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The Ultrastructure of Four Types of Human Spermatogonia*

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Summary. Electron microscopic examination of normal human testes has revealed the ultrastructure of four types of spermatogonia. These are the dark type A (AD), the pale type A (AP), the type B (B), and a new type A (AL). The subcellular criteria used in distinguishing between these four spermatogonial types include the shape of the nucleus, the density of the nucleoplasm, the type of nucleolus and its placement within the nucleus, the structure of the mitochondrial cristae, the association of the endoplasmic reticulum with the mitochondria, the amount of glycogen present within the cell, and the presence of previously undescribed filamentous structures in the cytoplasm of the AL and AD spermatogonia. Each spermatogonium is in contact with the tubular basal lamina; the amount of contact progressively decreases from the AL, a flat cell lying parallel to the basal lamina, through the AD and AP to the B spermatogonia, the latter being a pear-shaped cell with its long axis perpendicular to the basal lamina.

Key-Words: Spermatogenesis—Testis—Spermatogonial ultrastructure—Male reproduction—Germinal cells.

Introduction

The cells involved in human spermatogenesis can be conveniently grouped into three classes. The first class contains the most primitive cells, spermatogonia, which are in contact with the basal lamina. They divide mitotically to replace themselves and eventually give rise to the second class of cells, spermatocytes. Spermatocytes make up the interphase and prophase of meiosis and are separated from the tubular basal lamina. They divide to form two secondary spermatocytes which, in turn, divide to form the third class of cells, the spermatids. Spermatids reside along the lumenal edge of the tubule and undergo maturation to become mature spermatozoa. One other type of cell is contained within the tubule, the Sertoli cell. This cell extends between most of the cells of the germinal series and probably acts as a "nurse" cell.

The identification of the types of spermatogonia and the tentative outline of their sequential development in man was begun in the recent investigations on the normal human testes by Clermont (1963) and Heller and Clermont (1964). These studies, using light microscopy, identified three spermatogonial types in man; the A dark (AD), the A pale (AP) and the B spermatogonia. The main morphological features used in distinguishing between these three types of sperma-

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togonia at the light microscope level were the shape and staining characteristics of the nucleus, the placement of the nucleolus and the presence or absence of glycogen in the cytoplasm. The AD spermatogonium was suggested to be the least differentiated morphologically. The type B spermatogonium was considered to be the most differentiated of the three cells for two reasons: 1) it occurs in only four of the six cell associations and 2) daughter cells formed by its division are identified as spermatocytes. Both the AD and the AP spermatogonia are found in all six cell associations and are flattened against the basal lamina of the seminiferous tubule.

Roosen-Runge and Barlow (1953) carefully studied spermatogonial size and related their data to that of earlier workers. Although their conclusions were based on an erroneous premise (no organization in the human seminiferous epithelium) their measurements reflect the true variation in spermatogonial size. Clermont (1966), through painstaking mapping of the seminiferous tubules, worked out a model of spermatogonial development for the human male.

Electron microscopy of the human testis has, until recently, been restricted to observations from abnormal individuals (Smith *et al.*, 1967; Fawcett and Burgos, 1956a, b, 1960; De Kretser, 1967a, 1968; Leeson, 1966; Smith *et al.*, 1967; Gomez-Acebo *et al.*, 1968), tissue of unspecified origin or uncertain normalcy (Bawa, 1963; Nagano, 1962, 1966, 1969; Vilar *et al.*, 1962; Fawcett and Burgos, 1956a, b; Horstmann, 1961) and tissue of normal or aging human testes (De Kretser, 1967b, 1969; Vilar and Paulsen, 1967; Yasuzumi *et al.*, 1967; Tres and Solari, 1968). The majority of these investigations describe the fine structure of the interstitial cells, Sertoli cells or spermatids and only an occasional and incidental electron micrograph of a human spermatogonium has been published. Vilar and Paulsen (1967) have published an abstract on the fine structure of the human germinal cells. These workers reported two types of spermatogonia lying on the basal lamina and a third type surrounded entirely by Sertoli cell cytoplasm.

In contrast to man in whom only three types of spermatogonia have been described, six have been found in monkey testis (Clermont, 1969), six in mouse testis (Monesi, 1962) and seven in rat testis (Clermont and Bustos-Obregon, 1968). Very recently a less differentiated spermatogonium has been discovered and described for the rat (Clermont and Bustos-Obregon, 1968) and monkey (Clermont, 1969). We have also encountered a variant of the three recognized types of spermatogonia during this investigation. This spermatogonium, designated in this publication as A long (AL) has not been described previously in either light or electron microscopy studies in man. It may well be the precursor to the three recognized types.

