CHANGES IN FROG TADPOLE OVARIES RESULTING FROM TWO TYPES OF ORGAN CULTURE

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Introduction

To determine whether gonads from frog larvae would continue to differentiate *in vitro* the watch glass culture method of Fell and Robison (1929) was employed. Explants of gonads were supported by strips of rayon acetate fabric (Shaffer, 1956) on a solid medium of chick embryo extract and chicken plasma or on embryo extract and agar for periods up to 28 days (Foote and Foote, 1958a). It was reported that the cultured gonads showed maintenance and growth of non-germinal cells and of immature germ cells, but that the older ovocytes usually degenerated. In the present study modifications of the original methods for cultivation of amphibian gonads have been employed, and more quantitative studies made, in an attempt to clarify some aspects of the growth pattern and morphological changes which occur in the frog ovary grown in organ culture. Some cultures were maintained for longer periods of time, up to 56 days, and some were grown directly on the medium instead of on strips of rayon acetate fabric.

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Materials and Methods

Ovaries were removed from larvae of *Rana catesbeiana* and maintained in watch glass cultures by methods previously described (Foote and Foote, 1958a). The culture medium consisted of 13 parts reconstituted chicken plasma, 4 parts chick embryo extract from 11-day embryos, 2 parts glass distilled water, and 1 part penicillin (100-200 units). Transfers to new medium were made each fifth day. Cultures were kept at a constant temperature of 21° C. Explants were fixed in Zenker's solution, sectioned at 7 μ, and stained in Harris' hematoxylin and chromotrope-2R.

Comparisons were made between ovarian explants maintained for 10, 28, and 56 days directly on the medium with explants supported on rayon strips on similar medium for equivalent periods of time. Numbers of germ cells, in varying stages of development within the ovarian explants, were determined by projecting three areas from different sections of each explant onto a grid equivalent to 1 mm. square, divided into areas of 100 μ. Outline drawings of cells superimposed on the grid were made in order to make as accurate cell counts as possible and to determine the approximate size of each cell. Mean values were obtained

for each of the three areas of each explant and cells were classified into three size groups: cells larger than 100 μ in diameter, cells between 50-100 μ, and cells less than 50 μ. Data were then treated statistically to determine comparative number of cells, and comparative size of cells. Results are given in Table 1.

Results

Controls.

Cell types closely examined from ovaries of intact larvae were ovogonia, ovocytes, follicle cells, thecal cells, and stromal cells.

Ovogonia. These cells were found in groups of two to eight at the periphery of the ovary near the peritoneum. They were spherical in form with large rounded nuclei whose chromatin threads were in leptotene or zygotene stages of prophase.

Ovocytes. The largest number of germ cells in ovaries in this stage of differentiation were ovocytes of varying sizes. Most of these cells were between 50-100 μ in diameter, although some had a diameter greater than 100 μ. None reached a diameter of 150 μ. All of the ovocytes were in pre-yolk stages, the largest attaining a stage of development corresponding to stage 3 of Duryee (1950), and Y_0 of Kemp (1953). The cytoplasm of these cells took a basophilic stain.

Follicle Cells. These cells usually formed a single layer around the ovocytes at this stage of development, but in some instances more than a single layer of cells was present. The cells were somewhat flattened, with oval nuclei which appeared granular.

Thecal and Stromal Cells. Flattened epithelial cells formed the theca, enclosing loose connective tissue and blood vessels between it and the follicle cells.

Ovarian Explants Grown Directly On Medium.

