

The Syndrome of Arthrogryposis and Palatoschisis (SAP) in Charolais cattle; Abnormal motor innervation and defect in the focalization of 16 S acetylcholinesterase in the end-plates rich regions of the muscle. (1)

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Summary

The Syndrome of Arthrogryposis and Palatoschisis SAP is an hereditary disease in the *Charolais* cattle breed. We studied the neurobiological and neuropathological correlates of this disease in SAP calves, immediately after birth. We find that nerve-muscle interaction is abnormal; the focal accumulation of 16S AChE in end-plate rich regions is not observed as in control calf, and the motor innervation, evidenced by silver nitrate impregnation techniques, shows abnormal features, as frequent preterminal branching and ultraterminal sprouting.

Introduction

The Syndrome of Arthrogryposis and Palatoschisis (SAP) in *Charolais* cattle has been first described by LAUVERGNE and BLIN (1967). It is an hereditary disease, carried by an autosomal recessive gene with incomplete penetrance (see LEFORT *et al.* 1977 and also LAUVERGNE and FAUCON, 1976, and LAUVERGNE *et al.* 1979 for a review).

Its clinical expression is rather variable in severity, but is always characterized by fixture of joints (arthrogryposis) and severity of the disease is the consequence of different movement disabilities of the limbs, due to variable angles of fixture and also aberrant insertion of muscles or even lack of a given muscle. In man, arthrogryposis has been described as a congenital rigidity of joints in newborns, in the last decades of the nineteenth century. It is now considered as a symptom,

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or a syndrome, and not a definite disease, which may be due to different mechanisms, spinal radicular, or myogenic. In some cases, nerve and muscle biopsies performed in this syndrome revealed the features of a neurogenic process (BYERS and BANKER, 1961; HOOSHMAUD *et al.* 1971, YUILL and LYNCH, 1974). A few cases exhibited morphological abnormalities suggesting a myopathic process (BANKER *et al.* 1957). In many cases the muscle is surprisingly normal in biopsies, suggesting an early abnormality in the embryonic muscle development (DUBOWITZ, 1978). In all conditions, the joint and soft tissue changes are evidenced as secondary to the absence of a normal muscle activity in the limbs *in utero*.

In the search of primary causes of abnormal embryonic early differentiation, developmental genetics offers animal models of considerable value. GLUECKSOHN-WAELSCH has developed the study of embryonic differentiation by identifying lethal genes in the mouse (GLUECKSOHN-WAELSCH, 1963), leading either to a total, foetally lethal, arrest of the development of the mouse embryo (with rapid regression *in utero*) or to early defects, only lethal at birth. To the later case belongs one fascinating mutation for developmental neuroscientists: muscular dysgenesis (mdg) (GLUECKSOHN-WAELSCH, 1963). Muscular dysgenesis is characterized by death at birth, due to a total lack of muscle contractile activity and a delay (or arrest) in foetal differentiation (PAÏ, 1965*a, b*; BANKER, 1977). Mdg embryos also show early nerve defects, with profuse collateral and ultraterminal sprouting (RIEGER and PINÇON-RAYMOND, 1980).

One of the main characteristic of the mdg embryo occurs to be a pronounced arthrogryptic feature (often associated with cleft palate). Such a genetic model for the study of the consequences of early impairment of muscle activity *in utero*, has prompted us to set suitable neurobiological techniques for the study of mouse neonate nerve and muscle. These methods and the general neurobiological strategy is well adapted to study SAP neonate calves.

This preliminary report presents two abnormal, pathological features of SAP muscle and nerve. The first one is related to a molecular aspect of nerve-muscle interaction: one of the molecular forms of acetylcholinesterase (I6S) becomes highly concentrated in end-plate rich regions of mouse or rat muscles, during embryonic and especially post-natal development (HALL, 1973; VIGNY *et al.* 1976; KOENIG and RIEGER, 1980; RIEGER *et al.* 1980*b*), most probably under nerve influence. This is not realized in SAP calf muscles, where there is, apparently, no such focalization. The second one is an abnormal cytological aspect of the motor innervation: the motor innervation clearly presents frequent preterminal axonal branching and ultraterminal sprouting from nerve terminals.