Two difficulties have contributed to the paucity of information about the ultrastructure of human spermatogonia; 1) obtaining the appropriate well fixed normal biopsy material, and 2) properly identifying the germinal cells. We have overcome the first difficulty by obtaining biopsy specimens from testes of normal healthy young adults. The second was overcome by using three separate morphological approaches to insure accurate identification of the spermatogonial types.

Firstly, we examined paraffin embedded tissues with the light microscope in the classical manner to identify the three recognized types of spermatogonia. Secondly, we studied Epon embedded tissues sectioned at micron or sub-micron

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thicknesses by light microscopy and identified the spermatogonial types by making comparisons with paraffin embedded material. Thirdly, electron microscopic examination of thin Epon embedded sections adjacent to sections used for light microscopic examination was used to determine the ultrastructural characteristics. In this way we felt assured that the cells we designated as AL, AD, AP and B spermatogonia in paraffin sections corresponded to the cells we term AL, AD, AP and B spermatogonia in Epon sections studied with either the light or electron microscope. The identifying characteristics of human spermatogonia based on these comparative studies are described herein.

Materials and Methods

Biopsy specimens from testes of eight normal healthy adult volunteers were available for these studies. The men ranged in age from 29 to 50 years. None had a history of disease of the reproductive system. Individuals were selected for this study on the basis of a series of 18 or more weekly normal sperm counts and normal urinary and/or plasma levels of gonadotropins and testosterone. Bilateral testicular biopsies were performed as previously described (Rowley and Heller, 1966). One piece of tissue was immediately transferred to Cleland's fixative for subsequent paraffin embedding and one piece to glutaraldehyde fixative for Epon embedding. Tissues embedded in Epon were trimmed to blocks of 1 mm^3 during fixation. The glutaraldehyde fixative consisted of either 3% purified glutaraldehyde in 0.067 M phosphate buffer at pH 7.3 or 1% purified glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3. Tissues fixed in glutaraldehyde were post-fixed in OsO_4 (Millonig, 1961), dehydrated in ethanol, and embedded in Epon (Luft, 1961). Paraffin embedded material was sectioned at 4 μ and stained with iron hematoxylin-eosin. Epon embedded material was sectioned at 1 μ or less and stained with Richardson's stain (Richardson *et al.*, 1960). For electron microscopy, gray-silver sections of the Epon embedded tissues were stained with uranyl acetate and post-stained with lead citrate (Reynolds, 1963). Characteristics of the testicular material of the eight subjects were similar although slight variation was found in the depth of staining obtained in thin Epon sections.

Results

A. General Description of Spermatogonial Ultrastructure

Electron microscopy revealed that each human spermatogonium is in contact with the basal lamina. The degree of the contact varies from 30 μ to 2 μ in length (Figs. 2, 5, 6 and 9). The cell shapes are variable; ranging from a flattened cell containing a long irregularly shaped nucleus to a pear shaped cell containing a round nucleus (Figs. 2, 5, 6 and 9). The nuclei of spermatogonia have slightly granular nucleoplasm which is usually homogeneous. The more flattened cells, however, often have an area of rarefaction within the nucleus (Fig. 5). The density of this rarefied area is much less than the rest of the nucleus and resembles the cytoplasmic matrix. The rarefied area does not have a limiting membrane. Nucleoli are usually found along the nuclear membrane although occasionally (particularly in the pear shaped cell) they are in the center of the nucleus. The nucleolus contains a granular region (nucleolonema). The rounder nuclei also contain an amorphous region (Figs. 10–13).

Mitochondria are found singly, in pairs or in groups. Those occurring in pairs or groups appear to be connected by electron-opaque bars or plaques similar to that described by André (1962) and Nagano (1969), or by strands of rough endoplasmic reticulum (Figs. 14 and 15). Where mitochondria occur in groups, they

