Histochemical differentiation of the basement membrane of the mouse seminiferous tubule

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With 2 plates (figs 1 and 2)

Summary

The ground substance of the testis of the albino mouse is PAS-positive but not metachromatic, and probably highly aggregated. The basement of the seminiferous tubules is intensely PAS-positive, metachromatic, and possibly not so highly aggregated.

The reactivity of the ground substance to the PAS reaction and toluidine blue is tentatively ascribed to the presence of chondroitin sulphate C: this compound, previously known to contain N-acetyl-galactosamine, glucuronic acid, tyrosine and tryptophane, is associated with arginine.

The genesis of the basement membrane of the seminiferous tubule is shown to include the formation of a sheath of atypical elongated fibroblasts, the secretion of a PAS positive, metachromatic substance associated with arginine between this sheath and the seminiferous tubule, the appearance of mitochondria in the cells of the sheath, and lastly, the acquisition of alkaline phosphatase by these fibroblasts and its spread to the intervening ground substance. These changes are thought to be related to the structural and nutritional requirements of the seminiferous tubules.

In its intense positive reaction to PAS and in its metachromasy, the basement membrane of the seminiferous tubule agrees with the ground substance adjacent to sites of active protein metabolism, such as growing tumours, embryonic organs, hair follicles, and skin.

Introduction

INTERCELLULAR substances in the intertubular spaces of the testis, as in other connective tissues, fall into two principal groups, namely fibrillar structures and ground substance. The former comprise collagen, elastic, and reticular fibrils, which are thought to fulfil a largely mechanical function and whose distribution is well known. The ground substance consists of various colloids, crystalloids, gases, and water and is optically homogeneous when viewed with the light microscope. At a submicroscopic level it shows structural organization into a two-phase system (Gersh and Catchpole, 1960) and the terms 'colloid-rich, water-poor phase' and 'colloid-poor, water-rich phase' have been coined by these workers in an attempt to describe it as a physico-chemical system. The colloids are known to include hyaluronic acid, chondroitin sulphates A, B, and C, keratosulphate, and heparitin sulphate (Meyer, 1950): the water acts as a vehicle for ions, enzymes, hormones, vitamins, amino-acids, immune bodies, and proteins originating from the plasma. The phases of this heterogeneous colloidal system are in electrical and chemical equilibrium with one another. It is thought that the sol-gel consistency of the ground substance depends on the relative amounts of colloid and water present.

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and this in turn is known to be influenced by fibroblastic activity, depolymerizing enzymes, hormones, growth, ageing, and other physiological and pathological processes (Gersh and Catchpole, 1949).

The basement membrane may be defined as the region of specialized ground substance which intervenes between an epithelial structure and the ordinary ground substance. The present work is an account of the development of the basement membrane of the seminiferous tubule in the mouse testis between birth and puberty as revealed by various histochemical methods. From a functional point of view, any such description must include a consideration of the sheath of attenuated fibroblasts that surrounds the seminiferous tubule.

Material and methods

Thirty-six male Swiss White mice were used in preparation of the age series. The animals were killed in groups of 4 at weekly intervals between birth and the end of the eighth week of extra-uterine life. Two testes from each age group were fixed in a mixture of 90 ml water, 10 ml formalin, and 5 g mercuric chloride; in formaldehyde-calcium solution (90 ml water, 10 ml formalin, 1 g anhydrous calcium chloride); in Helly’s fluid; and in cold 70% ethanol.

The testes fixed in the first-named solution were dehydrated in cellosolve, embedded in estax (Watford Chemical Co.), and sectioned at 5 μ, a method found to preserve testicular morphology well (Baillie, 1960a). Sections were stained with haematoxylin and eosin, the McManus (1956) periodic acid / Schiff (PAS) technique (with methanol/chloroform and diastase controls), toluidine blue, methyl green, and Pyronin B for DNA and RNA, and also by a modified Sakaguchi reaction (Thomas, 1950) to show arginine. In addition, these stains were also controlled by digestion for 24 h with hyaluronidase (B.D.H.), buffered at pH 6.