Material and methods

We have studied two SAP calves and one control animal. The SAP calves were transported alive from the region of Nevers, to our laboratory still alive (immediately after birth). The normal control animal was obtained after caesarian of a sacrificed brucellic cow 2-3 weeks before giving birth and the calf immediately brought on ice to our laboratory. The SAP animals were sacrificed by bleeding, after Ketalar general anesthesia and dissected for anatomical observation. Sternomastoid, biceps and diaphragm were dissected out and immediately processed further for analysis of the molecular forms of acetylcholinesterase or metallic impregnation of the motor innervation.

— Analysis of the molecular forms of acetylcholinesterase (AChE) in end-plate rich and end-plate free regions: we performed, on the fresh muscles, a cytochemical reaction following a modified Koelle method at pH 7, to visually identify and dissect out end-plate rich or free regions of the muscles to be studied

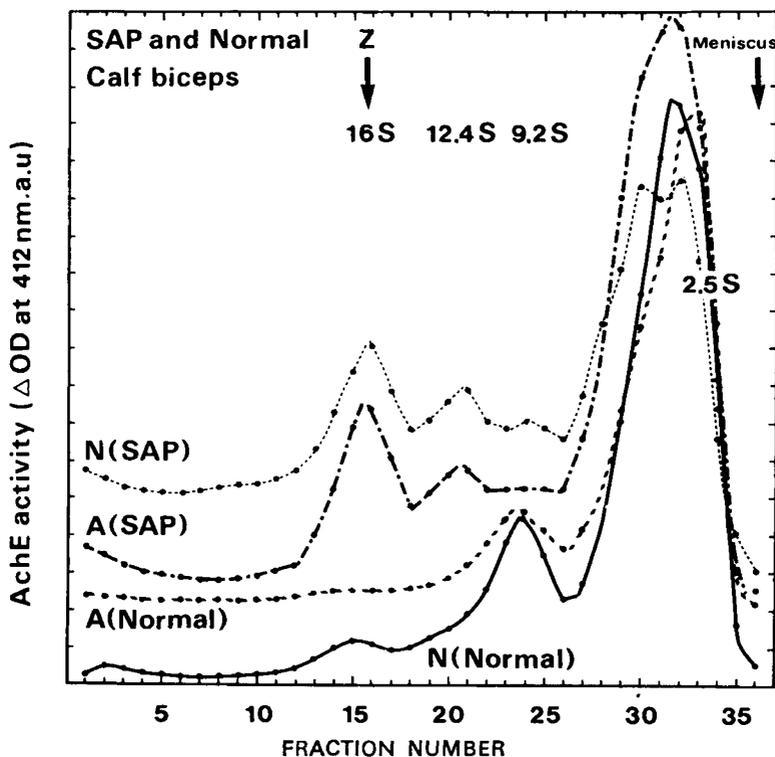


FIG. 1. — La forme 16S de l'Acétylcholinestérase est présente à la fois dans les zones neurales et aneurale du biceps du veau SAP.

La solubilisation, l'homogénéisation et l'ultracentrifugation des formes moléculaires de l'acétylcholinestérase (EC 3.1.1.7) ont été effectuées après la visualisation cytochimique de l'acétylcholinestérase totale accumulée aux jonctions neuromusculaires par une modification de la méthode de Koelle, à pH7 (KOENIG and RIEGER, 1980) et microdissection de tissus de zones neurale (N) et aneurale (A). Les activités AChE peuvent être directement comparées dans les 4 profils de sédimentation, la réaction enzymatique s'étant déroulée dans des conditions identiques.

Remarquons que le biceps contrôle (normal) a été prélevé chez un veau, juste avant la naissance, après césarienne d'une vache atteinte de brucellose. Des résultats analogues ont été trouvés dans d'autres muscles (diaphragme en particulier).