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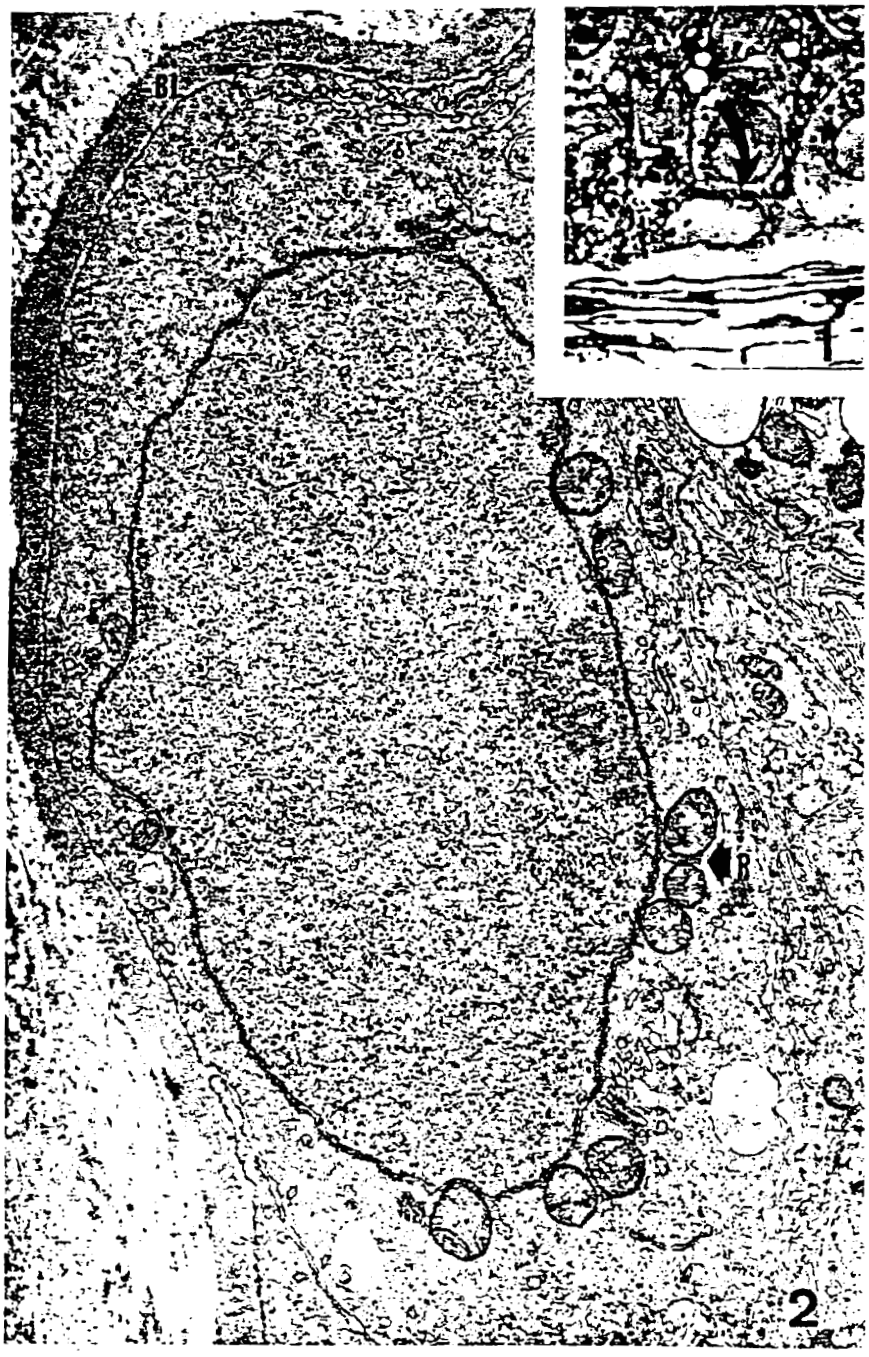

Fig. 1. Light micrograph of AL type spermatogonium embedded in Epon. The flattened cell, the irregular shape of the nucleus, and the placement of the mitochondria in the cytoplasm away from the basal lamina are distinguishing characteristics of the AL spermatogonia in micron-thick Epon sections. ×1,200

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are often closely associated with the nucleus and indented into the nuclear membrane (Figs. 2 and 5). In cells having only single mitochondria they are situated well away from the nucleus. Cristae within the mitochondria are either tubular or plate-like. Typical Golgi complexes are found surrounded by vesicles of uniform size and electron density (Figs. 5 and 9). Microtubules, coated vesicles and paired centrioles are also present in each type of spermatogonia. Glycogen, both as free particles and clustered in membrane-limited pockets is observed only in the flattened cells (Fig. 20).

A filamentous body corresponding to the crystalloid of Lubarsch (Lubarsch, 1896; Nagano, 1969) is seen only in the flattened cells. The filamentous body having dimensions of approximately $0.5 \times 2.0\ \mu$ is composed of parallel filaments (or microtubules). Each filament is 10–15 mμ in diameter and they are separated from each other by a distance of 20—30mμ. Rows of electron opaque spheres of 15 mμ in diameter are found between the filaments. In cross section each tubule has a wall 3–5 mμ thick with an electron transparent lumen 3–5 mμ (Fig. 16). The tubules are aligned in linear arrays so that the rows of electron-opaque spheres appear to be entrapped by a circle of tubules (Fig. 17).

Another body found in the cytoplasm of spermatogonia is a single membrane-limited structure having a diameter of 1–2 μ (Fig. 20). The bodies frequently possess internal membranes in a granular matrix. These structures may be lysosomes; however, in the absence of acid phosphatase incubations they are referred to hereafter as lamellar bodies.

