The Uptake of Intravital Dyes by the Testis

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With one plate (fig. 1)

SUMMARY

The vital staining reactions of the normal and ischaemic testis were investigated in 20 rats by the use of varying amounts of trypan blue and Unna's polychrome methylene blue. The experiments showed that it was possible to distinguish both true and reticulo-endothelial interstitial cell types. The implications of this subdivision are discussed and their importance in relation to neoplasms of the Leydig cells stressed. The distribution of mast cells in the testis is described.

INTRODUCTION

BOUFFARD (1906) was the first to describe the histological appearance of the presence of intravital dyes in the testis, but he failed to state whether they were to be found in all the intertubular cells, or merely limited to a particular group of them.

Goldmann (1909) not only gave a description of the cytological characteristics and situation of the macrophage cell in the testis, but was also the first investigator to recognize their widespread distribution in the body. He assigned a trophic role to them, claiming that they passed across the basement membrane of the seminiferous tubules from the intertubular tissue, and, having shed their granules of dye, united with either Sertoli cells or spermatids. These conclusions were in agreement with earlier work carried out by Plato (1896), Lenhossek (1897), Barbeleben (1897), and Friedmann (1898). Most of the later authors, except Mason and Shaver (1952), denied that migration of the macrophages took place under normal conditions; their opinion was based on indirect evidence since they did not observe the movements of these cells.

Research into the staining of intertubular cells by intravital dyes has created confusion as to whether the Leydig cells possess 'phagocytic' properties. Evans and Schulemann (1914), Esaki (1928), and Van Os and Ruyter (1939) believed that the interstitial cells of Leydig were able to 'phagocytose' these dyes during all, or at least certain, phases of their life cycle. Other investigators held the opposite view that the interstitial cells were wholly incapable of this activity (Addison and Thorington, 1917; Bratianu, 1930*a*; Guerriero, 1930; Stein, 1931). Whilst subscribing to the former opinion Takamori (1921) believed that intravital dyes coloured Leydig cells lightly after the administration of large amounts of these substances. This conclusion was criticized by Elek (1924), Natali (1925), Cannon and others (1929), and Cappell (1929) on the grounds that any cell loaded with stored lipoid material would fail to

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be stained at all strongly by intravital dyes; doubt has been cast on this by Schuleman (1917), who demonstrated that macrophages laden with vital dyes were still stainable in the normal manner.

Sheldon (1935) was of the opinion that the interstitial cells were rarely involved in haemochromatosis in man, so supporting the investigations of Polson (1929) in the rabbit. On the other hand, Nissim (1953 *a*, *b*; 1955) using massive doses of either ferric chloride caramelate or an iron-dextran complex ('Imferon', Benger Laboratories) in mice, found that Leydig cells took up these substances.

The divergence of opinion as to whether cells can exhibit an endocrine as well as a 'phagocytic' function is also found in relation to other ductless glands, notably the ovary (Borell, 1919; Cappell, 1929; Bratianu, 1930*b*).

Materials and Methods

Twenty healthy, sexually-mature, albino rats were used in this investigation. Two intravital dyes were employed; trypan blue and Unna's polychrome methylene blue. These were used either singly or in various combinations (table 1).

The medium used was a 0·2% solution in the dye, sterile distilled water being used as the diluting fluid. The standard dose was 1·5 ml of the solution per 50 g of body-weight; this was given as a single intraperitoneal injection.

In two of the experimental animals the amount of solution given was much greater, 30 ml and 150 ml respectively being injected as a single dose in the former, and in 5 equally divided doses on alternate days in the latter case. Autopsy on the rat which had received the larger amount of trypan blue revealed that some 5 ml of dark blue fluid remained in the peritoneal cavity, no evidence of peritonitis being discovered. It was found that if a scanty distribution of trypan blue granules was required, in order to obtain a clear picture of the cellular structure, the best results were obtained by halving the standard dose.

The animals were usually not killed before the 7th day following the last injection of dye, because the granules of dye were found to be much more clearly defined at this than at earlier periods.

It will be seen from table 1 that several additional procedures were also carried out:

(*a*) unilateral interruption of the testicular artery at a point before its anastomosis with the vasal artery.

