often difficult to draw clear boundary lines[10]. Thus I personally consider it important that an embryo that has not been transferred on the assumption that it is not normal should be carefully studied by scientific methods. In this way it would be possible to gather knowledge which is essential for deciding the fate of future embryos. This kind of research is already unacceptable for many. For obvious reasons the embryo has until now not been covered by legislation. Its position must therefore be examined and considered from the beginning. Various endeavors have been made to define the beginning of human life, and different conclusions have been reached depending upon outlook and intention. It is certain that the zygote already contains all the genetic information that is needed for an individual with his or her own characteristics and abilities to develop. On the other hand it has to be seen that nature is not squeamish about deciding the fate of normal and abnormal embryos[3].

No manipulation can be permitted which may affect the genome of gametes and embryos obtained for IVF and ET. We still know too little about the structure of our genetic material and have no way of making selective changes in the human genome. So long as this is the case there remains the possibility of transplanting genes to wrong segments so that these would no longer be subject to natural control. This could have fatal consequences in later life. Further, one cannot foresee the consequences of gene manipulation for future generations.

The status of the embryo is not clear. The ways of thinking in society with regard to abortion, birth control, prenatal diagnosis and IVF show that public opinion is only beginning to form. Therefore it is very important that the medical, ethical and other aspects of IVF and ET, and especially their consequences, should be discussed by the general public to promote a better understanding of the methods involved and to ensure that an appropriate discussion and dissemination of information can take place. Scientists and other experts should inform not only their colleagues but also the general public about the value of their procedures and their implications. We must therefore provide each other with explanations of complex situations and each must listen patiently to the views of the other. Only in this way is it possible to find a practicable solution for our society.

1 Angell, R.R., Aitken, R.J., van Look, P.F.A., Lumsden, M.A., and Templeton, A.A., Chromosome abnormalities in human embryos after in vitro fertilization. Nature *303* (1983) 336–338.

2 Beier, H.M., and Lindner, H.R. (eds), Fertilization of the human egg in vitro. Biological basis and clinical application. Springer-Verlag Berlin, Heidelberg, New York, Tokyo 1983.

3 Creasy, M.R., and Alberman, E.D., A cytogenetic study of human spontaneous abortions using banding techniques. Hum. Genet. *31* (1976) 177–196.

4 Edwards, R.G., Steptoe, P.C., and Purdy, J.M., Establishing full-term human pregnancies using cleaving embryos grown in vitro. Br. J. Obstet. Gynec. *87* (1980) 737–756.

5 Jones, H.W., Acosta, A.A., Garcia, J.E., Sandow, B.A., and Veeck, L., On the transfer of conceptuses from oocytes fertilized in vitro. Fert. Steril. *39* (1983) 241–243.

6 Lopata, A., Successes and failures in human in vitro fertilization. Nature *288* (1980) 642–643.

7 Lopata, A., Johnston, I.W., Hoult, J.J., and Speirs A.L., Pregnancy following intauterine implantation of an embryo obtained by in vitro fertilization of a preovulatory egg. Fert. Steril. *33* (1980) 117–120.

8 Müller, Hj., and Brückner, Ch., Richtlinien der Schweizerischen Akademie der medizinischen Wissenschaften über artifizielle Insemination und In-vitro-Fertilisation/Embryotransfer, in: Spermakonservierung, Insemination und In-vitro-Fertilisation. Eds. Ballmann and Schill. Urban und Schwarzenberg, München 1985.

9 Schweizerische Akademie der medizinischen Wissenschaften, Medizinisch-ethische Richtlinien für die In-vitro-Fertilisation und den Embryotransfer zur Behandlung der menschlichen Infertilität. Schweiz. Ärzteztg. *65* (1984) 1416 and *66* (1985) 1127–1129.

10 Thomson, K.S., Research on human embryos: Where to draw the line? Am. Scient. *73* (1985) 187–189.

11 Warnock, M., Report of the committee of inquiry into human fertilization and embryology. Her Majesty's Stationery Office 9314, London 1984.