Two forms of membraneous specialization occur on the internal side of the nuclear envelope. The first specialization is a membraneous layer closely appressed to the internal face of the nuclear envelope. Because of the appearance of this specialization when viewed in cross-section we refer to these structures as nuclear envelope stripes or simply "stripes" (Fig. 18). A three dimensional view may reveal that these structures are plaque-like formations. The stripes are composed of at least two membranes and an intermembraneous space. In some instances another layer of the stripe has been observed and one finds three electron-transparent regions, all being membrane-limited. Electron-opaque regions occur on the inner sides of each membrane-limited space. Thus from the cytoplasmic side of the nuclear envelope moving inward (Fig. 18) there is the outer membrane of the nuclear envelope, the intermembraneous space (perinuclear space), the inner membrane of the nuclear envelope, an electron-opaque region, the external membrane of the stripe, an intermembraneous region within the stripes, the inner membrane of the stripe, and another electron-opaque region. The different membraneous components of stripes are apparently continuous with the nuclear envelope. The nucleolus is frequently associated with the stripes.

The second specialization associated with the nuclear envelope is a region surrounded by double membrane (Fig. 19). Its appearance suggests a "bleb" and

Fig. 2. Electron micrograph of AL type spermatogonium. The nucleoplasm is homogeneous. The mitochondria (*M*) are linked by rough endoplasmic reticulum (*R*) and electron-opaque bars and show a preferential location away from the basal lamina (*BL*). Particularly note the length of the cell on the basal lamina (*BL*). We are unable to take a photograph of the entire cell as it is too long. ×14,300

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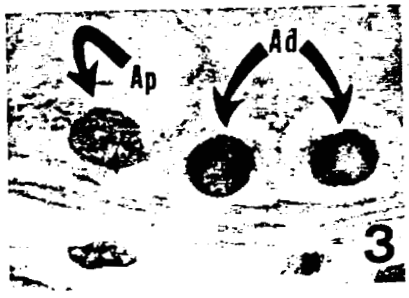

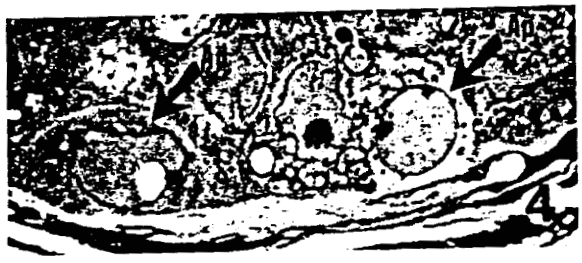

Figs. 3–5

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we refer to such a structure as such. When closely appressed to the nuclear envelope the outer membrane of the bleb is separated from the internal membrane of the nuclear envelope by an electron-opaque region. The nuclear envelope protrudes into the cytoplasm when associated with this membrane-limited structure. The contents of the blebs are either particulate in nature, averaging 15 mμ in diameter, and resemble the fine particulate material found in the nucleoplasm, or the contents of the membrane-limited body are electron-transparent with fine filaments being present.

Spermatogonia are often in direct cell-cell contact with each other, although Sertoli cell cytoplasm can extend between them to the basal lamina. Sertoli cell-spermatogonia and spermatogonia-spermatogonia contacts were seen about equally.

B. Division of Cells into Types

The characteristics of spermatogonia described above appear in some spermatogonia and not in others. Some characteristics seen in one cell type may have a somewhat different appearance in another cell type. From the variations between cells we believe that spermatogonia can be divided into four types based upon their ultrastructural characteristics alone. The cell types are abbreviated AL, AD, AP and B and listed below with specific characteristics belonging to each.

A Long (AL). The AL is a long, very flat cell attached to the basal lamina for as much as 30 mμ; it usually has a long irregularly shaped nucleus; the mitochondria often occur in groups of up to ten connected by "bars" or strands of rough endoplasmic reticulum; the mitochondria contain tubular cristae and are often associated with the nucleus. The nucleolus is of the diffuse nucleolonema type and always located on the nuclear membrane; a filamentous body (crystalloid of Lubarsch) may be present; glycogen is present as is the lamellar body (Figs. 1, 2, 10, 17 and 20).

