Continuous culture of bovine retinal cells

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Cells from the retinas of cattle eyes have been growing continuously in tissue culture for more than one year in our laboratory. The culture medium used is defined as modified 199, with 20 per cent calf serum. The glass surfaces of Blake bottles, toxin bottles, cover slips, and synthetic membranes provide the substrate for these monolayer cultures. The cell population doubles every 7 to 14 days. The cells can be transferred and divided by trypsinization. Cultures can be maintained in the same vessel for periods of one to three months or longer, with no cell deterioration. Small, medium, and large cells resembling neurons and glia cells are observed. The number of these cells varies with the age of the culture. The morphology, staining properties, and growth rate of these retinal cells are described.

The vertebrate retina is a complex structure including the pigment epithelium, the rods and cones, the external limiting membrane, the outer nuclear layer, the inner molecular layer, the ganglion cell layer, the nerve fiber layer, and the internal limiting membrane. The nervous layers comprise the rods and cones, the bipolar cells, and the ganglion cells.

Tissue culture techniques and microanalytical methods are being developed to study the growth, structure, and chemistry of the neurons and ganglion cells. Liss and Wolter recently reported the growth, in plasma clots, of human embryonic retinal (4 to 5 months) ganglion cells in tissue culture. There have been other reports of maintenance of rabbit and chick retinal cells and frog and the growth of nerve cells in tissue culture. The continuous long time cultivation of adult vertebrate retinal cells has not been reported. Therefore, in this investigation, we would like to report a study of the continuous culture and growth of bovine retinal cells.

Materials and methods

The eyes were obtained from freshly killed cattle and kept in the cold and in the dark for several hours, until used. The retinas were dissected aseptically in a laboratory cold room, in dim red light. The isolated retina was minced and placed in a Petri dish containing medium 199 with the addition of 20 per cent calf serum, and then incubated, not under CO₂, in the dark at 37°C. The synthetic medium 199 described by Morgan, Morten, and Parker and modified by Youngner, Ward, and Salk was used. To each 100 ml of medium, 3 ml of a 2.8 per cent sodium bicarbonate solution was added. The calf serum used was also obtained from the slaughter house and was inactivated at 56°C for one half hour. In addition, the following antibiotics were used: dihydrostreptomycin sulfate 200 µg per milliliter, Neomycin, 100 µg per milliliter, and penicillin G,
400 units per milliliter. The pH of the medium is 7.3 to 7.4, and rises to 7.8 to 8.0 upon incubation and during the course of growth the pH falls to 7.0 to 6.8 because of cell metabolism. All solutions used were sterilized by Seitz filtration under positive pressure.

Trypsin solution, made from Bacto-Trypsin* in a concentration of 0.25 per cent by weight with phosphate buffered saline pH 7.5, was used to remove the cells from the walls of the bottles.

**Observations**

The initial culture from one bovine retina was made on June 30, 1961. The nutrient medium was changed at 10, 12, and 14 days, and on the eighteenth day the cells were trypsinized and a new cell culture made (subculture 1). Three days later, a solid sheet of clear stellate cells was present, and these were again trypsinized and an additional cell culture made (subculture 2). Again, in another 3 days, the bottle surface was completely covered with elongated, contiguous cells, many of which were in division. From these cells, continuous subcultures were made. Since this time, the cells have been continuously subcultured as described, with the time of subculturing varying from 7 to 14 to 21 days. At present, the cells are in their thirty-third subculture. A total of 20 ml. of packed centrifuged cells has been collected from this single retinal culture. Although the growth rate is slow, it is significant that the cell population doubles every 10 to 14 days. For example, for an inoculum of 0.025 ml. (1.5 x 10⁶ cells) in 60 ml. of medium, after 14 days' incubation, 2.5 x 10⁶ cells were collected.

Other cattle retinas obtained at different periods of time, as well as the cells from the visual cortex of the brain are now being cultured. These also show the same morphological cell types and growth. During this 14 month period, observations of the living retinal cells (Figs. 1, 2, and 9), as well as many histologic preparations (Figs. 3-8), have been made.

The cultures can be maintained in the same vessel for long periods of time (6 months is the oldest culture to date) and the cells show no degenerative changes, but continue to grow until at least a two-layered sheet is formed. The oldest fixed and stained material was grown for 98 days on coverslips, with a weekly change of medium (Figs. 8 and 9).

At least three sizes (diameters) of cells can be seen in this continuous cell line; large (< 100 μ), medium (< 30 to 60 μ), which predominate, and small (< 10 to 20 μ). The nuclei are oval or slightly rounded and contain 2 to 9 nucleoli (Figs. 2 and 3). Many fat droplets surround the nuclei and the cytoplasm is extensive and full of tiny granules (Figs. 1, 2, and 3).

At the end of the second week, and always during the third week of cultivation in the same vessel, small bulbous bodies appear in the cytoplasm of some of the cells. From these bodies, fiber-like processes appear, which eventually interlace the culture (Figs. 3 and 8). In very old preparations, there is a network of this material which seems to have its origin in areas where the cells are close together. From these areas the "fibers" grow out and surround either single cells or small groups of cells (Figs. 8 and 9). The older the culture, the thicker this material becomes, and random swellings develop along the "fibers" (Fig. 9). These fibrous structures have appeared in all of the subcultures to date. They resemble the supporting fibers illustrated by Last.