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Full Papers

Fetal rat brain hemisphere tissue in nonadherent stationary organ culture

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Summary. A simple organ culture system for brain tissue is described. Fragments of fetal rat brain hemisphere tissue are explanted to multiwell dishes base-coated with semisolid agar. In this system nonadherent organ culture can be performed for at least 50 days. Cell migration, biochemical and morphological differentiation and the formation of a layered architecture seem to mimic some of the phenomena occurring in the developing rat brain in vivo. The fragments may therefore be a useful organ culture model for nervous tissue.
Key words. Rat brain fragments; stationary, nonadherent organ culture; cell differentiation and migration.

Introduction

In recent years there has been a rapid development of different modifications of cell and organ culture systems for the maintenance of nervous tissue in vitro for prolonged periods[14,17,24,30].

Basically there are three main types of such culture methods:

1. Dissociated neural cells grown in monolayer cultures[18,35,36,42].
2. Nervous tissue grown as solid pieces adherent to a surface, for example in Maximow double coverslip assemblies and roller tubes[16,34].
3. Nervous tissue reconstructed from single cell suspensions, either in gyratory shakers[12,26,33,41], or in stationary cultures on a nonadherent surface[2].

These culture systems offer numerous possibilities for the investigation of morphological, biochemical and electrophysiological properties of neural cells, and they have been valuable tools in neurobiological research. What has been lacking, however, is a three-dimensional tissue culture system where the histiotypic structure of the tissue is not artificially disturbed either by means of dissociation and reaggregation or by interaction with a substratum. There has also been a need for brain tissue model where cellular migration in the developing brain may be investigated and which can serve as host tissue in studies on interaction between normal and malignant brain cells outside the body.

In this article we present a modified organ culture system which has been especially designed for such studies and which is simple and easy to perform.

Materials and methods

Animals. Rats of the inbred BD IX-strain were used[13].

Fragments. Pregnant rats were subjected to caesarean section under ether anesthesia at the 18th day of gestation.

Whole brains were dissected out from the fetuses and placed in sterile petri dishes containing phosphate-buffered saline (PBS). The brain lobes were dissected free, the meninges carefully removed and the lobe tissue cut into approximate cubes, measuring about 800 µm in all dimensions.

Culture conditions. Dulbecco's modification of Eagle's medium (DMEM, Flow laboratories, Glasgow, Scotland), supplemented with 10% heat-inactivated newborn calf serum, 4 times the prescribed concentration of nonessential amino acids, L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) was used. The fragments were grown in multiwell dishes (Nunc plastic, Denmark) base-coated with 0.5 ml 0.75% semisolid agar (Agar Noble, Difco Laboratories, Detroit, USA). One fragment was transferred to each well and 1 ml of the medium was added. The cultures were maintained at 37 °C in 5% CO_2 in air with 100% relative humidity. They were observed daily under a phase contrast microscope. The medium was changed every second day. The culture period was 50 days and the fixation times were day 0, 1, 2 and 5 and every 5th day thereafter until day 50.

Viability. At the different observation times some fragments were transferred to wells without agar. Cellular outgrowths observed at the bottom of the wells within 24 h were regarded as an expression of viability.

Size measurement. The size of the fragments was measured every second day, from the start of the culture period until day 50. They were measured using a phase contrast microscope with a calibrated grid in one of the oculars. The size was expressed as half the sum of two diameters at right angles to each other.

Light microscopy (LM). Fragments were fixed for 24 h in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer and dehydrated in graded ethanol concentrations up to 100%. After 2 × 10 min in propylenoxide and 12 h in a 1:1 mixture of propylenoxide/Epon 812, the fragments were embedded in pure Epon 812. The final polymerization was carried out at 60 °C for 3 days. 2-µm sections were cut on an LKAB microtome (LKAB, Bromma, Sweden) and stained with toluidine blue. Identification of the different cell types in the brain tissue fragments was done according to the criteria used by Ling et al.[21] and Peters et al.[29].

Scanning electron microscopy (SEM). The procedure for fixation and dehydration was the same as that described for LM. Critical point drying was carried out according to the technique described by Anderson[1], and the fragments were mounted on stubs using tape and silver paint. The specimens were coated with a gold layer, approximately 50 nm thick, in a vacuum evaporator (Polaron E 5000 SEM coating unit, Watford, England), and examined in a Philips SEM 500 microscope. Kodak Tri-X-pan professional film was used.