A Dark (AD). The AD is also a long flat cell, a little less so than the AL: its long oval shaped nucleus has a regular nuclear membrane and is the only spermatogonia to have a region of rarefaction within the nucleus. Nuclear "stripes" and nuclear "blebs" are present and it has a nucleolonema type of nucleolus seen peripherally on the basal lamina; the mitochondria are found in groups connected by electron-opaque bars, many are found attached to the nucleus; cristae are

Fig. 3. Light micrograph of an AD type spermatogonium and an AP type spermatogonium embedded in paraffin. Note the "vacuole" in the nucleus of the AD. ×1,200

Fig. 4. Light micrograph of an AD type spermatogonium and an AP type spermatogonium embedded in Epon. Note the rarefied (*RR*) area in the AD nucleus and the length of these cells on the basal lamina (*BL*). ×1,200

Fig. 5. Electron micrograph of an AD type spermatogonium. Contact of the spermatogonium with the basal lamina (*BL*) is evident. The nucleus contains granular nucleoplasm, a region of rarefaction (*RR*), and a peripheral nucleolus. Mitochondria (*M*) are shown in close proximity to the nuclear envelope and are also joined to each other. Smooth endoplasmic reticulum is shown in the region of the centriole (*C*) and the Golgi complex (*Go*). ×9,100

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Fig. 6. Electron micrograph of an AP type spermatogonium. The cell is in contact with the basal lamina (*BL*). The nucleus is spherical, the nucleoplasm homogeneous, and the nucleolus peripheral. The cytoplasm contains no glycogen and is somewhat lighter in density than the cytoplasm of either of the other A spermatogonia. The mitochondria (*M*) are frequently joined in pairs. × 9,500

mainly tubular and a few are plate-like: the fibrillar body (crystalloid of Lubarsch) is sometimes present: glycogen is present: smooth and rough endoplasmic reticulum is present in small amounts and occurs as single strands (Figs. 3–5 and 11).

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Fig. 7. Light micrograph of B type spermatogonium embedded in paraffin. × 1,200
Fig. 8. Light micrograph of B type spermatogonium embedded in Epon. × 1,200

A Pale (AP). The AP is a rounded cell with even less contact with the basal lamina and has a round or slightly ovoid nucleus: the nucleolus consists of a nucleolonema and a pars amorpha and is located peripherally on the nuclear mem-

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Fig. 9. Electron micrograph of B type spermatogonium. The pear-shaped cell has a minimal, yet definite, contact with the basal lamina (arrow). The nucleolus is centrally positioned and flakes of chromatin are shown on the internal face of the nuclear envelope. The mitochondria (*M*) are not joined together and occur throughout the cytoplasm. Scanty amounts of both smooth and rough endoplasmic reticulum and a Golgi complex (*Go*) are present. ·9,100

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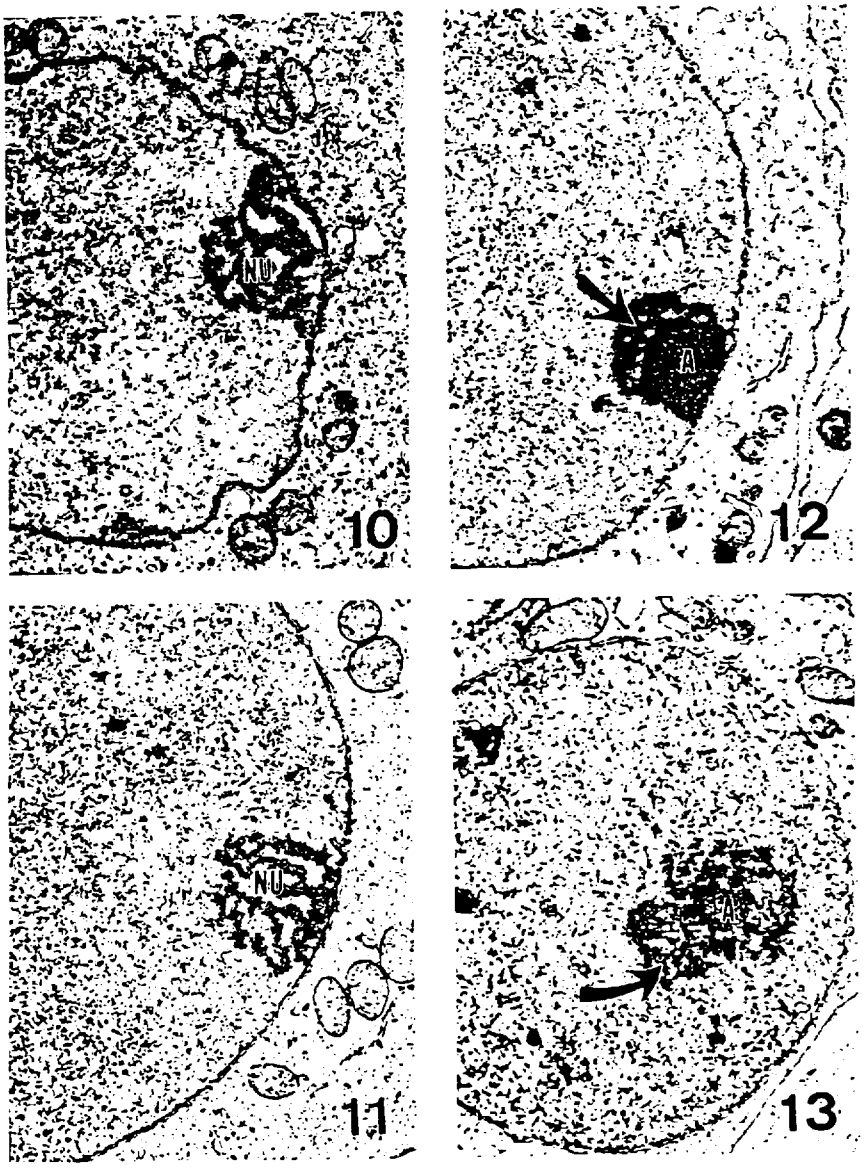