16S AChE is present in both neural (N) and aneural (A) regions of SAP biceps muscle.

Solubilization, homogenization and subsequent centrifugation of muscle acetylcholinesterase (EC 3.1.1.7) have been performed as described in "Material and Methods" after staining of end-plate accumulated AChE by a modification of the method of Koelle, at pH7. Control and SAP muscles have been dissected out from end-plate rich (Neural: N) and end-plate free (Aneural: A) regions. AChE activities are comparable in the 4 sedimentation profiles (identical incubation times). We should note that the control muscle is not, strictly speaking, the real, necessary control, because it has been dissected from a calf just before birth from a brucellic mother, for evident economical reasons. We found comparable results in control and SAP diaphragms.

(KOENIG and RIEGER, 1980). The dissected tissues (after washing of the reaction mixture in a Krebs-Ringer solution) were homogenized (in a 1 to 10 weight to volume proportion) solution containing 1 M NaCl, 0.001 M EGTA, 1 p. 100 triton X 100 and 0.01 M Tris pH 7.2 (standard medium) or in a standard medium with an antiprotease cocktail (0.25 mg bacitracin, 0.1 mM benzethonium chloride, 0.2 mg/ml benzamidine, 0.02 mg/ml pepstatin). No difference was found between these two media, suggesting no particular proteolytic instability of the homogenate. AChE activity was measured by the method of ELLMAN *et al.* (1961). Continuous sucrose gradient centrifugation was performed by loading 75 μ l aliquots of the supernatants of low speed centrifugation (SORVALL RC2; 20 000 g for 15 mn) on top of preformed 12 ml 5. 20 p. 100 sucrose gradients, made with the standard medium and runned in a SW 4r BECKMAN rotor, in a L8 BECKMAN ultracentrifuge for 15 hours at 4 °C and 38 000 rpm. 35 to 40 fractions were collected, assayed for AChE activity and sedimentation coefficients were estimated by comparison with alcohol dehydrogenase (ADH: 4.8 S₂₀, S_{20,w}) and β -galactosidase (Z : 16 S₂₀, S_{20,w}).

— *Neurofibrillary staining*: We used a modification of the original method of BIELCHOWSKY-GROS: after fixation of the muscles in a mixture of Arseniate anhydride at saturation (1 vol.) 95^o, Ethanol (1 vol.) and 20 p. 100 neutral formalin (1 vol.) for 1 hour, the tissues were kept 48 hours in 20 p. 100 neutral formalin and washed for 30 min. in bidistilled water. 50 μ m frozen sections were made stained in 20 p. 100 silver nitrate during 10 to 60 min. After a rapid wash in 20 p. 100 neutral formalin, a brown staining is obtained by plunging the sections into ammoniacal silver nitrate, immediately followed by immersion in 20 p. 100 pure ammonia for 30 min. After several washes in bidistilled water. the sections are mounted in aqueous glycerol.

A modification of the method of BARKER and IP (1963) was sometimes used to specifically stain the neurofibrillary network in teased preparations.

Results

Solubilization of acetylcholinesterase in the presence of highthionic strength buffer (1 M NaCl) and detergent (1 p. 100 Triton \times 100) is effective, Yielding 90-95 p. 100 of the total tissue acetylcholinesterase. There is no loss of activity after incubation of the tissues in the KOELLE and FRIEDENWALD histochemical

FIG. 2. — *Anomalies de la plaque motrice et branchements axonaux préterminaux chez le veau SAP.*