Testes fixed briefly in formaldehyde-calcium solution were embedded in gelatin and sectioned at 10 μ on the freezing microtome. Some sections were coloured with Sudan black to demonstrate the total lipids present; others were subjected to Hayes’s (1949) modification of Feulgen and Voit’s true plasmal reaction to show acetal phosphatides and possibly atypical α-ketols (Boscott and others, 1948). Bennett’s (1940) reaction was also employed on frozen sectioned material. 2,4-dinitrophenyl hydrazine was used, notwithstanding the author’s experience of this reagent (Baillie, 1959).

Specimens fixed in Helly’s fluid were sectioned at 4 μ and stained with toluidine blue and acid fuchsin to demonstrate mitochondria. Material fixed in 70% ethanol was sectioned at 5 μ in wax: alkaline phosphatase was demonstrated by a modified Gomori technique (Lillie, 1954).

Results

Haematoxylin and eosin. At birth the seminiferous tubules are surrounded by an incomplete sheath of spindle-shaped mesenchymal cells, which have large, oval nuclei: the limits of the cytoplasm are clearly defined. Round some
tubules the sheath of mesenchymal cells is complete; its nuclei are becoming elongated and stain more densely, and the cytoplasm is becoming attenuated and closely applied to the wall of the seminiferous tubule (fig. 1, A). Eosinophil intercellular material is not present at this age. At the end of the first week of life the sheath-cells resemble atypical fibroblasts having greatly attenuated cytoplasm. Their nuclei, when seen in profile, appear as densely staining rods. Eosinophil material has appeared in the intertubular extracellular spaces and also between the sheath cells and the seminiferous tubules. For this reason cell boundaries are indistinct. This staining method does not reveal any further changes in the connective tissue of more mature testes. Hyaluronidase has no effect on this picture.

The PAS reaction. At birth the intertubular extracellular spaces contain no PAS-reactive material. While the majority of seminiferous tubules have no PAS-positive basement membrane, there is a fine PAS-positive membrane beneath the mesenchymal sheath of the tubules, surrounded by a complete layer of fibroblasts (fig. 1, B). At the end of the first week the PAS-positive membrane, though exceedingly thin, is constantly present in the form of a red, refractile line surrounding all the seminiferous tubules. The extracellular spaces at this stage contain traces of PAS-positive material. With increasing age the PAS-positive basement membrane becomes slightly broader, and abundant PAS-positive ground substance becomes visible: the general ground substance does not stain so intensely with PAS as does the basement membrane of the tubules. Large staining defects occur at places in the ground substance (fig. 1, C); these resemble cartilage lacunae in shape and size but do not contain interstitial cells. Extraction with chloroform-methanol and diastase completely abolishes the PAS reactivity of the basement of the seminiferous tubules and also the reactivity of the general ground substance (fig. 2, A).

Toluidine blue. In the neonatal testis, beneath the mesenchymal sheath of the seminiferous tubules and surrounded by a complete layer of cells there is a very fine membrane which stains metachromatically with toluidine blue. This membrane is present in all older testes. The general ground substance of the neonatal testis does not stain at all with toluidine blue, while the ground substance of older testes does stain, but not metachromatically. Hyaluronidase extraction removes the metachromatic properties of the basement membrane and diminishes the affinity of the general ground substance for toluidine blue.

Methyl green and pyronin B. The PAS-positive basement membrane and ground substance, whose distribution is described above, show a general weak affinity for these stains, giving a pale greenish-pink result. This affinity is reduced by hyaluronidase digestion but not by extraction with perchloric acid. This staining method reveals no other features of note.

α-naphthol. The distribution of material demonstrated by this reagent closely parallels the reactivity of the ground substance and basement membrane to PAS. Thus at birth a few seminiferous tubules have a basement membrane
containing arginine lying beneath a layer of fibroblasts. The seminiferous
tubules of all older testes possess some arginine in their basement membranes
(fig. 2, b). The extracellular spaces in the neonatal testicular interstitium are
devoid of material stainable with alkaline β-naphthol. Later testes have extra-
cellular material which stains faintly with alkaline β-naphthol; it is distrib-
uted in the same manner as the PAS-positive material. Hyaluronidase
extraction removes those components of the basement membrane and ground
substance that stain with alkaline β-naphthol, but has no effect on the affinity
of spermatozoa and other cells for this reagent.

**Sudan black.** At no time between birth and puberty does the basement
membrane of the seminiferous tubules or the general ground substance colour
with Sudan black. The fibroblasts which ensheathe the tubules contain a few
sudanophil droplets.