Fig. 10. Nucleolus (*Nu*) of AL type spermatogonium. The nucleolus consists of only nucleolonema. ×10,500

Fig. 11. Nucleolus (*Nu*) of AD spermatogonium. A nucleolonema is associated with the nuclear envelope. ×10,500

Fig. 12. Nucleolus of an AP type spermatogonium. The amorphous zone (*A*), in this micrograph, is associated with the nuclear envelope and the nucleolonema (arrow) lies in the nucleoplasm. ×10,500

Fig. 13. Nucleolus of B type spermatogonium. The amorphous zone (*A*) is surrounded by the nucleolonema (arrow). The nucleus is not associated with the nuclear envelope. ×10,500

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Fig. 14. Electron-opaque bars (*B*) joining mitochondria (*M*) together in an AL type spermatogonium. A strand of rough endoplasmic reticulum (*R*) is associated with a mitochondrion. ×35,200

Fig. 15. Electron-opaque bar (*B*) continuous with rough endoplasmic reticulum (*R*) in AL spermatogonium. Note the association of the bar with a mitochondrion (*M*). ×49,300

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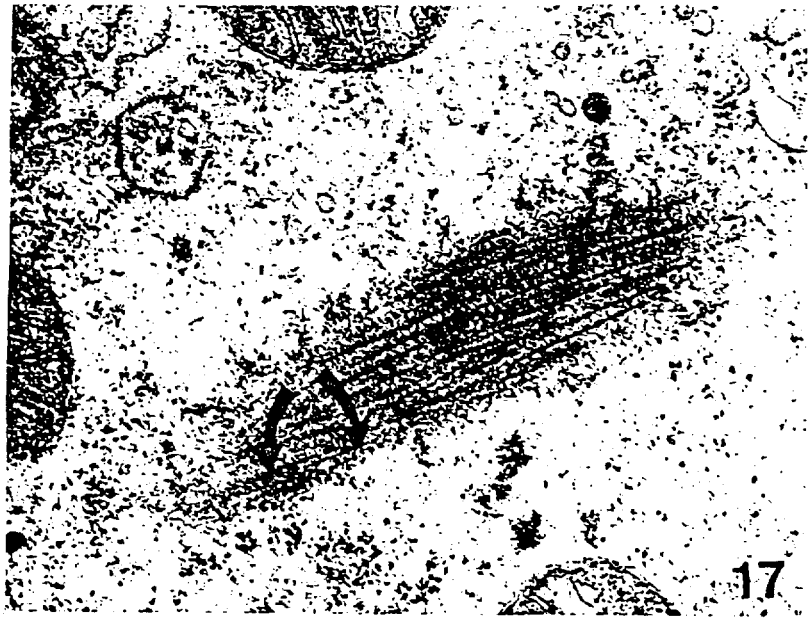

Fig. 16. Cross-section of a filamentous body (*FB*) in the cytoplasm of an AL type spermatogonium. Electron-opaque spheres (*Sp*) can be observed within the encircling linear array of tubules. ×42,500

Fig. 17. Longitudinal section of a filamentous body (*FB*) in the cytoplasm of an AL type spermatogonium. Electron-opaque spheres (*sp*) are shown between the parallel filaments. 45,000