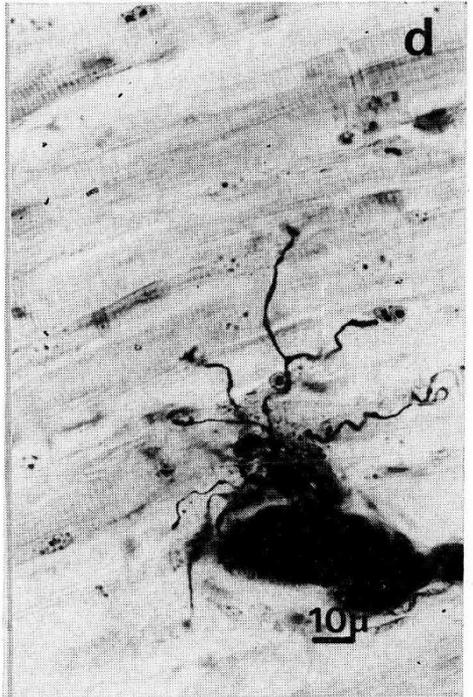
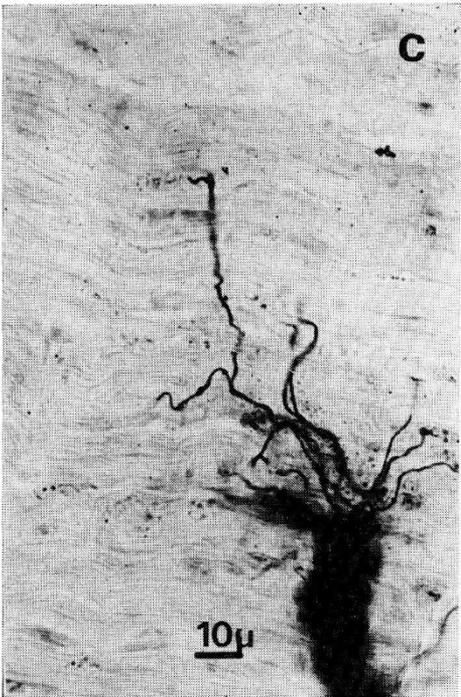
ab Des gonflements terminaux sont très fréquemment observés au niveau des branches terminales des terminaisons nerveuses chez le veau SAP. La microphotographie représente une place motrice assez typique, provenant de tissus prélevés du sternomastoïdien et traités par la méthode de BIELCHOWSKY-GROS.

b, c, d: Divers et fréquents branchements axonaux préterminaux (encadré en *b*: un faisceau d'axones, comprenant le branchement *b*, apparaissant à faible grossissement).

Abnormalities of the motor end-plate and axonal preterminal branching in SAP.

a: Swelling of many motor end-plates (with fine distinct, dissociating neurofibrils) are found in SAP muscles. The micrograph is a typical swollen aspect of a SAP motor end-plate, from sternomastoid muscle. Note the hypolemmal nuclei (BIELCHOWSKY-GROS method).

b, c, d: Frequent terminal branching with innervation of different myofibers (insert of *b*: low magnification of the bunch of axons to which belongs the branching axon).



mixture for 5 to 15 min. (end-plates are made visible after only a few minutes, as white spots under binocular observation) and, as already evidenced for mouse or rat tissues, the sedimentation profiles remain unchanged after incubation, washing and subsequent homogenization and centrifugation compared to untreated (unrevealed fresh) tissues.

Figure 1 shows that in biceps (and we found same results for diaphragm) control muscle does not possess any 16S AChE contribution to total activity in end-plate free regions, when end-plate rich regions contains 2 to 5 p. 100 of 16S AChE. This shows that focalization and concentration of 16S AChE is already realized in end-plate regions in calf muscle at birth. This is not at all the case for any of the SAP muscles analyzed. 16S AChE can be found (in biceps as well as in diaphragm) in both regions, approximatively in equivalent proportion and its level is proportionnaly higher in SAP muscle than in control muscle.

BIELCHOWSKY silver nitrate impregnation technique allow to visualize for optic microscopy the pattern of motor innervation of the muscles. Figure 2 shows two abnormal aspects of the terminal arborization: very frequent swellings with small excrescences of the SAP motor end-plate and especially abnormally frequent preterminal branching of the axons.

Figure 3 gives some evidence of some ultra-terminal sprouting, from small sprouts slightly extending the motor end-plate region to long sprouts running along the length of the muscle fiber. These aspects have been confirmed by using the technique of BARKER and IP (which is more specific for neurofibrillary structures and stain less other structures like myelin or nuclei).