Transmission electron microscopy (TEM). The procedure for fixation and dehydration was the same as described for LM. Representative fragments from each observation point were cut on an ultra microtome (Reichert ultramicrotome, 'Om U3'). The thin sections were mounted on copper grids and stained with uranyl acetate for 30 min and lead citrate for 8 min. Sections of the fragments were studied under a transmission electron microscope (Philips EM 300) at a magnification of 6200.

Immunohistochemical procedure

Glial fibrillary acidic protein (GFAP). GFAP is considered to be characteristic for astroglia, and is connected to the intermediary filaments[11]. GFAP antibodies were kindly supplied by Elisabeth Bock, The Protein Laboratory, University of Copenhagen, Denmark. GFAP antigen was prepared from white substance in normal human brain[9]. Antisera against human GFAP were produced in rabbits and tested for specificity as described elsewhere[27]. Immunization and isolation from pooled antisera were performed according to standard procedures[19].

Neuron specific enolase (NSE: 14.3.2). NSE is an isoenzyme of enolase specific for neurons[8]. Polyclonal antibodies were kindly supplied by Kenneth Haglid, University of Gothenburg, Sweden. They were produced in rabbits and purified as described by Moore[25]. Further details are given elsewhere[3,4].

Indirect immunoperoxidase technique. Activity of GFAP and NSE was tested on 6-µm paraffin-embedded sections from fragments at the time of explantation and after 20

and 40 days in culture by the indirect immunoperoxidase technique. The sections were rehydrated in toluene (2 × 5 min) and 96% ethanol (4 min). They were incubated for 15 min in methanol with H_2O_2 (3 ml 35% H_2O_2 in 200 ml methanol) and for 2 min in 96% and 70% ethanol and then washed for 3 × 10 min in Tris-HCl buffer (pH = 7.4, 2.5 M NaCl) with 0.01% non-IDET added. Incubation with antisera diluted in Tris-HCl buffer (GFAP 1:1000, NSE 1:2000) was performed in moist chambers at 4 °C. The incubation time was 48 h. Serum from non-immunized rabbits was used as negative control. The sections were then washed for 3 × 5 min in Tris-HCl buffer, incubated for 30 min with peroxidase labeled swine anti-rabbit IgG (Dako, Denmark) diluted in Tris-HCl buffer and normal swine serum (Dako, Denmark) (0.1 ml immunoglobulin + 5 ml buffer + 1 ml swine serum) and washed again for 3 × 5 min in buffer. After 9.5 min in a solution of 75 mg 3'3-diaminobenzidine tetrahydrocloride (DAB, Sigma, USA) in 150 ml buffer without NaCl and 10 min in a similar mixture with 75 µl 35% H_2O_2 added, the peroxidase formed the brown insoluble reaction product which identified cells as neurons or astrocytes depending on the antibody employed. The sections were serially mounted. Every other section was labeled with GFAP and NSE antibodies, respectively.

Results

General. The fragments behaved in a constant manner. All the reported findings are based on verification of the same phenomena in at least 5 separate fragments. For LM morphology we used 8–12 fragments at each observation point. They came from 5–8 separate experiments, each comprising one litter of 3–8 fetuses. For SEM and TEM we used 5–7 fragments on each observation day, where 2–3 were from one litter of fetuses. Immunohistochemical investigation was done on 6 fragments at each stage, and they came from 2–3 different experiments.

Phase contrast microscopy. The shape of the fragments changed from cubic to spheric. This process started after a few hours in culture, and after 5 days most of the fragments were perfectly spherical. This shape was retained during the rest of the culture period (see section on SEM).

There was also a considerable decrease in the size of the fragments. The greatest decrease occurred between day 0 and day 20. The size reduction was 65% during this period. The decrease between day 20 and day 50 was only 2.5% (fig. 1). In all cases the fragments reached a steady state with diameter about 300 µm (fig. 1). This was independent of the initial size of the fragments in the range between 600 and 1200 µm.

Light- and transmission electron microscopy. The following description is based on light microscopy of the architecture and cell identification by TEM: *Day O:* Sections at this stage showed brain lobes with the four zones described by the Boulder Committee[40]. The cortical plate could also be identified. The great majoritiy of the cells were undifferentiated. Some cells in the cortical plate, however, had the characteristic morphology of neurons. Macroglial cells could not be identified (fig. 2). Mitoses were found in all sections examined. Their number varied from 1 to 5 per 0.5 mm^2 section, and they were situated in the ventricular and subventricular zones (fig. 2).

