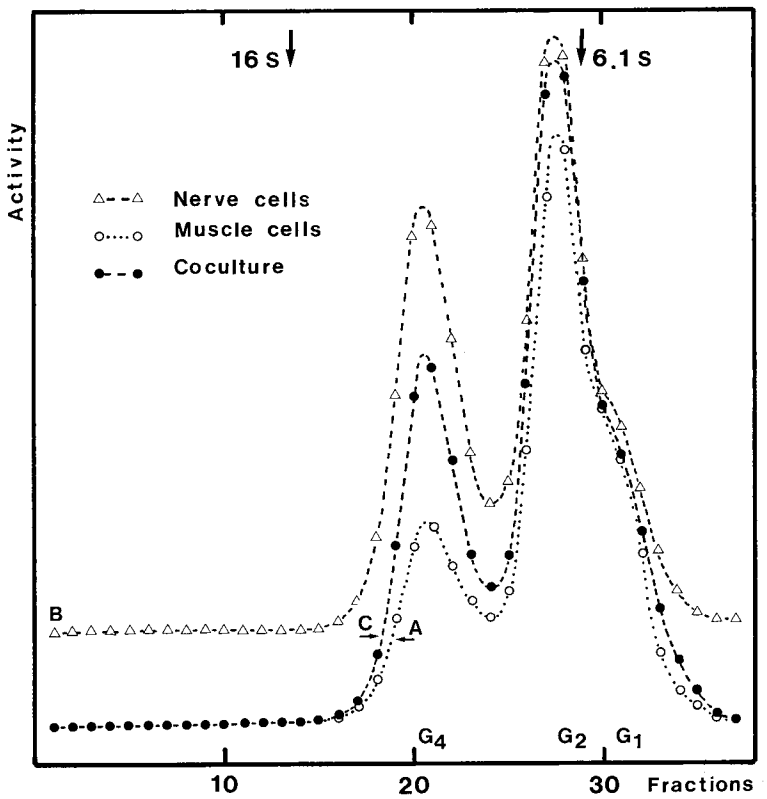

FIG. 3. — *AChE molecular forms produced in 5 day-old myoblasts cultures.* A : Muscle cells from 5 day-old chick embryo leg muscles : 2.10^6 cells/dish (= 10 cm), 6 days in culture. AChE activity : 0.23 OD/min/dish. AChE specific activity : 0.1 OD/min/mg protein. B : Nerve cells from 6 day-old chick embryo spinal cord : 2.10^6 cells/dish, 6 days in culture. AChE activity : 0.34 OD/min/ dish. Specific activity : 0.31 OD/min/mg protein. C : Coculture of 2.10^6 muscle cells and 2.10^6 nerve cells. Total AChE activity : 1.05 OD/min/dish. Specific activity : 0.41 OD/min/mg prot.
Cells were homogenized in high salt buffer containing 1 % T × 100. The extracts were analyzed in 5-20 % sucrose gradients centrifuged for 19 h at 36 000 rpm in a SW41 rotor.

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 C_2 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 G_1 (3S) and G_4 (10.5S) forms of AChE. In type 2 and 3 cultures, A forms were produced (A_{12} : 16.4S, A_8 : 13S) in addition to G_1 and G_4 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 A_{12} form (17.4S *vs* 16.4S in the control sample) and A_8 form (14.2S *vs* 13S) were increased as a result of the partial digestion of the collagen tail and that the amount of G_4 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 G_4 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