Discussion

The origin, or primary cause, of the SAP mutation is unknown and is expected to be extremely difficult to establish. Only a multidisciplinary approach has a chance to find the first expression of the mutation at its early, probable, time of appearance. The anatomical aberrations (lack of a whole muscle), suggest abnormalities of somites differentiation and migration. It will be certainly difficult to find at so early times of embryogenesis the defective element (precursor of muscle or neuronal cells) which has to be identified and isolated in order to study the cellular or molecular expression of the SAP gene deficiency.

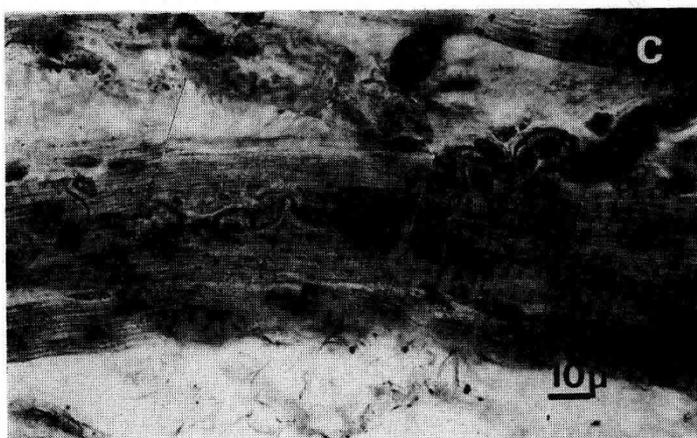
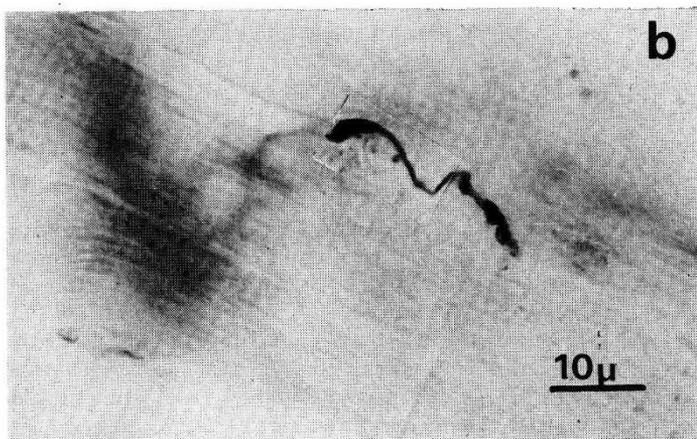
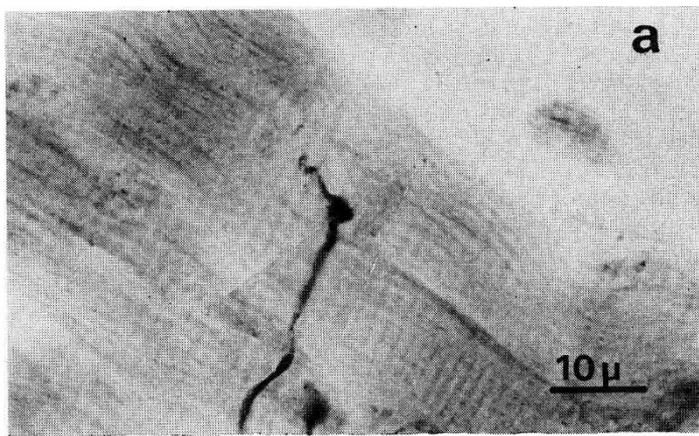
FIG. 3. — *Bourgeoisement ultraterminal dans les terminaisons nerveuses SAP.*
Les fibres nerveuses donnent des bourgeons de différents aspects:

- a: fines neurofibrilles,
 - b: une plaque motrice très allongée,
 - c: long bourgeoisement ultraterminal.
- a, b, c: sont représentés au même grossissement que dans la figure 2a.

Ultraterminal sprouting in SAP Muscle.