(*b*) unilateral interruption of the testicular artery, with removal of the contralateral testis, as described by Harrison (1953). This was performed 7 days after the injection of the standard amount of dye.

(*c*) 2·5 mg of testosterone propionate were given by intraperitoneal injection on alternate days for the periods of time indicated.

(*d*) unilateral orchidectomy was carried out, with aseptic precautions, on 2 rats, 7 days after they had been injected with the standard amount of dye.

(*e*) laparotomy was performed on 2 rats and, after the testes had been

delivered into the wound, 0·5 ml of trypan blue solution were injected underneath the tunica albuginea of each testis.

The figures given in the right-hand column of table 1 indicate either the number of days' survival after injection or, when a further procedure has been carried out, the survival period after this.

It was noted that the above-mentioned intravital dyes were non-toxic and did not produce sensitization when given in the amounts and in the dilution indicated in these experiments.

TABLE 1

Number of rats	*Dye used*	*Amount of dye*	*Operation performed or drug used*	*Days elapsing between experimental procedure and killing of animal*
2	Polychrome methylene blue (Unna)	S.D.	..	7
3	Trypan blue	S.D.	..	4, 7, 60
1	Trypan blue	4 ml	..	7
1	Trypan blue	30 ml	..	7
1	Trypan blue	150 ml	..	7
2	Trypan blue	S.D.	Testosterone propionate 2·5 mg on alternate days	20, 35
4	Trypan blue	S.D.	Interruption of testicular artery	1, 5, 10, 15
2	Trypan blue	S.D.	Interruption of testicular artery and unilateral orchidectomy	7, 11
2	Trypan blue	0·5 ml	Injection of dye direct into testis	7
2	Trypan blue	S.D.	Unilateral orchidectomy (left)	7, 35

S.D. = standard dose = 1·5 ml per 50 g of body-weight.

Trypan blue was most frequently employed as this dye was found to give the most satisfactory intravital staining during the preliminary tests.

On removal from the body the testis was placed in Heidenhain's 'Susa' fixative for 1 hour, after which time a segment was removed from the equator of the testis with a sharp razor and replaced in the fixative. Five-micron sections, parallel to the equator of the testis, were lightly counterstained with Heidenhain's iron haematoxylin and eosin (Gurr, 1953). In the case of the testis of the rats subjected to unilateral orchidectomy, serial sectioning (1 in 25) of the whole organ was carried out.

The seminal vesicles with the coagulating glands and a narrow bridge of prostatic tissue were removed at autopsy, and weighed, after having been dried over calcium chloride for 48 h.

Results

Trypan blue stains the following four types of cell:

cells which are morphologically indistinguishable from the interstitial cells of Leydig (fig. 1, G); certain of the fibroblasts, the majority of these being applied to the outer surface of the basement membrane of the seminiferous tubule (fig. 1, C); certain of the perivascular cells (fig. 1, B); small cells with irregular outlines, about 10 to 20 μ in diameter, the structure of which is obscured by the nature of the staining with the intravital dye. In the preceding three types of cell the dye is aggregated into well-defined granules, whereas in this type of cell, the granules are more widely separated and show a lack of definition (fig. 1, A).

Variations in the amount of dye injected do not produce any difference in either the number or type of intertubular cells which are stained by the dye.

The distribution of mast cells in the testis was investigated by the methods described above. They are found to be present within the tunica albuginea (fig. 1, E), immediately underlying this structure (fig. 1, F), and in the connective tissue of the epididymis (fig. 1, D), but are absent from the intertubular tissue.

Under normal conditions cells containing dye are not found within the basement membrane of the seminiferous tubules. After the production of ischaemia of the testis by ligation of the testicular artery, there is a marked passage of these cells from the intertubular tissue into the seminiferous tubules. During the first 48 h after operation some of these cells lie close to the inner surface of the basement membrane of the tubules, whilst others are situated either among the actively dividing spermatogonia, or on the surface of the desquamated mass of tubular cells (fig. 1, G). By the 3rd day they are widely scattered throughout the tubular contents (fig. 1, H), and the intracellular dye, with the exception of that contained within the cells in the subepididymal region of the testis, has lost its granular form, staining both the cytoplasm and nucleus in a diffuse manner.