**The plasmal reaction.** The neonatal testis is devoid of intercellular
materials stainable with this reaction. The ground substance and basement
membranes of the tubules in later testes stain pale pink with Schiff's reagent
after oxidation with mercuric chloride. This coloration is not removed by
acetone.

**2,4-dinitrophenyl hydrazine.** This reagent and the plasmal reaction appear
to be staining the same structures between the tubules.

**Mitochondria.** At birth the mesenchymal cells surrounding the semi-
ferous tubules possess few mitochondria. Mitochondria are plentiful in these
fibroblasts at the end of the first week and in all subsequent testes; they take
the form of minute granules which are located in small groups in the cyto-
plasm at each end of the nucleus.

**Alkaline phosphatase.** There is no alkaline phosphatase demonstrable by
the Gomori method in the basement membrane of the seminiferous tubules
during the first 14 days of extra-uterine life. Twenty-one days after birth the
rod-shaped nuclei of the fibroblast sheath possess demonstrable alkaline
phosphatase. All older testes are characterized by a black line, indicative of
enzymatic activity, bounding the seminiferous tubules (fig. 2, c). Closer
inspection suggests that much of this enzymatic activity is located in the
PAS-positive basement membrane which intervenes between the fibro-
blasts and the seminiferous tubules, although some occurs in the nuclei and
cytoplasm of the fibrocytes. In interpreting these findings it must be remem-
bered that negative results with the Gomori phosphatase / cobalt sulphide
method after alcoholic fixation and paraffin embedding are of doubtful sig-
ificance, and that alkaline phosphatase in nuclei is widely regarded as an
artifact.

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**Fig. 1 (plate).** A, neonatal testis; H and E. Mesenchymal cells (mes) are condensing to form
a sheath for the seminiferous tubule.

B, neonatal testis; PAS and haematoxylin. A fine membrane (m), reactive to PAS, is just
visible in places round one seminiferous tubule.

C, testis, 4 weeks old; PAS and haematoxylin. The basement membrane of the tubule is
prominent: staining defects (sd) are visible in the intertubular ground substance at one point.
Discussion and conclusions

From the foregoing description it is apparent that the intercellular material of the testicular connective tissue conforms to Gersh's (1951) definition of ground substance in that it is optically homogeneous when viewed with the light microscope, gives a positive PAS-reaction, and (in places) stains metachromatically with toluidine blue. For a time it was widely believed that the ground substance matrix was a colloidal carbohydrate/protein complex which was aggregated to a variable extent and it was held that the amount of metachromasia and the intensity of the PAS-reaction exhibited were in inverse proportion to the degree of aggregation of the colloid. Recent studies (Gersh and Catchpole, 1960) indicate, however, that the staining propensities of basement membranes are due to a preponderance of the 'colloid-rich, water-poor phase' in a very finely corrugated layer of ground substance.

The central difficulty in assessing the present findings lies in the conflict of opinion regarding the interpretation to be placed on the results of the staining methods used. Meyer (1950) claimed that metachromatic, PAS-positive substances fall into five categories, namely, hyaluronic acid, hyaluronic acid monoester sulphate, and the 3 chondroitin sulphates, A, B, and C; but several recent workers have suggested that the metachromatic and the PAS-positive components of tissues are naturally separate, or can be separated by experimental manipulations. Thus, Einbinder and Schubert (1951) showed that pure chondroitin sulphate, which is a strong chromotrope, reduces periodate only very slowly. Further, Glegg and others (1954), by differential alcoholic precipitation of alkaline tissue-extracts, separated PAS-positive fractions of several tissues from metachromatic fractions. Moreover, Braunstein and Buerger (1959) made a clear separation in vitro of metachromatic material from PAS-staining material in amyloid.

It has been established (Meyer, 1950) that hyaluronidase hydrolyses hyaluronic acid, hyaluronic acid monoester sulphate, and two of the chondroitin sulphates, chondroitin sulphate B being resistant to enzymic extraction. Since hyaluronidase abolishes the PAS-reactivity of the seminiferous tubular basement membrane and intertubular ground substance, it may be concluded that the PAS-positive material is probably an acid mucopolysaccharide and that chondroitin sulphate B is not present in the testis in histochemically demonstrable amounts, since it would have survived hyaluronidase extraction. Chondroitin sulphate A may also be excluded as the basis of the PAS-reactivity of the testicular intertubular ground substance, since this mucopolysaccharide is probably peculiar to hyaline cartilage. Further, it is generally considered

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Fig. 2 (plate). A, testis, 4 weeks old, after hyaluronidase digestion; PAS and haematoxylin. The reactivity of the basement membrane and ground substance to PAS has been abolished by enzymic hydrolysis.

