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Immunohistochemical Study of the Ontogenetic Development of Smooth Muscle in the Mouse Embryo

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Summary. This work, performed by immunofluorescence on the mouse embryo, makes use of the remarkable specificity of human pathological anti-striated and anti-smooth muscle auto-antibodies. It makes it possible, on the one hand, to date the appearance of smooth muscle Actomyosin during embryogenesis, and, on the other hand, to witness the appearance of immunological distinctions between contractile proteins of smooth and striated muscle.

Most immunohistological studies of the embryogenesis of muscle and contractile protein have been performed on striated muscle (Romanovsky, 1964a, 1964b; Piantelli, 1964; Ogawa, 1962; Bergman, 1962; Biro *et al.*, 1963). Usually, these immunohistological studies were carried out by means of heteroantibodies reacting with various muscle components. The matter of smooth muscle embryogenesis has rarely been approached.

This work, utilizing immunofluorescence in the mouse embryo, takes advantage of the remarkable specificity of human pathological anti-striated and anti-smooth muscle auto-antibodies. Its purpose is twofold:

Establishing the developmental stage at which the smooth muscle actomyosin appears.

Observing the appearance of immunological distinctions existing between contractile proteins of smooth and striated muscle.

Material and Methods

Human Pathological Anti-Smooth Muscle Auto-Antibodies. Anti-smooth muscle antibodies (asmAb) have been mainly reported in chronic active hepatitis (Johnson *et al.*, 1965; Ironside *et al.*, 1966; Whittingham *et al.*, 1966a, 1966b; Doniach, 1966). There are at least two types of these asmAb: Type A (asmAb A) and type B (asmAb B) (Herbeuval *et al.*, 1972); they are not species-specific and react only with smooth muscle (Ironside *et al.*, 1966). The asmAb A reacts with smooth muscle actomyosin (SMA), a myosin and actin complex, at moderate or high ionic strength, but reacts neither with myosin nor with globular-actin used separately (Montagne *et al.*, 1972). Here, we used only the asmAb A.

Hyperimmune Rabbit Anti-smooth Muscle Actomyosin Serum. SMA was extracted from bovine intestinal smooth muscle at high ionic strength (Laszt *et al.*, 1961) and purified by precipitation at low ionic strength and ultracentrifugation (Sparrow *et al.*, 1970).

SMA purified solution was kept at $-25°C$ with an equal volume of glycerol (Archer *et al.*, 1971) and has been used to obtain a hyperimmune rabbit serum (Montagne *et al.*, 1972).

Abbreviations. asm Ab (Anti-smooth muscle antibodies); asm Ab A (Anti-smooth muscle Type A antibodies); asm Ab B (Anti-smooth muscle Type B antibodies); ASM Ab (Anti-striated muscle antibodies); MG (Myasthenia gravis); NHS (Normal human serum); SMA (Smooth muscle actomyosin).

Human Pathological Anti-Striated Muscle Auto-Antibodies. Some individuals with myasthenia gravis (MG) have a serum globulin factor that reacts with alternate striations of skeletal muscle (Strauss *et al.*, 1960). This anti-striated muscle antibody (ASMAb) is not species-spécific and reacts not only with skeletal muscle but also with cardiac muscle (Beutner *et al.*, 1962) and some thymic myoid cells (van der Geld *et al.*, 1964; Strauss *et al.*, 1966). The component of striated muscle reacting with ASMAb is still uncertain. The A (Myosin or possibly actomyosin (Strauss *et al.*, 1961; Hale *et al.*, 1965; Nastuk *et al.*, 1966); I (Troponin or Tropomyosin) (Vetters, 1965; Strauss, 1967) and Z discs (Nastuk *et al.*, 1966) of the sarcomere have all been thought of as possible sites of antibody binding.

Mouse Embryos. In order to obtain a series of mouse embryos of various known ages, we used a group of female mice (*Mus musculus*, albinos, Swiss) whose estrous cycles were studied by vaginal smears.

Vaginal smears were performed according to the techniques of Papanicolaou (1948) for taking and fixation of vaginal secretions, Papamiltiades (1953) for nuclear staining with $ZnSO_4$-hematoxylin, Pundel (1966) for cytoplasmic staining with Shorr's3 stain and Isaac and Wurch (1951, 1952) for smear setting. A female mouse was placed with the male when the smear was characteristic of estrus or heat. The first day of gestation was fixed as the day following estrus. Embryos 5 to 20 days old were thus obtained. The error in age determination did not exceed a few hours. Generally, embryos were taken in an intact uterus and immediately deep-frozen in liquid nitrogen at $-196°C$; then, the embryos were kept at $-70°C$ until used as immunofluorescence substrates.

Immunofluorescence Staining. Fluorescent staining was carried out according to Coon's indirect method (Coons *et al.*, 1950). Fluorescein-labelled anti-human globulin reagent was obtained from the Institut Pasteur. 4 μ-cryostat sections were examined under a Leitz fluorescence microscope. Results were labelled $++++$ to—according to the intensity of the observed fluorescence. Negative tests (normal human serum (NHS) with smooth, cardiac and skeletal embryonic muscles as substrates) were performed for each stage of embryogenesis. ASMAb and asmAb A were used with adult mouse muscles as substrates for positive tests. All sera were tested at 1/8 dilution in pH 7.2 buffer containing 0.85% sodium chloride.

