THE IN VITRO CULTIVATION OF MAMMALIAN SERTOLI CELLS*

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The microscopic detail of spermatogenesis and its cycle is quite well known in some mammals such as the rat[1] and the mouse,[2] but the underlying molecular mechanisms are not at all understood. The lag of progress in this area is largely due to the fact that such complex cellular activities are extremely difficult to subject to experimental analysis. If, however, a method could be found whereby the component elements of the germinal epithelium might be isolated and grown *in vitro* independent of other cells, such a method would no doubt facilitate the use of techniques which are not applicable to the cells *in vivo* and which may advance our understanding of the subcellular events in spermatogenesis. Such considerations prompted us to undertake extensive studies on the *in vitro* cultivation of testicular cells of some mammals. This report describes a part of these studies, namely, those that dealt with Sertoli cells. Our work on the cultivation of germ cells will be reported elsewhere.

Materials and Methods.—Modern cell-culture studies have introduced a variety of methods for the cultivation of mammalian somatic cells. Some of these methods were tried on the testis cells of rats of the Wistar and Sprague-Dawley strains at various ages, and from these trials there emerged two methods whereby Sertoli cells may be cultured *in vitro* exclusive of all other testicular cells. One of these methods will be referred to as the tubule method and the other as the free-cell method. With a little modification, these techniques work well with mice, bulls, rabbits, and presumably also with other mammals.

Tubule method: Testes are removed from the animal, freed from the albuginea, and cut into as small pieces as possible in a Petri dish. Then the pieces are immersed in a small amount of medium (see below), and while being observed under the microscope, they are transferred by a Pasteur pipette to a Leighton tube containing a cover slip and approximately 14 ml of medium. Precautions are taken to keep interstitial tissues and free cells from entering the tube. It is then tightly stoppered and incubated in a slightly slanted position, so that the tubules rest on the cover slip. The optimal range of temperature is 34–35°C. The pH of the medium should be approximately 7.3. The tube is kept static for at least 48 hr, during which time the tubules adhere to the glass, and Sertoli cells begin to migrate out of the tubules. Periodic observations show that Sertoli cells continue migration and eventually form monolayers surrounding the tubules. Germ cells, on the other hand, are immobile and stay either within the tubules or, if pushed out, near the open ends. Unlike Sertoli cells, germ cells are unable to adhere to the substrate and are easily removable from the culture. While in migration and in monolayer formation, Sertoli cells multiply by mitosis.

The lamina propria that bounds the tubule consists of the basement membrane and a layer of closely apposed cells. In culture these cells neither migrate nor undergo mitosis. They keep the layer intact.

Leighton-tube cultures have been used primarily for observing the behavior and morphologic details of various tubule cells and not for establishing long-term mass cultures of Sertoli cells. The tubule method may be used, however, for the latter purpose by following the foregoing procedure and using an Erlenmeyer flask instead of Leighton tubes. Thus, a large number of tubules are cultured in a flask, and when Sertoli cells have covered the surface, the flask is shaken to suspend tubules and free germ cells, and the suspension is replaced by fresh medium. Repetition of this process eventually eliminates all tubules and germ cells, giving rise to a pure culture of Sertoli cells. Although this technique is workable for starting a mass culture, it is not satisfactory as a routine method because of the considerable time required for preparing a culture and for

Sertoli cells to migrate from tubules, to grow, and to undergo active mitosis. For preparing mass cultures, the following free-cell method which has no such shortcomings is preferable to the tubule method.

Free-cell method: Testes are freed from albuginea and cut into small pieces as in the previous method. Tubules as well as interstitial tissues are transferred to an Erlenmeyer flask containing medium and a magnetic bar. The flask is stoppered, and its content is agitated on a magnetic stirrer for 45 min at room temperature. As soon as intact tissues precipitate after the cessation of agitation, the cell suspension is transferred to another flask, its pH is adjusted to 7.3, and the culture is incubated. In 5 or 6 days, numerous small aggregates are formed on the glass. They consist of germ and Sertoli cells associated in completely random fashion. Furthermore, they are anchored to the glass by Sertoli cells at the surface facing the substrate. These Sertoli cells proliferate and spread over the glass forming monolayers. When the glass is covered sufficiently with cell layers, the flask is shaken vigorously to suspend aggregates and free germ cells, and the suspension is replaced by fresh medium. By repeating this process, aggregates and germ cells are eventually completely eliminated from the culture. Sertoli cells may be transferred then to a culture bottle and serially passed thereafter.