The injection of trypan blue directly under the tunica albuginea of the testis results in similar histological findings to those described above, except

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Fig. 1 (plate). A, interstitial cell showing lack of definition of intravital dye granules and irregularity of cellular outline. (Trypan blue; counterstained with haematoxylin and eosin.)

B, section of blood-vessel demonstrating presence of dye granules in one perivascular cell. (Same technique as A.)

C, two peritubular fibroblasts, only one of which contains dye. (Same technique as A.)

D, mast cells in connective tissue of the epididymis. (Unna's polychrome methylene blue.)

E, mast cells in tunical albuginea of the testis. (Same technique as D.)

F, mast cells underlying the tunica albuginea of the testis. (Same technique as D.)

G, two interstitial cells, only one of which contains granules of intravital dye. (Trypan blue; azo-carmine counterstain.)

H, cells containing dye are widely scattered among contents of a seminiferous tubule. (Same technique as A.)

I, cells containing dye are seen among the spermatogonia and on surface of desquamated mass of tubular cells. (Same technique as A.)

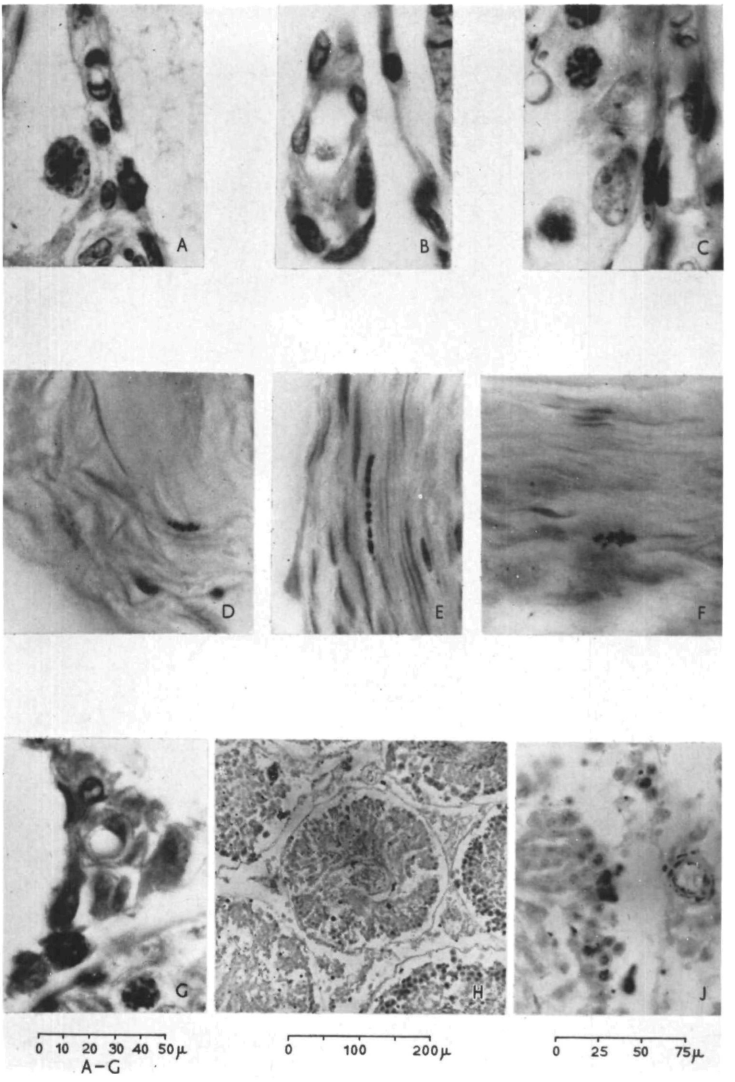

FIG. 1

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that the dye is confined to a circumscribed area of this organ, and that all the cells in this area contain massive quantities of dye.

The administration of testosterone propionate leads to a gradual diminution in the number of interstitial cells not intravitally stained, and to an increase in the amount of intertubular fluid. It has little or no effect on the intravitally stained Leydig cells. No significant difference is noted between the weights of the seminal vesicles of the animals which have been injected with dye and those of the control series. In the former group of rats, signs of castration are invariably absent.