- Nerve fibers are giving ultraterminal sprouts of different aspects :
- a: small outgrowth,
 - b: elongated elaborated motor end-plate,
 - c: long ultraterminal sprouting, with a very thin secondary branch.
- a, b, c: same magnification than in figure 2a.

The aim of our present study was to obtain preliminary neurobiological data on possible abnormal nerve-muscle interaction, by studying the SAP calves neuromuscular system, at birth. Preliminary anatomical and histological observa-



tions on muscle do not show any evident fiber type modification, atrophy or delay in their cyto-enzymologic differentiation. However a differentiation process, which is believed to be under neural control the concentration and focalization of 16S AChE seems to be ineffective in SAP calf muscle. The real meaning of this observation is difficult to assess, because the mechanisms underlying the accumulation of AChE in end-plate regions, though under intensive studies, are not yet understood (RIEGER *et al.*, 1980a; KOENIG and RIEGER, 1980; RIEGER *et al.*, 1980a). But a comparison with the results obtained with *mdg* mice could shed some light on certain aspects of SAP mutation. In *mdg* mouse muscle the 16S AChE is not present. This is because of the total lack of muscle activity (which is permissive for the biosynthesis of 16S (RIEGER *et al.* 1980a). In SAP calf muscles 16S AChE is present, even in higher amounts than in control muscle. This suggests that muscle activity in itself is not involved in the pathologic process. The non focal accumulation of this 16S means, on the other hand, that some kind of nerve-muscle uncoupling is involved in the disease as expressed in the neonates.

Such an abnormality in the focal accumulation of 16S AChE may have a potential value for the recognition of SAP gene heterozygote carriers: a partial expression of this abnormal feature could be searched for, as in $+/mdg$ heterozygote adult mice, which show appreciable differences in AChE forms relative content in skeletal muscles.

This nerve-muscle abnormal interaction receives a cytological illustration from the observation of the terminal arborization of the motor innervation. We have observed systematic aspects of ultraterminal sprouting from abnormally looking end-plates and, often, preterminal branching of axons which very rarely occurs in normal cattle (SWATLAND, 1973). Increased terminal branching is usually interpreted either 1° as a change in the normal ratio of motor neurons to myofibers, often accompanied by denervation-reinnervation (after a loss of motor neurons or myofiber hyperplasia) or 2° as suggestive of multiple innervation of the myofibers. The first possibility is usually accompanied by histochemical fiber type grouping, which has not been observed in preliminary experiments. The second possible explanation can only be evidenced by single fiber isolation techniques, to obtain evidence of several foci of AChE or ACh Receptors accumulations at nerve-muscle contacts. We found, in whole SAP diaphragm muscles, several AChE stained, end-plates like rich bands per muscle fasciculus, observation may be related to the occurrence of multiple innervation. In brief, the SAP mutation is mainly characterized by spectacular muscle impairment affecting muscles and joints; the neurobiological correlates of this arthrogryptic condition are suggestive of defects of the nerve-muscle interactions (with the lack of effective focal accumulation of 16S AChE in the region of the neuromuscular junctions) and primary and/or secondary nerve changes and modifications, with possible multiple innervation of myofibers.

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Résumé

Le syndrome d'Arthrogrypose et de Palatoschisis (SAP) du bétail Charolais innervation motrice anormale et défaut dans la focalisation de l'acétylcholinestérase 16S dans les régions riches en terminaisons nerveuses

Le syndrome d'Arthrogrypose et de Palatoschisis (SAP) réalise une atteinte neuromusculaire héréditaire congénitale de la race bovine *Charolaise*. Une étude neurobiologique et neuropathologique préliminaire de veaux SAP nouveau-nés a montré que l'interaction nerf-muscle est établie de façon anormale; il n'y a pas d'accumulation préférentielle de la forme 16S de l'acétylcholinestérase dans les zones riches en terminaisons nerveuses. L'innervation motrice, après imprégnation métallique, présente des caractéristiques anormales : branchements préterminaux axonaux fréquents et bourgeonnements nerveux ultraterminaux.

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