Since it is difficult to estimate the total number of Sertoli cells suspended in the medium after the agitation because of the presence of germ cells, the concentration of these cells cannot be easily adjusted according to the surface area of the culture flask. This difficulty has been eliminated, however, by our finding that if the body weight is above 100 gm, one rat and, if below, two rats usually yield enough Sertoli cells for a 125-ml flask. The optimal amount of medium for this flask is 100 ml.

Culture medium: Various media commonly used for somatic-cell cultures[3-6] were tried for Sertoli cells, and it was found that whereas those from immature rats grew well in some of these media, those from mature animals grew very slowly and often only a short time. Studies were undertaken, therefore, to modify Eagle's medium[3] in order to improve it for faster and longer growth of these cells regardless of the animal's age, and from these studies a medium with the desired quality was obtained (see Table 1). For the most consistent and luxuriant growth of Sertoli cells of the rat, calf serum should be added to the medium in 2:3 ratio. This mixture has been tested for mice, bulls, and rabbits with satisfactory results. Addition to the mixture of 100 units of penicillin and 100 μg of Kanamycin per ml is recommended.

Results.—Morphology of cells in tubule culture: Sertoli cell monolayers display a typical pavement-like pattern characteristic of epitheloid cells in monolayer culture (Fig. 1). The nuclei of these closely contiguous cells exhibit the morphologic characteristics observed in Sertoli cells *in vivo*. They stain fairly densely and always show one quite prominent nucleolus, nucleolar karyosomes, and heterochromatic granules of varying shapes and sizes. Some nuclei display wrinkles, which are one of the unique characteristics of Sertoli cells *in vivo* (Fig. 3). The

TABLE 1

COMPOSITION OF MEDIUM FOR SERTOLI CELL CULTURE*

L-arginine HCl	120	L-asparagine	20	Biotin	0.2
L-lysine HCl	120	L-tryptophane	10	Vitamin A	0.2
L-histidine	50	L-cystine	24	Calciferol	0.2
L-leucine	52	L-cysteine HCl	90	Vitamin B$_{12}$	0.1
L-isoleucine	52	L-tyrosine	36	Ascorbic acid	50
L-methionine	15	L-glutamine	300	α-Tocopherol	0.2
L-phenylalanine	32	Thiamine HCl	2	NaCl	8000
L-threonine	48	Pyridoxal HCl	2	KCl	400
L-valine	46	D-Ca-pantothenate	2	MgSO$_4$-7 H$_2$O	150
L-alanine	40	Nicotinamide	2	CaCl$_2$-2 H$_2$O	100
L-proline	40	i-Inositol	2	NaH$_2$PO$_4$-H$_2$O	200
L-serine	40	Choline chloride	2	NaHCO$_3$	2000
L-glutamic acid	40	p-Aminobenzoic acid	0.2	Dextrose	1000
L-aspartic acid	40	Folic acid	1	Phenol red	20
Glycine	40	Riboflavin	1		