Results and Discussion

Negative Tests. No fluorescent staining was observed when NHS was reacted with adult mouse muscles. The same negative results were obtained with smooth cardiac and skeletal embryonic muscles, whatever the age of the embryo. In the youngest embryos (7, 6 and 5 days old), it was not always possible to identify all primitive muscles, but, in these stages, no structure reacted positively.

Positive Tests. ASMAb of MG serum reacted with alternate striations of adult mouse cardiac and skeletal muscles, but did not react with smooth muscle (Table 1). On the contrary, asmAb A reacted with smooth muscle, of course, but also, although less strongly, with alternate striations of adult mouse cardiac and skeletal muscles.

So, Ironside's organ specificity (Ironside, 1966), true for man and many animals (Herbeuval *et al.*, 1972) is not so for mice; there is a component of adult mouse cardiac and skeletal muscles, which is distributed in alternate striations and possesses a cross-antigenicity with the SMA.

Embryonic Smooth Muscle (Table 2). Intestinal smooth muscles in embryos at least 14 days old, reacted with asmAb A and the observed staining was the same as with adult mouse intestinal smooth muscle. The smooth muscles of an embryo 11 days old reacted also, but faintly. In younger embryos, no fluorescence was observed at all, although it was possible to identify the primitive gut as early as the 9th day.

Table 1. Observed fluorescent staining with positive tests

Substrates	ASMAb	asmAb A	
	13381	9637	15507
Adult mouse			
Smooth muscle	–	+++	+++
Striated muscle	+++	++	++
Cardiac muscle	+++	++	++

Table 2. Auto-antigenicity of mouse embryonic smooth muscle

Substrates	ASMAb	asmAb A	
	13381	9637	15507
Adult mouse	–	+++	+++
Embryos			
19 days	–	+++	+++
14 days	–	+++	+++
11 days	–	+f.	+f.
10 days	–	–	–
9 days	–	–	–
8 days	?	?	?
7 days	?	?	?
6 days	?	?	?
5 days	?	?	?

A comparable study, performed with a hyperimmune rabbit serum anti-SMA, showed that it was not only the appearance of the auto-antigenicity A that could be dated but truly the appearance of the SMA.

Therefore the SMA or an embryonic actomyosin, immunologically identical to adult actomyosin, appears in the primitive gut of the mouse embryo "in utero" between the 10th and the 11th day of its growth. This appearance coincides with the formation of the primary intestinal loop (Snell *et al.*, 1968). The mouse embryo's gut is, at that time, in the same developmental stage as a 29 day old human embryo (Otis *et al.*, 1954).

The embryonic smooth muscles never reacted with ASMAb. There is therefore no immunological affinity between the smooth muscle proteins and the striated muscle auto-antigen that reacts with ASMAb of a MG serum, and this is true for all developmental stages.

Embryonic Skeletal Striated Muscle (Table 3). The striated muscles of limb rudiments reacted with ASMAb, like those of the adult, as early as the 11th day of growth. In the embryo 10 days old, immunofluorescent staining was still only faintly positive. There was no reaction before the 10th day. The asmAb A reacted with embryonic skeletal muscles, as with embryonic smooth muscle, from the 11th day onward. There was no fixation before the 11th day.

Table 3. Auto-antigenicity of mouse embryonic skeletal striated muscle

Substrates	ASMAb	asmAb A	
	13381	9637	15507
Adult mouse	+++	++	++
Embryos			
19 days	+++	++	++
14 days	+++	++	++
11 days	+++	+f.	+f.
10 days	++	−	−
9 days	−	−	−
8 days	?	?	?
7 days	?	?	?
6 days	?	?	?
5 days	?	?	?

Table 4. Auto-antigenicity of mouse embryonic cardiac muscle

Substrates	ASMAb	asmAb A	
	13381	9637	15507
Adult mouse	+++	++	++
Embryos			
19 days	+++	++	++
14 days	+++	++	++
11 days	+++	++	++
10 days	+++	++	++
9 days	++	−	−
8 days	−	−	−
7 days	?	?	?
6 days	?	?	?
5 days	?	?	?

Thus the antigen of mouse skeletal striated muscle, immunologically related to SMA, appears at the same time as SMA but later than the antigen of skeletal striated muscle reacting with ASMAb.

Embryonic Cardiac Muscle (Table 4). It was possible to observe in cardiac muscle, an identical delay between the appearance of antigen reacting with ASMAb and the later appearance of antigen related to SMA. But these two components appear earlier in cardiac muscle than in skeletal striated muscles, since they can be detected by immunofluorescence, respectively as early as the 9th and 10th days, instead of the 10th and 11th days.

The acquisition of adult striated muscle antigenicity by cardiac muscle corresponds with the differentiation of epimyocardium into myocardium and epicardium (Snell *et al.*, 1968). The heart of the mouse embryo is then at the same developmental stage as the 23-day human embryo (Otis *et al.*, 1954).

Conclusions

The smooth muscle actomyosin or an embryonic actomyosin appears in the primitive gut, between the 10th and the 11th day of mouse growth "in utero".

In the mouse, the skeletal and cardiac striated muscles of adult and embryo possess an antigen immunologically related to the smooth muscle actomyosin. This component is distributed in alternate striations and always appears later than the antigen with which anti-striated muscle auto-antibodies react.

The myocardium gains the antigenicity of adult striated muscle between the 8th and the 9th day, *i.e.* earlier than skeletal muscles.

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