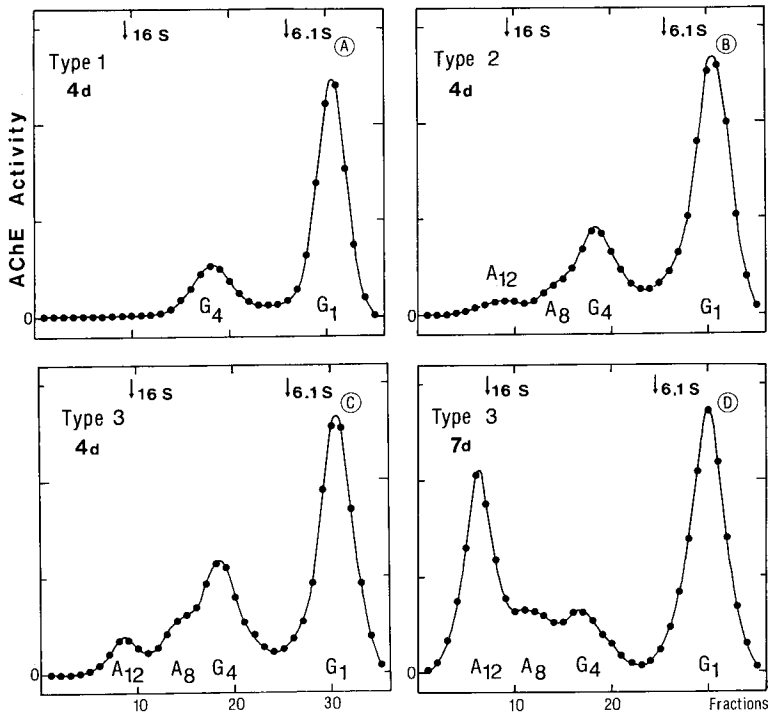

FIG. 4. — *Sedimentation analysis of AChE molecular forms produced by C_2 cell* in vitro. A : Type 1. Cells were cultured for 7 days in Ham's F_{12} medium + 20 % fetal calf serum then 4 days in the same medium. Myoblasts remain in the cell cycle AChE specific activity : 0.03 OD/min/mg prot. No A form. B : Type 2. Cells were cultured for 7 days in Ham's F_{12} medium + 20 % fetal calf serum then 4 days in DMEM + transferrin + insulin + 0.2 mM Ca^{2+}. Cells are withdrawn from the cell cycle but do not fuse. Specific activity : 0.03 OD/min/mg. A forms (A_{12} + A_8) represent 8 % of the total activity. C : Type 3. Same as in B but the second medium contains 2 mM Ca^{2+}. Cells fuse. AChE specific activity : 0.03 OD/min/mg. A forms (A_{12} + A_8) represent 18 % of the total activity. D : Type 3. Same conditions as in C but cells are cultured 7 days in the DMEM medium. AChE specific activity : 0.15 OD/min/mg. A forms represent 50 % of the total activity.
Extracts obtained in high salt medium containing 1 % T × 100 were loaded on 5-20 % sucrose gradient in high salt buffer containing 1 % Triton × 100 and centrifuged for 20 h at 40 000 rpm in Beckman SW41 rotor. *E. coli* β-galactosidase (16S) and alkaline phosphatase (6.1S) were included as internal sedimentation standards.

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

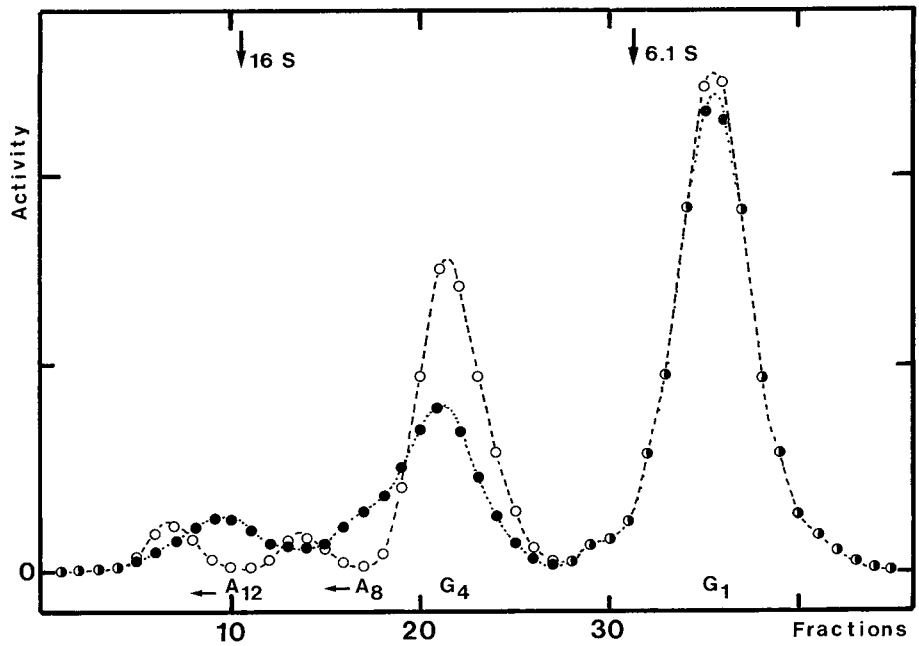

FIG. 5. — *Collagenase sensitivity of asymmetric forms in C_2 cell culture of type 2.* Cells were extracted in Triton-saline buffer (1 M NaCl, 1 % T × 100). 300 µl of this extract were incubated with 100 µl of collagenase solution for 1 h at 20 °C. (○) or 100 µl of buffer (●) and centrifuged for 20 h at 40 000 rpm at 4 °C in a Beckmann SW41 rotor. Collagenase induced an increase of the sedimentation coefficients of A_{12} form (16.4S 17.6S) and A_8 form (13S → 14.2S) and the appearance of a G_4 dissociation product.

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 semimembraneous 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 A_{12} and G forms (Toutant *et al.*, 1983). Similarly, murine C_2 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 C_2 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_2 mononucleated cells were also shown to accumulate muscle specific contractile proteins (C. Pinset, personal communication). This observation *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 elles dérivent sont prélevés dans des muscles embryonnaires accumulant cette forme *in ovo* (c'est-à-dire après le 5e 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 5e 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_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.

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