Long Term Cultures of Neural Retina and Pigment Epithelium from Newborn Rabbits

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Z. Naturforsch. **38 c**, 141 – 145 (1983); received August 3/November 3, 1982

Long Term Cultures, Neural Retina, Pigment Epithelium

In vitro cultures of neural retina, obtained after dispersion and trypsinization of tissue fragments, were composed of 3 morphologically distinct types of neural cells, as demonstrated by silver impregnation. They resembled ganglion or receptor cells, horizontal or amacrine cells, and bipolar cells of the intact retina. Pigment epithelium was cultured without trypsinization. Both kinds of techniques may prove helpful for long term experiments in neurobiology and neurovirology.

Introduction

Retinopathies caused by neurotropic viruses, which often grow slowly and have the ability to persist, are the subject of considerable interest [1]. In Borna Disease virus infection [2], for example, a multifocal retinopathy with vasculitis and pigment epithelitis in rabbits and primates has been observed and analysed, following experimental animal infection [3–5]. Before *in vivo* investigations can further elucidate such disease processes, *in vitro* studies on the interaction of these neurotropic viruses with retinal cells are necessary. In recent years, knowledge about nerve cell cultures including those of the neural retina has been greatly improved [6] and its application in a variety of scientific fields is widely recognized [7–13]. *In vitro* studies, however, often require establishment of long term cultures to follow morphological, biochemical and, eventually, electrophysiological alterations of persistently infected cells.

In this article conditions are presented for the growth of neural retina and pigment epithelium cells of newborn rabbits, and for the maintenance of such cultures for at least 2 months.

Neural Retina Tissue Cultures

Newborn rabbits were decapitated and the eyes were immediately enuclated under sterile conditions. Under stereomicroscopic conditions the iris was cut out and the lens and vitreous body removed. The neural retina was torn from the pigment epithelium with fine tweezers and cut into small pieces. After two washes with prewarmed PBS, the tissue fragments were treated with 0.25% trypsin at 37 °C for 10 min and then washed with growth medium, consisting of 75% Eagles's Medium (Dulbecco's Modification), 10% fetal calf serum, 10% horse serum, 5% glucose (5 g/100 ml in distilled water) and antibiotics.

After pipetting the retina-suspension five to ten times through a Pasteur pipette, 0.2 ml of the suspension was placed on a collagen-coated cover-slip [14] (22 mm, Matsunami, Japan) in a 35 mm petri dish (NUNC, Denmark). One eye usually provided enough cells to seed three cover slips. Cultures were incubated at 36.5 °C in a 10% CO_2 atmosphere. After 6 days *in vitro*, 0.5 ml of fresh growth medium was added. Five days later, the medium was replaced by another growth medium containing 10 mg/l arabinosylcytosine (Sigma, Germany) [15] which was replenished seven days later by the above growth medium without arabinosylcytosine, and then twice weekly. Observations were routinely made with a reversed microscope. Cultures were fixed and stained with haematoxilin and eosin, or submitted to silver impregnation.

The use of the silver impregnation technique following the procedure given by Sevier and Munger [16] has the advantage in that a controlled development of the blackening and delineation of neurites, especially small fibers, can be achieved. This technique, which is based on the Bielschowsky silver technique has been modified and improved by Richardson [17] and is favorable for the demonstration of neural elements in the peripheral, autonomic and central nervous system [16, 18]. In the procedure reported here, the cover slip grown cell sheets were fixed in 10% neutral buffered formalin and then car-

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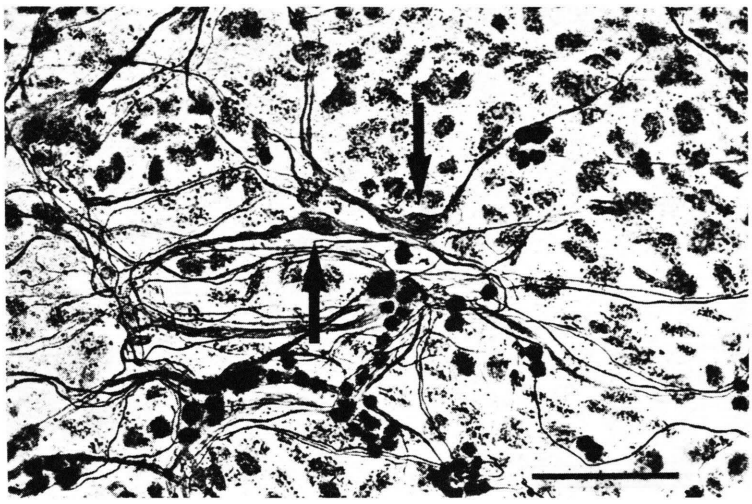