10 days in culture. Explants maintained directly on the medium for 10 days did not appear, superficially at least, to be much changed from control ovaries. The general form of the explant and the position of the cells appeared to be the same with no great amount of outgrowth. However, cell counts and detailed observations showed that some changes had occurred. The major alteration was a decrease of 50% in total number of germ cells. Statistical analysis indicated that there had been a significant decrease (84%) in number of ovogonia and small ovocytes (Table 1), and a 34% decrease in number of larger ovocytes. Some vacuolation of the cytoplasm was apparent in larger ovocytes and some of these had shrunk away from the surrounding theca, particularly in those the greatest distance from the medium. Of non-germinal cells the most marked changes occurred in follicle cells. The nuclei of some of these cells had large aggregates of chromatin and the form of the cells changed to that of a fibroblast-like cell. Many of these cells were undergoing mitotic divisions and increasing in number. Thecal cells had the same appearance as those of control ovaries, although the chromatin of the nuclei of some appeared granular. There was some increase in the amount of stroma due apparently to a shrinkage of the larger

TABLE 1.—Number and size of germ cells in one square mm. of ovarian tissue maintained in culture.

	No. Counts	Days In Vitro	Over 100 μ		50-100 μ		Under 50 μ		Totals	
			No.	SEm	No.	SEm	No.	SEm	No.	SEm
Controls	21	..	41.5	±3.06	25.0	±5.08	36.6	±2.38	101.0	±4.44
On medium	15	10	27.2	±2.34	17.6	±2.67	5.8	±0.96	50.6	±3.32
Difference			—34%		—30%		—84%		—50%	
Controls	21	..	41.5	.....	25.0	.....	36.6	.....	101.0	.....
On strips	9	10	33.3	±2.05	11.6	±1.40	6.5	±1.80	51.4	±1.15
Difference			—20%		—54%		—82%		—49%	
On medium	15	10	27.2	.....	17.6	.....	5.8	.....	50.6	.....
On strips	9	10	33.3	.....	11.6	.....	6.5	.....	51.4	.....
Difference			—18%		+52%		—11%		—2%	
Controls	21	..	41.5	.....	25.0	.....	36.6	.....	101.0	.....
On medium	21	28	6.7	±1.91	16.6	±2.16	8.8	±1.67	32.1	±2.28
Difference			—84%		—34%		—76%		—68%	
Controls	21	..	41.5	.....	25.0	.....	36.6	.....	101.0	.....
On strips	36	28	9.3	±1.46	40.0	±3.28	17.1	±2.99	66.3	±5.10
Difference			—77%		+60%		—53%		—34%	
On medium	21	28	6.7	.....	16.6	.....	8.8	.....	32.1	.....
On strips	36	28	9.3	.....	40.0	.....	17.1	.....	66.3	.....
Difference			—28%		—59%		—48%		—52%	

ovocytes together with their investing follicular cells.

28 days in culture. For explants in culture for 28 days one of the more marked changes was a decrease in overall size. This was apparently due not only to a significant loss in number of germ cells (68%), but also to a decrease in size of the larger ovocytes (Table 1). This reduction in size of these ovocytes appeared to be the result of a loss of cytoplasmic material from the cell, but without subsequent necrosis. As noted in Table 1 there was a significant decrease in number of germ cells except for those 50-100 μ in diameter. While there were fewer of these than in controls the number was equal to that in explants from 10 day cultures. However, this stability in number of cells of this size was probably a reflection of the reduction in size of many cells which were originally over 100 μ in diameter.

56 days in culture. Only an occasional ovocyte could be identified in explants maintained directly on the medium for 56 days, but the total mass of the explant was approximately one-half that of the original explant. It was spherical in shape and composed of a mass of cells resembling mesenchymal connective tissue, most of these cells being of the same type and form. There were some acidophilic masses indicating the positions of degenerating ovocytes.

Ovarian Explants Grown On Rayon Strips.

10 days in culture. Explants supported by strips of rayon on the solid medium showed more marked changes in form than explants placed directly on the medium. They tended to flatten out, thus becoming thinner and showing a greater surface area. There was much outgrowth from the explant, particularly on the side adjacent to the medium, and the dispersion of cells contributed to its general change in form. Apparently more cells of the explant were able to obtain nutriment from the medium and less vacuolation occurred in the larger ovocytes. The decrease in total number of germ cells (49%) and in cells under 50 μ in diameter (82%) was almost identical with that observed in explants grown directly on the medium, while ovocytes over 100 μ decreased less in number. Non-germinal cells followed the same pattern of change as they did in the case of explants grown directly on the medium.