* All figures are in mg/liter.
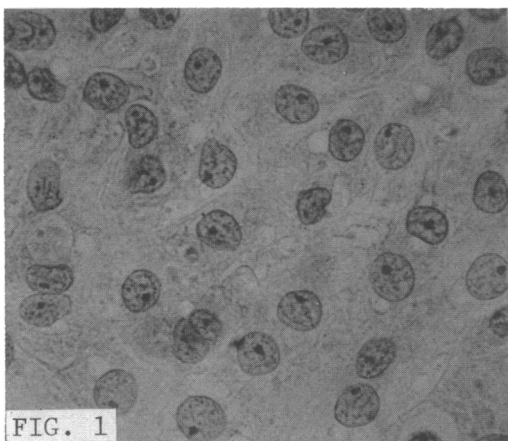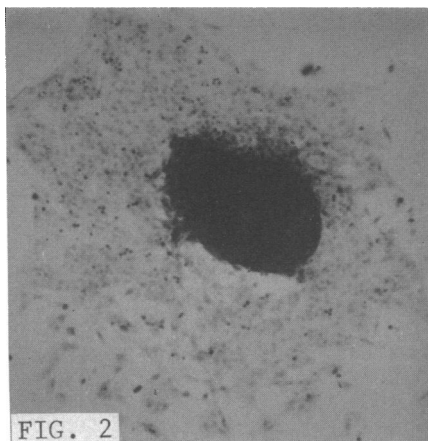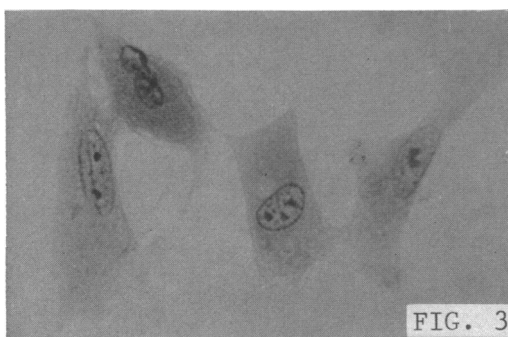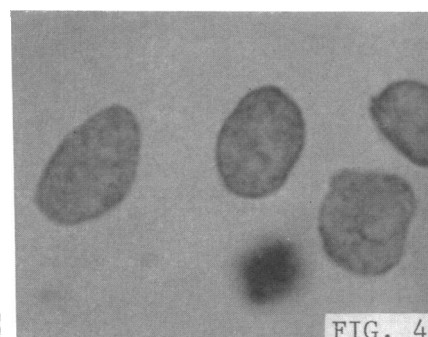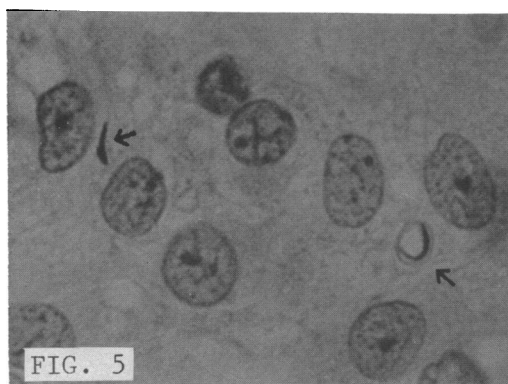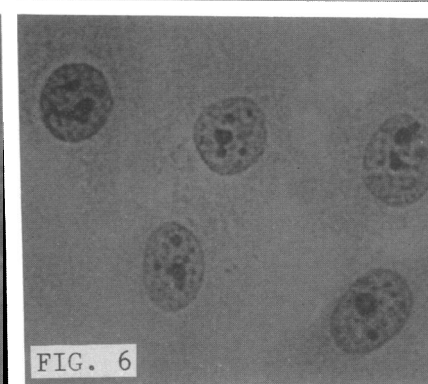
FIG. 1.—Sertoli cells in monolayer in a tubule culture.   500×.

FIG. 2.—Sertoli cells in monolayer surrounding the tubule (the dark mass) from which they migrated.   Some cells are dissociated and dispersed.   120×.

FIG. 3.—Sertoli cells dissociated from a monolayer in a tubule culture.   Note nuclear wrinkles in some of the cells.   500×.

FIG. 4.—Lamina cell layer of a tubule after Sertoli cells have migrated away.   900×.

FIG. 5.—Sertoli cell monolayer in a tubule culture.   Note two cells harboring spermatids (*arrows*).   See text for explanation.   700×.

FIG. 6.—Sertoli cells in monolayer formation in a free-cell culture.   700×.

All figures were photographed from acetic orcein preparations.

cytoplasm contains inclusions in the form of granules and droplets. The latter are especially prominent during the early stages of cultivation and gradually become invisible as the culture ages. They are extractable with lipid solvents. Lipid droplets and granules are a characteristic feature of Sertoli cells *in vivo* of mature animals. In culture even those of immature animals produce lipid droplets.

The mosaic pattern of Sertoli cell layers disappears as soon as cells dissociate and migrate away from each other (Fig. 2). The migrating cells display a wide variety of shapes, as would be expected of cells in active amoeboid movement. They are flat and large and usually have one or more cytoplasmic processes (Fig. 3). Mitosis is observed frequently. In such cultures a small number of cells display amitotic cell division. In each such cell, cleavage takes place through the resting nucleus, dividing it into two segments of about equal size and giving rise to a binucleated cell. Later this cell may undergo cytokinesis, producing two small cells. Occasionally, cleavage divides the nucleus into three or more segments of different sizes, producing a multinucleated cell. Cytokinesis does not follow in such a cell, but instead some of the nuclear segments are expelled from the cell. The expelled nuclei are free of cytoplasm, and they soon disintegrate. The abnormal cells produced by amitosis or nuclear diminution survive only a limited time. In the same relatively old culture, another abnormal cellular behavior is observed occasionally: A cell extends out a long cytoplasmic process, and then the distal part of the process is broken off. Similar behavior has been observed in cultured macrophages.[7]

Sertoli cells, which are able to move freely, exhibit active pinocytic and phagocytic activities. Undulation of the distal ends of cytoplasmic processes and the formation of pinocytic vesicles are observed frequently in such cells. Phagocytosis has been demonstrated experimentally in the following manner: Tubule cultures were prepared from 22-day-old rats as described above. As soon as Sertoli cells began to migrate from tubules, sterile carbon particles were placed on the cover slips, and the cultures were reincubated for 6 more days. Sertoli cells migrated over the glass where the carbon particles had been placed. The cover slips were then fixed and stained. These preparations showed many Sertoli cells which had ingested carbon particles.