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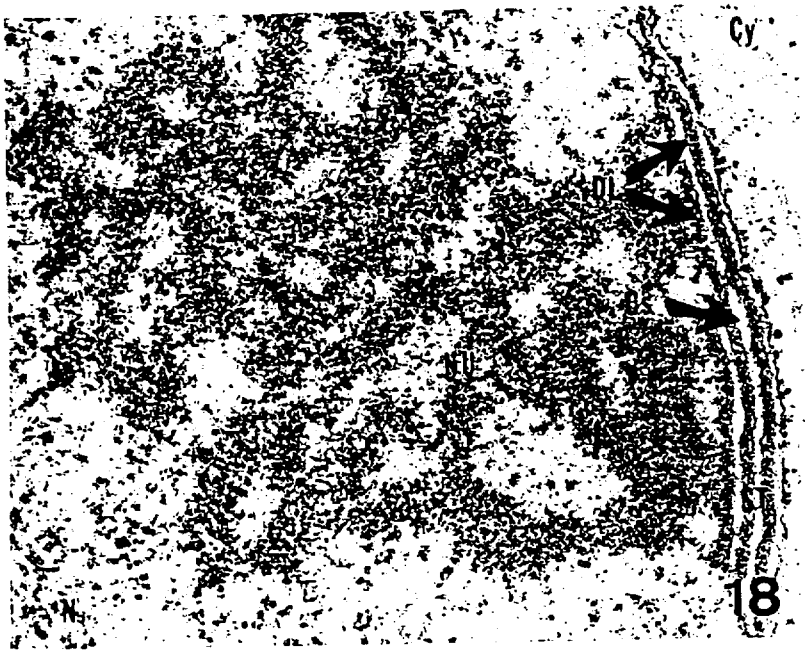

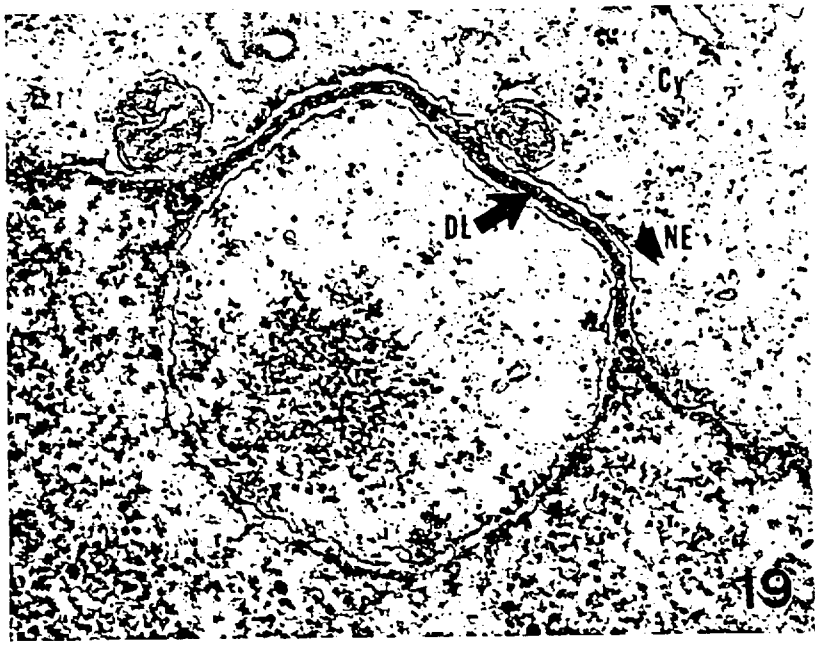

Fig. 18. Nucleolus (*Nu*) associated with stripe (*St*) on the nuclear envelope of AD spermatogonium. The nuclear envelope is continuous and the stripe is composed of a membrane-limited cisterna (*Cs*) and dense layers (*DL*) on the internal side of both the nuclear envelope and the cisterna. The nucleoplasm (*N*) and cytoplasm (*Cy*) are indicated. × 22,000

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Fig. 20. Two AL nuclei (N_1 and N_2) in a common cytoplasm. Both the cytoplasm and the nuclei possess the characteristics of AL spermatogonia. Note lamellar body (L). The cell lies on the basal lamina (BL). × 8,300

Fig. 19. "Bleb" associated with the nuclear envelope (NE) of an AD type spermatogonium. The nuclear envelope is continuous and protrudes into the cytoplasm (Cy). The membrane specialization (bleb) is composed of a double membrane-limited body having particulate and filamentous contents. Note that a dense layer (DL) separates the nuclear envelope from the bleb. × 20,000

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brane; mitochondria are connected by electron-opaque bars and are found in pairs only, they are never found attached to the nucleus, as they are in AL and AD: cristae are usually plate-like; smooth and rough endoplasmic reticulum are present in single strands and myelin-like arrays sometimes are present (Figs. 2, 4, 6 and 12).

B. The B is a pear shaped cell having the least contact with the basal lamina (as little as 2 mμ); the nucleus is spherical and has chromatin granules within and along the nuclear membrane; the nucleolus is centrally located and consists of a nucleolonema and pars amorpha; mitochondria are single and scattered throughout the cytoplasm; cristae are plate-like; scanty amounts of smooth and rough endoplasmic reticulum are present; Golgi is frequently more elaborate than in the other three types of cells; myelin-like arrays are present (Figs. 7–9 and 13).