B, testis, 5 weeks old; alkaline α-naphthol. The basement membrane (bm) of the seminiferous tubules contains arginine.

C, testis, 6 weeks old; alkaline phosphatase. The basement membrane (bm) of the tubule contains much alkaline phosphatase.
that aqueous fixation and an aqueous PAS method, such as that employed in the present investigation, do not preserve hyaluronic acid and hyaluronic acid monosulphate (Lillie, 1954). Hotchkiss (1948) used alcoholic solutions and obtained different results. These observations suggest that chondroitin sulphate C is probably the PAS-reactive component of the basement membrane of the mouse seminiferous tubule, but it is difficult to reconcile this with Leblond’s (1957) statement that acid mucopolysaccharides fixed with chromate do not give the PAS reaction under the usual histochemical conditions (that is, with brief periodic acid oxidation). Possibly the formaldehyde / mercuric chloride fixation employed in the present study increases the reactivity of acid mucopolysaccharides to PAS. Alternatively, hyaluronidase may digest Leblond’s (1957) heteropolysaccharides in addition to acid mucopolysaccharides.

The above observations indicate that the genesis of the basement membrane of the seminiferous tubules involves a number of stages which may be arbitrarily distinguished from one another. First, indifferent mesenchymal cells metamorphose into atypical, elongated, ensheathing fibroblasts with attenuated cytoplasm and densely staining, rod-shaped nuclei. Secondly, a PAS-positive substance, associated with arginine and metachromatic material, appears between the cytoplasm of the seminiferous tubule and that of the fibroblast sheath. While this complex is being elaborated mitochondria appear in the cytoplasm of the sheath fibroblasts. Lastly, alkaline phosphatase seems to appear in the nuclei of these cells, and maturation is completed by the spread of this enzyme to the cytoplasm of the sheath fibroblasts and also to the ground substance of the basement membrane.

These changes in the mouse are largely completed by the end of the animal’s fourth week of extra-uterine life, the time at which spermatozoa appear in the tubules. Clearly the structural changes are related to the increased support requirements of the growing tubule, and the mitochondrial changes possibly reflect fibroblastic synthesis of the polysaccharide complex which forms the PAS-positive membrane. The enzymic changes may parallel the increase in the nutritional requirements of the tubule, particularly glucose. While the majority of the seminiferous tubules in the mouse acquire their PAS-positive membranes after birth, the seminiferous tubules of the sheep have well defined PAS-positive basement membranes long before birth (Baillie, 1960b).

At the periphery of invasive tumours and in rapidly growing embryonic organs the ground substance of the related connective tissue becomes intensely metachromatic and PAS-positive during the phase of active growth (Gersch, 1951). A comparable phenomenon has been described in the ground substance of the dermal papilla of an actively growing hair follicle and also in the connective tissues involved in the repair of dermal damage (Montagna, 1956). In each of these various sites, cessation of growth is followed by progressive aggregation of the carbohydrate-protein complexes, with the concomitant loss of metachromasia and PAS reactivity. Similarly, the basement membrane of growing and functioning seminiferous tubules is highly PAS-positive and
metachromatic, and this suggests by analogy with the foregoing that the
degree of aggregation of connective tissue mucopolysaccharides is a generalized
reflection of active protein metabolism.

From the Sudan black, plasmal, and 2-4-dinitrophenyl hydrazine tests it is
clear that free neutral fats, acetal phosphatides, and atypical α-ketols (Boscott
and others, 1948) are not present in the basement membrane of the mouse
seminiferous tubule. The faint coloration of the basement membrane with
the plasmal test and 2-4-dinitrophenyl hydrazine, with resistance to acetone
extraction, may possibly be a weak pseudoplasmal reaction of the type de-
scribed by Cain (1949) and attributable to aldehydes known to be present in
the elastic fibres of rodents (Pearse, 1960).

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References

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