Fig. 1a

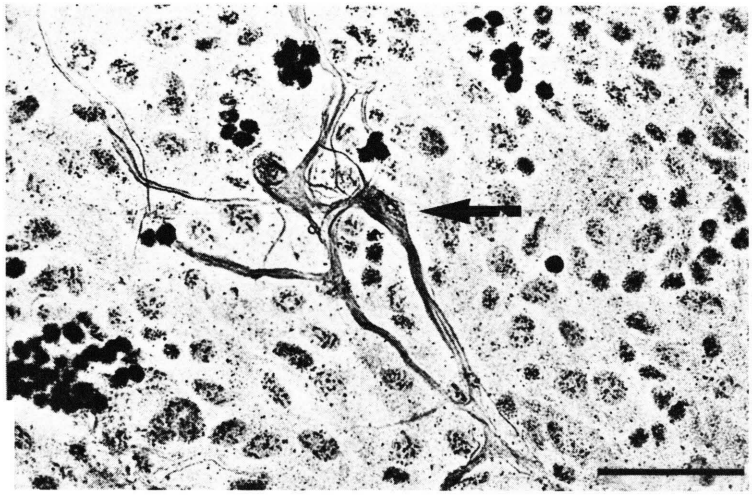

Fig. 1b

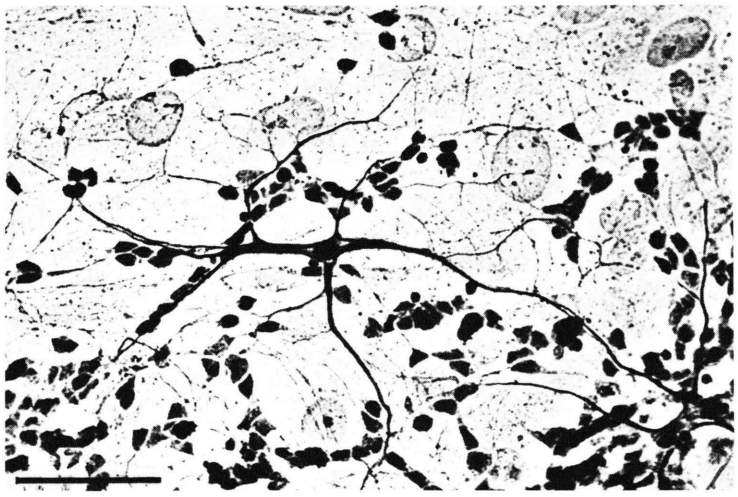

Fig. 1c

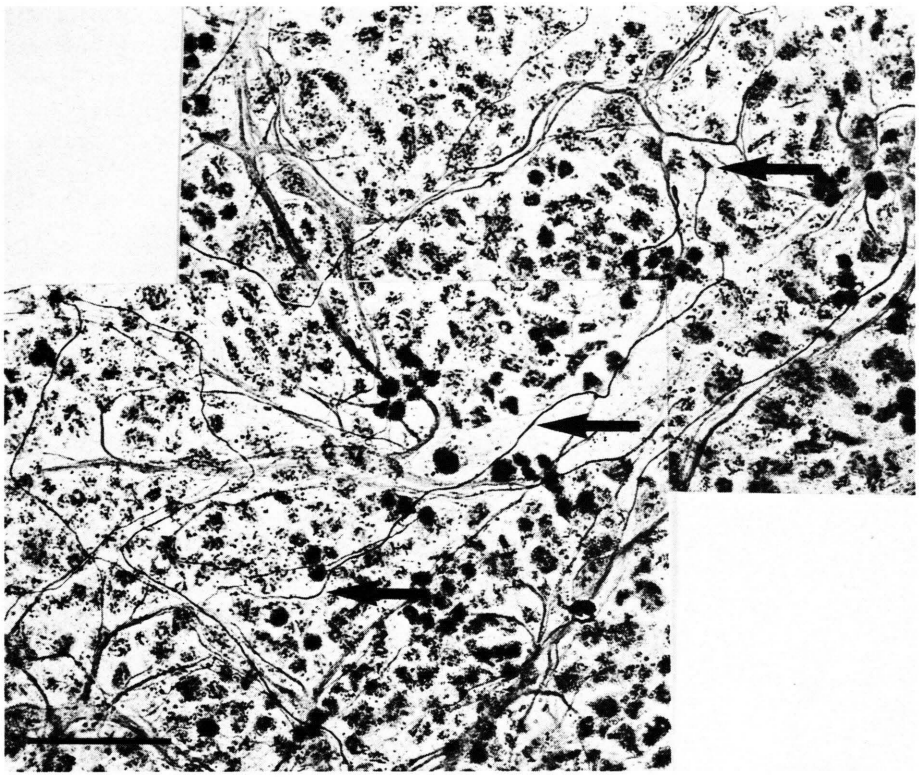

Fig. 1 d

Fig. 1. Neural retina cultures stained by silver impregnation. a) Two neuronal cells (arrows) which are assumed to be ganglion cells; day 55. b) A neuronal cell (arrow) morphologically similar to a receptor cell; day 55. c) A horizontal or amacrine cell; day 22. d) Various bipolar cells with long processes; day 55. One is marked by arrows. Scale: 100 μm.

ried through the impregnation procedure using ammoniacal silver, exactly following the conditions outlined in detail by Sevier and Munger [16].