Lamina cells: The nuclei and cytoplasm of lamina cells are comparable in size to those of Sertoli cells, but their microscopic structures are entirely different. Unlike those of Sertoli cells, the nuclei of lamina cells stain very lightly and display neither nucleoli nor karyosomes. Heterochromatic granules are also very indistinct. In contrast to the highly heterogeneous cytoplasm of Sertoli cells, the lamina cell cytoplasm appears homogeneous and shows no inclusions such as granules and droplets, and the cell boundaries are usually not recognizable (Fig. 4).

Cultivation of Sertoli cells harboring spermatids: The cells which migrate from tubules and grow in monolayer have been identified as Sertoli cells on the basis of the size and morphology of their nuclei and cytoplasm, by which Sertoli cells are readily distinguishable from other intratubular cells. Although the morphologic identification leaves little doubt that they are Sertoli cells, an unequivocal proof has been obtained from the following experiment: Tubule cultures were prepared in Leighton tubes from 42-day-old rats. Stained squash preparations of tubule samples showed a large number of spermatids at various stages of differentiation, including ones with fairly well-developed tails. This indicated that the cultured

tubules contained Sertoli cells harboring spermatids. Starting at the fifth day of cultivation, cultures were terminated periodically, and the cover slips were turned into permanent stained preparations. In all preparations Sertoli cells were in monolayer as expected, and many of them had spermatids in their cytoplasmic pockets. Figure 5 shows an area of a monolayer where two cells harboring spermatids are observed. It is clearly evident that the cells which grow in tubule cultures are the Sertoli cells.

Morphology of cells in free-cell culture: Since, in the free-cell method, tubules and interstitial tissues are agitated together in the same flask, the resulting suspension might seem to contain cells from both tissues. This has been found not to be the case. Cell samples taken from initial and subsequent cultures have never been found to contain cells which could be attributed to extratubular origin, except erythrocytes, which were always found in initial cultures. The Leydig and other interstitial cells are not expected to remain intact after colliding with cells and tubules for 45 min in a rapidly whirling medium. When much shorter agitation such as for 3 min was tried, the resulting suspension did contain, in addition to germ and Sertoli cells, some granulocytes, fibroblast-like cells, and a small number of large cells which appeared to be Leydig cells. Such cells have never been observed after agitation for 45 min. A considerable number of germ and Sertoli cells are also destroyed by agitation, but being far greater in number than interstitial cells, many of them survive the destructive action of agitation.

After germ cells are completely eliminated, the cells remaining in free-cell cultures are homogeneous and morphologically identical with Sertoli cells of tubule cultures (Fig. 6). Furthermore, like Sertoli cells in tubule cultures, these cells display amoeboid movements, pinocytosis, and phagocytosis. From these observations it has been concluded that after germ cells are completely eliminated, free-cell cultures consist entirely of Sertoli cells.

Karyotype shifts: Sertoli cells *in vivo* are diploid, and in culture they maintain the normal chromosome number for an unusually long time, but not indefinitely. Sooner or later, cultures undergo gradual shortening of cell-generation time and shifting of karyotype from diploid to heteroploid, giving rise eventually to permanent cell strains. The nuclear and cytoplasmic morphology and cellular behavior are apparently not altered when karyotype shifts occur *in vitro*.

Discussion.—Sertoli cells of the mammal, or of any of the lower vertebrates, have never been cultured *in vitro* for a long period of time, although there are a few reports on growth of these cells in culture. For example, Dux[8] found that certain cells of the seminiferous tubule of newborn rats grew in sheets, and that they appeared to be Sertoli cells. Michailow[9] observed active migration of Sertoli cells from tubules into medium in cultures of testes from immature rabbits. No attempts were made, however, to culture these cells continuously. The present report is, therefore, the first on the successful long-term cultivation of Sertoli cells, and its significance lies in the fact that our cultures not only continue indefinitely, but also become completely free of germinal and other cells of the testis.