28 days in culture. While there was a decrease in number of germ cells between 10 and 28 days (34%), this decrease was less than in explants maintained directly on the medium (68%). The loss in number of cells occurred primarily in ovocytes over 100 μ in diameter (77%). This was due not to a complete degeneration of these cells, but to an apparent loss of cytoplasm which produced an increased number of germ cells in the 50-100 μ diameter group. Some loss (53%) occurred in the group of germ cells less than 50 μ in diameter.

56 days in culture. While the number of germ cells decreased markedly there were individual large ovocytes which took a basophilic stain. The thickness of the cellular mass was greater than in the original explant and covered the entire strip of rayon

acetate. The thickening of the mass was due to a tremendous increase in number of mesenchymal type cells. These cells did not have the appearance of any of the cell types which could be identified in control ovaries or in those explants in culture for shorter periods of time. These cells more nearly approximated the form of the stromal cells of the control ovaries.

Discussion

This study indicates that explants on rayon strips in culture are maintained in better condition for longer periods of time than are those grown directly on the medium. This supports the results obtained by Shaffer (1956) who maintained tissues from chick embryos on rayon strips. Cells of explants on these strips have greater freedom to produce outgrowths and as the surface area expands cells are brought nearer the medium and the obtaining of nutriment is facilitated. However, the change in the organ is sometimes so extensive that the original form of the organ is lost. Explants directly on the medium tend to round up and form a capsule of connective tissue which not only restricts the outgrowth of cells but interferes with exchanges of material between explant and medium. According to Wolff (1957) such encapsulation is essential for the maintenance of the structure as an organ in culture.

In attempting to determine the fate of cells of ovaries maintained *in vitro* several possibilities may be considered. The cells could continue to differentiate, or maintain themselves without change, or dedifferentiate, or degenerate or retrogress. For each of these possibilities each specific cell type must be considered. It appears that few ovogonia continue their process of ovogenesis through the prophase stages. While some of the ovogonia maintain their characteristic form for some time there is eventually a great decrease in number of these cells. It appears that some of the younger germ cells transform into fibroblast or mesenchymal type cells, but some no doubt undergo cytolysis and eventually degenerate. Large ovocytes do gradually decrease in size and finally disappear as definitely formed cells. This gradual decrease in cell size seems to result from a loss of cytoplasmic material from the cell, perhaps being withdrawn by the follicular cells for their nourishment.

It is felt that while the non-germinal cells do not retain their characteristic form indefinitely there is much less degeneration among these than among the germ cells. The follicle cells maintain themselves for a time and increase greatly in number, as on synthetic media (Foote and Foote, 1958b), but eventually lose their characteristic form. The stromal and thecal cells maintain themselves for a longer period of time than other types of cells but do not increase in number as extensively as follicle cells. Most of the non-germinal cells appear to assume the form of mesenchyme-like cells, and it is suggested that some germ cells may do so, also.

Summary

1. Ovarian explants from larvae of *Rana catesbeiana* were placed in

organ cultures, either directly on a solid medium or supported on the medium by strips of rayon acetate fabric.

2. Explants were kept in culture for 10, 28, or 56 days.
3. In all explants there was a progressive decrease in number of germ cells the longer they remained in culture, but non-germinal cells increased in number.
4. Comparative studies indicate that the decrease in number of germ cells was less extensive in explants supported on rayon strips for 28 days.
5. A possible explanation is that explants directly on the medium rounded up and encapsulated, while those on rayon strips expanded in area and brought cells nearer the medium.
6. Few germ cells were present in explants maintained for 56 days in culture and most of the cells were mesenchyme-like.
7. It is suggested that while many germ cells, particularly the large ovocytes, degenerate some ovogonia and small ovocytes transform into the mesenchyme-like cells.

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