After several days *in vitro* axons could be seen to extend from the trypsinized neural retina tissues. These tissues are first observed in a multi-cell layer, but after a few days the cells are dispersed on the fibroblasts. The fibroblasts grow much faster than the retinal cells, and thus, serve as a feederlayer for this dispersion process. After approximately 14 days, each kind of neural retina cell can be observed in this network lying on the fibroblasts. These cells can be maintained *in vitro* more than 60 days without any degenerative changes. However, when arabinosylcytosine is eliminated from the medium, neuronal cells degenerate and disappear within 3 to 4 weeks.

In these cultures three predominant kinds of cells are recognized *in situ*, namely, fibroblasts, neuronal cells which have long processes, and small cells with scanty cytoplasms.

Through the use of silver impregnation, neuronal cells and fiber networks become distinguishable, and several kinds of neuronal cells can be grouped based on morphological differences.

Group 1: Gross cell bodies with a large nucleus and bipolar type formation. These cells are speculated to be, in part, ganglion cells, which have long processes (Fig. 1 a) and, in part, receptor cells which are coniform on one side and harbor short processes on the other side (Fig. 1 b).

Group 2: Middle gross cell bodies with multipolar processes which are relatively short. It is probable that these cells represent horizontal or amacrine cells (Fig. 1 c).

Group 3: Cells with scanty cytoplasm of the bipolar type. Their processes are very long. These cells are considered to be bipolar cells (Fig. 1 d). Axons or dendrites of receptor cells, bipolar cells and ganglion cells are known to be short in length within the architecture of the retina, but once the cells are trans-

ferred to *in vitro* conditions they can disperse freely. and seem to acquire the ability to develop and extend their processes.

Pigment Epithelium Cultures [19–21]

After removal of the retina, the pigment epithelium was torn from the sclera as an intact segment. This pigment epithelium monolayer was cut into four pieces, two of which were put on a collagen-coated cover slip, and smoothed out, in sheet-like fashion. The medium was the same as that utilized for the neural retina cultures, but arabinosylcytosine was omitted. Pigment epithelial cells grew significantly in size, but no cell division occurred. For two months, they were well preserved with a honeycomb structure, as *in vivo* (Fig. 2).

Outlook

The neural retina has many kinds of cells, including receptor cells, bipolar cells, ganglion cells, horizontal cells and amacrine cells, which exist as neuronal elements [22]. When neural retina is transferred to an *in vitro* system, the original structure and morphological character of each cell type change, and it becomes difficult to differentiate between them. However, as stated above, several kinds of neuronal cells grow *in vitro* and can be grouped and related to those established *in vivo* on the basis of their morphological similarities. The important goal of this research, was not so much to identify the cells, but to demonstrate that cultured retina cells can sufficiently differentiate and preserve their morphology for a long time *in vitro.*

Although the characterization of individual cells with additional markers or monoclonal antibodies [23–25] awaits further investigation, the above described technique and results yielding successful long term cultures of rabbit retina and pigment epithelial cells should prove very useful for neurobiological and neurovirological studies. These results form the basis for continuing research in this laboratory concerning morphological and functional alterations of retina cells *in vitro*, following persistent infection with conventional slow growing viruses. Preliminary results suggest that such neural retina and pigment epithelium cells can be selectively infected with the neurotropic Borna Disease virus, spread it within the culture and maintain the infection over several weeks (manuscript in preparation).

Acknowledgement

The help and suggestions of Tine Leiskau, Dr. Krey, Dr. Gosztonyi, and Jan Lewis are gratefully acknowledged. The work was supported by the Deutsche Forschungsgemeinschaft (SSP: "Persistierende Virusinfektionen: Molekulare Mechanismen und Pathogenese") and the Alexander von Humboldt-Stiftung.

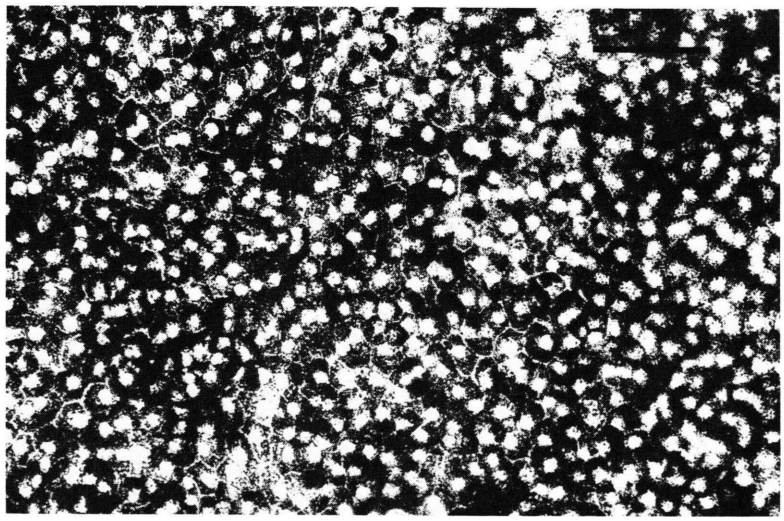

Fig. 2. Pigment epithelium 28 days *in vitro;* no staining. Scale: 100 μm.

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