C. Progression

This is the first time four spermatogonial cell types have been differentiated in the human being.

Since these four cell types were recognized by their ultrastructural characteristics alone we felt that direct comparisons should be made with the cells currently described using light microscopy. If the cells identified ultrastructurally are the same as those identified by light microscopy we believe the terminology already in use should be retained. The three types of spermatogonia currently recognized are based on their light microscopy descriptions and are the A dark, the A pale and the type B spermatogonia. Based on the work of Clermont (1963) the AD is considered to be the most primitive and the B the most differentiated. Therefore, as described previously, careful comparisons were made between paraffin and epoxy embedded tissue using the light microscope. The same cells identified as AD, AP or B in the thicker Epon sections were then examined using electron microscopy.

We found that those cells positively identified as AD, AP or B were the same cells separated out as different types on the basis of ultrastructural characteristics alone. Therefore in the previous section the characteristics for these cells are listed under the headings AD, AP and B. However, we found one group of cells which were different from any of these three types. Its characteristics are listed in the previous section under the heading AL (L for long length of the cell). Although this cell could conceivably be a different form of, perhaps, the AD: we suspect it is a separate and more primitive cell than any of the other three. The evidence confirming the cellular progression from AD to AP to B and the addition of the AL before the AD is listed below by developmental progression of each characteristic.

1. Cell shape and attachment to basal lamina (Figs. 2, 5, 6 and 9). *AL*, very long irregularly shaped cell, BL attachment up to 30 mμ; *AD* medium long smoothly shaped cell, attachment up to 20 mμ; *AP*, cell rounder, attachment relatively small; *B* cell pear shaped, attachment as little as 2 mμ.

2. Shape of nucleus and nuclear chromatin (Figs. 2, 5, 6 and 9). *AL*, long very irregular nucleus having irregularly dense nucleoplasm; *AD*, long oval nucleus sometimes having rarefied area within its otherwise homogeneous nucleoplasm;

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AP, round to slightly ovoid nucleus, nucleoplasm light and finely granular; *B*, round nucleus, nucleoplasm pale with chromatin lining the nuclear membrane.

3. Nucleolus (Figs. 10–13). *AL*, diffuse nucleolonema on nuclear periphery; *AD*, dense nucleolonema on nuclear periphery: *AP*, nucleolonema and amorphous regions on nuclear periphery: *B*, nucleolonema and amorphous regions centrally positioned within the nucleus.

4. Mitochondria (Figs. 2, 5, 6, 9, 14, 15 and 20). *AL*, clumped in groups, connected by bars or strands of rough endoplasmic reticulum, sometimes in apposition to the nucleus, cristae tubular; *AD*, clumped in groups, connected by bars, strands of rough endoplasmic reticulum sometimes associated with a mitochondria, often associated with the nucleus, cristae tubular and plate-like; *AP*, clumped in pairs or singly, connected by bars, never associated with nucleus, cristae usually plate-like; *B*, single scattered away from nucleus, cristae plate-like.

5. Glycogen (Fig. 20). *AL*, and *AD* present scattered throughout cell although occasional single membrane-bound pockets have been seen; *AP*, not present; *B*, not present.

6. Fibrillar body (crystalloid of Lubarsch) (Figs. 16 and 17). *AL*, present; *AD*, present; *AP*, not usually present (seen only once); *B*, not present.

7. Lamellar body (Fig. 20). *AL*, present; *AD*; not present; *AP*, not present; *B*, not present.

8. Nuclear "stripes" and "blebs" (Figs. 18 and 19). *AL*, not present; *AD*, present: *AP*, not present: *B*, not present.

We have found instances of binucleate AL, AD and AP spermatogonia (E.g., see Fig. 20) and we interpret these cells as having completed karyokinesis and of partially completing cytokinesis. Fawcett (1961) has shown that spermatids are joined by intercellular bridges. This is a condition which we believe exists throughout spermatogenesis. These binucleate cells would substantiate Clermont's mapping studies (1966) where pairs of spermatogonial types were found together and tend to confirm the occurrence of "equivalent" mitotic division in spermatogonia. We have never observed nuclei of different spermatogonial types within a common cytoplasm. Our present concept is that each cell divides mitotically to form two of the next more mature spermatogonia. If this concept is correct the finding of AL spermatogonia joined by a bridge containing spindle fibers, and apparently finishing division, would add strength to the concept of the AL as a separate and distinct cell type.

The AL may be analagous in structure and function to the A zero (A_0) of other species (Clermont and Bustos-Obregon, 1968). However, because of the uncertainty of the analogy the cell was termed AL rather than A_0. Very little study has been done on human spermatogenesis when compared to that done on other species, therefore it is not strange that additional spermatogonia have not been previously described with confidence in the human being.

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