Day 1–day 2: The most characteristic feature at this stage was the rounding process. The sections showed an expansion of the outer parts of the fragments. This event was not discernible in the ventricular zone, but increased with increasing distance outwards. The eventual result was a spheroid where ventricular and subventricular cells and neurons constituted the center, and intermediate and marginal cells the periphery. Along with this, there was a change in cellular organization. The 5 layers observed at day 0 could no longer be demonstrated. Instead, most of the fragments at this stage were two-layered structures. The central layer had a high cellular density, especially in the periphery, and contained both the most mature and the most immature cells in the fragment. Young neurons were present. Numerous mitoses were also observed, most of them peripherally, in this inner zone. The outer layer was totally dominated by cells with small amounts of cytoplasm and granular nuclei. They were larger than the more centrally located, dark nucleated, cells. A few neuron-like cells were also seen in this outer area. Macroglia, macrophages or microglia were not observed.

Day 5–day 10: At this stage it was not possible to see separate layers. Signs of cellular organization could, however, be observed (fig. 3). The majority of the most

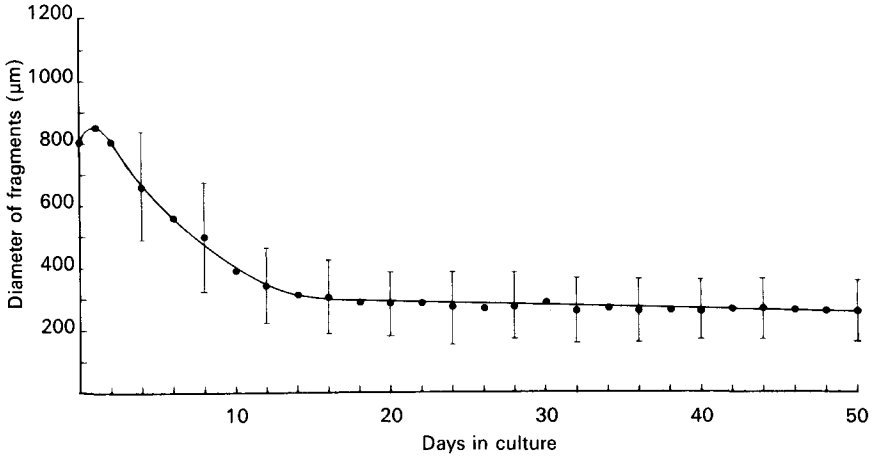


Figure 1. Mean diameter of brain fragments at the different stages of culture. Each point is the mean of 90–108 separate fragments from altogether 4 replicate experiments ± SE. For practical reasons SE is only given for every 4th observation point.

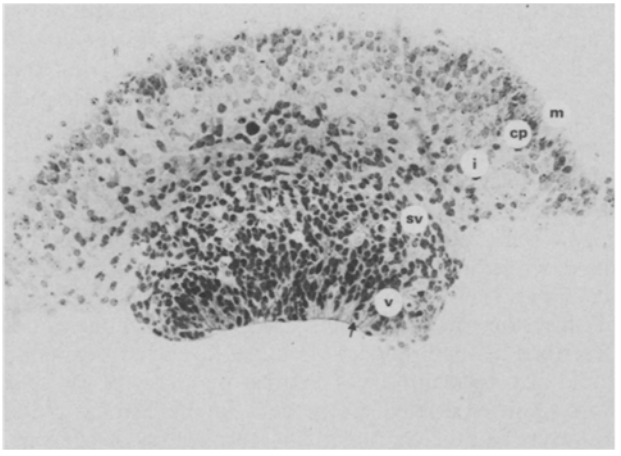


Figure 2. Fragment at day 0, i.e. at the time of explantation, showing the characteristic five layers of fetal rat brain cortex. Abbreviations are: v, ventricular zone; sv, subventricular zone; i, intermediate zone; cp, cortical plate; m, marginal zone; arrow, mitosis. Semithin section. Toluidine blue stain, × 208.