Discussion

It is clear from the results of these experiments that interstitial cells are divisible into a group consisting of those which are stained by trypan blue (reticulo-endothelial interstitial cells) and a group which are not (true Leydig cells). By definition the former belong to the reticulo-endothelial system as first described by Aschoff. The mechanism by means of which colloidal vital dyes gain access into these cells is uncertain, but it has been suggested that 'ultra phagocytosis' or 'colloidoplexy' are responsible (Robertis, Nowinski, and Saez, 1950), whatever these terms may imply. Soluble dyes like methylene blue or neutral red, which are not colloidal but which nevertheless, like trypan blue, stain cells intravitally, manifestly cannot be taken in by phagocytosis or colloidoplexy.

The occurrence of these two morphologically identical but functionally different types of cell corresponds to the two varieties of benign interstitial cell-tumour. Although it has been stated that the reason why these neoplasms do not usually give rise to manifestations in post-puberal patients is because the secondary sexual characteristics are already established, this may equally well be due to the occurrence of reticulo-endothelial interstitial cells in these cases. As Leydig cell-tumours are associated with precocious puberty in children (Stewart, Bell, and Roehlke, 1936), it would appear that pre- and post-puberal benign interstitial cell neoplasms arise from true and reticulo-endothelial Leydig cells respectively.

Maximow (1899) describes numerous large Leydig cells undergoing mitotic division around the periphery of experimentally produced testicular injuries. These cells are, without doubt, the reticulo-endothelial type of interstitial cell, which are also probably the ones which Testa (1929) refers to when he describes interstitial cells as playing an active role in the formation of granulation tissue. It is suggested that if one postulates that the reticulo-endothelial and true Leydig cells multiply by mitotic and amitotic division respectively, the discrepancy between the work of Maximow (1899) and Testa (1929), and that of Barbeleben (1897), Ancel and Bouin (1903), Stieve (1922), and Stein (1931) is explicable on the grounds that the two first-mentioned authors based their observations on material in which the reticulo-endothelial interstitial cells predominate.

It is suggested that the difference between the results obtained in the present series and those reported by Nissim (1955) is due to the use of iron-dextran complex Imferon, which in the amounts used would lead to the formation of widespread fibrous tissue. Goldberg, Fee, and Martin (1955) point out that the amounts of Imferon used by Nissim (1955) produce a metallic iron concentration of 500 mg per kg of body-weight in the mouse, whereas in man the amount of iron is unlikely to exceed 50 and is usually about 20 mg per kg of body-weight. This, it is submitted, in long-term experiments, would result in the obliteration of the greater number of the interstitial cells by fibrous tissue, and give rise to an appearance which suggests an uptake of the iron-dextran complex by the majority of the intertubular cells.

The presence of four morphologically-distinct cell types in the testis, all of which are stained by intravital dyes, as opposed to the majority of other organs which contain only two, may be explained by the transition of one cell type into another (Ranvier, 1890, 1900; Möllendorff and Möllendorff, 1926; Esaki, 1928).

The passage of reticulo-endothelial interstitial cells from the intertubular tissue to the seminiferous tubules after the production of testicular ischaemia is most readily explained by the assumption that in the ischaemic testis the latter are the only routes by which these cells can be removed. In support of this it has been shown by Grant (1955) that cellular debris from the seminiferous tubules can be seen in the ductus epididymidis under these circumstances, and that the decrease in testicular volume following interruption of the testicular artery is so rapid that it is explicable only in this way. A second possible explanation is that there is a greater liberation of 'leucotaxine' (Menkin, 1938) from the cells of the seminiferous tubules, than from those of the intertubular tissue. Both these processes may operate at one and the same time.

The nature of the small cells with the crenated outlines is difficult to determine because, as has been previously mentioned, their morphological features are frequently obscured by dye. The diffusion of the granules of dye implies that these cells are undergoing degenerative changes, the irregular outline of the cytoplasm confirming this. These characteristics indicate that the cells correspond to the decrepit type of interstitial cell (Regaud, 1900).

The distribution of mast cells described above is of importance, since Duthie and Barker (1955) suggest that these cells may have a function in the reaction to trauma; their absence from the greater part of the testis may explain the poor regenerative powers exhibited by this organ.

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