There exists in the seminiferous tubule a system for the transportation of sperms which are not motile when released from Sertoli cells. Although the nature of this system is not fully understood, since its existence is certain, there must exist also a counteracting mechanism for preventing immature spermatids from being trans-

ported away from the seminiferous epithelium. Such a mechanism is provided by Sertoli cells; by harboring immature spermatids in their cytoplasmic invaginations, Sertoli cells ensure the development of these germ cells to maturity within the germinal epithelium.

Entrance of a cell into another cell usually leads to the death of either or both cells. Exceptionally, both cells may survive apparently unaltered as in the case of lymphocytes in the cytoplasm of other cells.[10] In contrast to these cases, spermatids harbored by Sertoli cells not only survive, but also undergo transformation to an entirely different form of cell. This unusual phenomenon poses a question: Do Sertoli cells merely hold spermatids *in situ* while the transformation occurs autonomously in the spermatids, or do Sertoli cells hold spermatids and at the same time induce the transformation of the latter? In considering this question which has not been answered satisfactorily, it may be pointed out that there are some indications that Sertoli cells do something more than mechanically retain spermatids in the epithelium. For instance, it has been reported that Sertoli cells produce a lipid at puberty in the human, and mature sperms are produced simultaneously,[11] pointing to the possibility that the lipid plays a role in the maturation of spermatids. Another indication comes from the following observation: While harboring spermatids, Sertoli cells do not grow in culture, whereas when free of spermatids, they do grow and divide. Furthermore, if spermatids have already been produced, spermatid-free Sertoli cells are not able to grow as rapidly as those from animals not yet producing spermatids. The declined potentiality of growth would seem to indicate that when spermatids appear in the tubule, Sertoli cells are physiologically differentiated, possibly in order to function as nurse cells.

On the basis of these and other observations, it is not unreasonable to assume that Sertoli cells have a nutritional function for developing germ cells. Some authors further assumed that the lipid found in Sertoli cells *in vivo* was a nutritive substance and tested its chemical identity.[12] However, tests had to be limited to those of histochemistry by which a decisive identification was not possible. With the development of the methods described in the present report, by which Sertoli cells can be isolated and cultured, it should now be possible to study the foregoing problem using techniques of greater precision. Since there are indications that steroids might be produced by these cells *in vivo*,[13, 14] an investigation is under way in collaboration with Dr. David Berliner to find out if normal Sertoli cells of the rat cultured *in vitro* possess steroidogenic capacities.

Sertoli cells in culture are able to engulf inert particles as shown by our experiment. That they are also able to ingest and destroy live microorganisms was observed in a culture which was contaminated with bacteria. This property provides Sertoli cells with an important function, namely, the protection of germ cells against microbial invasion.

There has been a widespread opinion that Sertoli cells in adult mammals form a syncytium. Even when fixed and stained, the cell boundaries are usually not discernible. A contrary opinion has been expressed, however, although the supporting evidence was indecisive.[15] The present study has shown that Sertoli cells in the tubule are able to migrate away from each other as individual cells. This fact settles the question, indicating clearly that Sertoli cells do not form a syncytium.

When spermatozoa complete their development, they are released from Sertoli

cells, and then and only then a new cycle of spermatogenesis is initiated by spermatogonial mitosis. This orderly sequence of events in the spermatogenic cycle seems to indicate that the events are initiated and regulated by molecular communications between Sertoli cells and specific germ cells such as spermatogonia and spermatids. It has not hitherto been possible, however, to find out if such intercellular communications actually take place within the seminiferous tubule. With the development of our system of isolation and cultivation of Sertoli cells, it is now possible to investigate this important problem. Such a study, which is in progress, may bring to light the nature of molecular mechanisms underlying morphogenesis of male reproductive cells of the mammal.

Summary.—Techniques have been developed for isolating and cultivating mammalian Sertoli cells *in vitro*. Under appropriate conditions, pure cultures of Sertoli cells continue indefinitely, giving rise eventually to heteroploid cell strains. Diploid as well as heteroploid cells display typical nuclear and cytoplasmic morphology of Sertoli cells, active amoeboid movements, pinocytosis, and phagocytosis. On the basis of observations made during cultivation of Sertoli cells, their possible functions *in vivo* are considered to be (1) harboring immature spermatids to prevent them from being transported away from the germinal epithelium, (2) nursing undeveloped spermatids for maturation to sperms, and (3) protecting germ cells from microbial infection by phagocytizing invading microorganisms.

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