The fixatives and stains used so far identify these fibers as protein, e.g., they stain yellow with galloycyanin or with naphthol yellow after Carnoy fixation. With other fixatives and stains such as Bouin and hematoxylin and eosin, the cytoplasmic material stains red and the fibers blue. Some nerve cells and fibers have an affinity for methylene blue and (in 95 per cent alcohol and methylene blue) the cytoplasmic material and the fibers are stained blue (Figs. 3, 4, and 5). Osmic acid (OsO₄) vapor, which is a fixative for lipids, stains the cytoplasmic material black and the fibers

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*Difco Corporation, Detroit, Mich.
Fig. 1. Living cells, 2 day culture. From subculture 10. (×275.)
Fig. 2. Living cells, 3 day culture. From subculture 26, phase contrast. (×325.)
Fig. 3. Cells fixed in 95 per cent alcohol and stained with methylene blue; 15 day culture. From subculture 4. (×330.)

Fig. 4. Cell in division, fixed in 95 per cent alcohol and stained with methylene blue; 28 day culture. From subculture 7. (×650.)

Fig. 5. Cell in division, fixed in 95 per cent alcohol and stained with methylene blue; 7 day culture. From subculture 5. (×650.)
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Fig. 6. Cells fixed and stained with osmic acid (OsO₄) vapors; 37 day culture. Subculture 13. Note the many dendritic-like processes. (x145.)

Fig. 7. A cell fixed in 10 per cent formalin and with Bodian silver stain; 28 day culture. Subculture 9. (x1,350.)

Fig. 8. Cells fixed with Carnoy and gallocyanin stain; 98 day culture. Subculture 10. Note the long interconnecting fibers. (x220.)

Fig. 9. Living cells, phase contrast; 98 day culture. Subculture 10. Note the highly refractile fibers. (x205.)
pale gray to black (Fig. 6). Using formalin and Bodian's silver stain, the cytoplasmic material stains black, and only dots of silver outline the fibers (Fig. 7). With formalin and Mallory's phosphotungstic acid hematoxylin, the cytoplasmic material is unstained, but in some places along the fibers there is a faint purple color. The cytoplasmic material and the fibers are unstained with formalin fixation and Sudan black B.

Discussion

It is difficult to compare these cultured cells with histological sections of the intact nervous cell layers of the retina. In many respects these cells resemble the illustrations in the papers of Liss and Wolter and Geiger. Recently, Bodian discussed the confused terminology which characterizes certain descriptions of the "typical" or generalized neuron.

The ganglion layer of the retina contains glia cells which act as connective and supporting tissues. There are different types of glia cells. For example, there are the fibers of Müller, Golgi's spider cells, astrocytes, and microglia. The fibers of Müller are long, narrow, complicated structures, which pass through the whole thickness of the retina from the internal to the external limiting membranes. Golgi's spider cells are small glial cells with a round or oval nucleus and numerous cytoplasmic processes. Astrocytes or star cells are found at random throughout the ganglion cell layer; pigment granules are also to be found in these cells. Microglia are phagocytic, wandering cells, and may be round, oval, or rod-shaped.

The point here is that these tissue-cultured cells can be continuously grown for long terms (14 months to date) and that mitotic divisions can be seen in the very young as well as in the oldest cultures. The differences seem to be that as the cells age, they become more closely packed and that the "fiber-like" material becomes thicker and more extensive. It is significant that the cells have not lost the potential for producing the peculiar "fibers" after 14 months in culture and after so many trypsinizations.

In addition the outer segments of the retinal rods do not completely degenerate and have been observed after 28 days in culture is also of interest.

An important question then is: Do these cells structurally fulfill the electrophysiological requirements? If so, they should respond to light. Rushton indicates that these should be large cell bodies of the order of 30 to 50 μ, with branches that spread in all directions in a circle of at least 1 mm. The large and the more numerous medium sized cells that we observe fulfill the size requirement. These cells could be the neurons with their dendritic processes. The smaller cells are probably a mixture of glia type cells. It has been suggested that such neuroglia cells transfer energy rich compounds (adenosine triphosphate) to the nerve cells. Lasansky employing the electron microscope, indicates a morphologic association of neuroglia and neurons in the toad retina. Therefore, it is not surprising that these two types of collaborating cells grow simultaneously in our cultures. The examination of different cell types of the nervous system in respect to cell behavior, which can be expected because of the differences in histologic types, becomes then, increasingly important.

Since these retinal cells can now be grown in quantity for analysis, it is possible, and therefore necessary for us to know whether they react to light, what function they play in energy transfer, and their other physicochemical properties, which at this time are still unknown.

REFERENCES

10. Last, R. J.: In Wolff's Anatomy of the eye and orbit, ed. 5, Fig. 124, Philadelphia, 1961, W. B. Saunders Company, p. 96, Fig. 124.