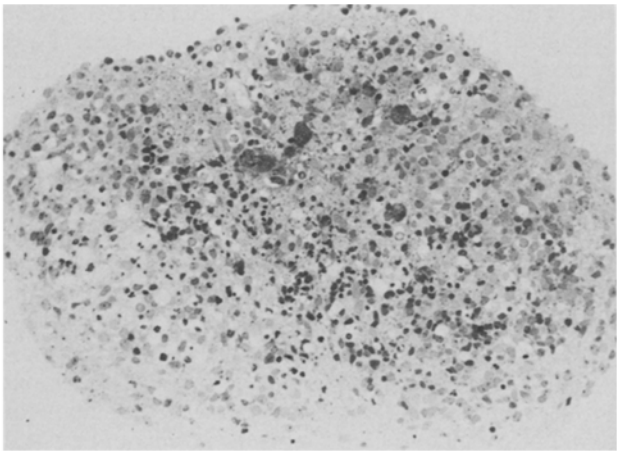


Figure 3. Fragment after 10 days in culture showing no typical layers. Semithin section. Toluidine blue stain, × 208.

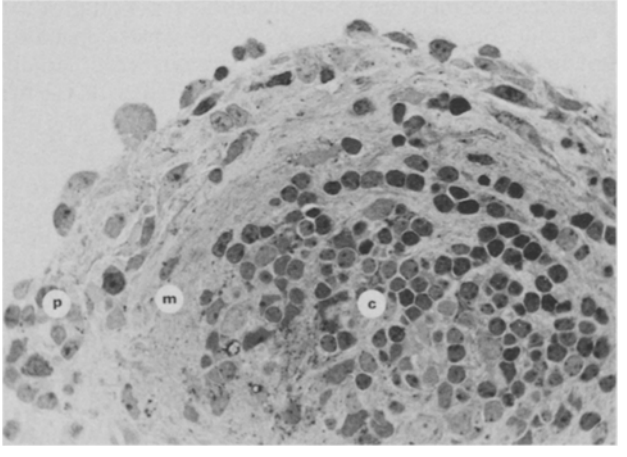


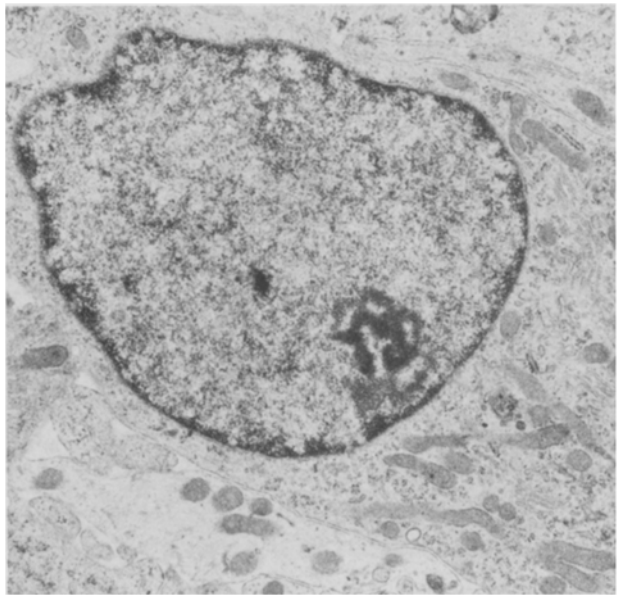
Figure 4. Fragment after 35 days in culture showing the reappearance of defined layers. Abbreviations are: c, central layer; m, middle layer; p, peripheral layer. Semithin section. Toluidine blue stain, × 524.

centrally located cells were neurons. Among them were smaller cells with little cytoplasm and dark, granular nuclei. Their incidence increased towards the periphery where they constituted the dominant cell type. Single neurons were observed at some distance from the center, but not in the most peripheral parts of the fragment. Macroglia appeared during this period. They were situated in the peripheral parts of the fragments. Mitoses were observed in all sections examined, 1–2 per 0.5 mm^2. Macrophages also appeared during this period. They were polychromatic with lipid droplets and granula in their cytoplasm. Their number varied considerably, and they could be found anywhere in the fragment. Microglia were observed in a majority of the sections without any specific location. Numerous necrotic cells were observed in all sections examined. They were randomly distributed all over the fragments.

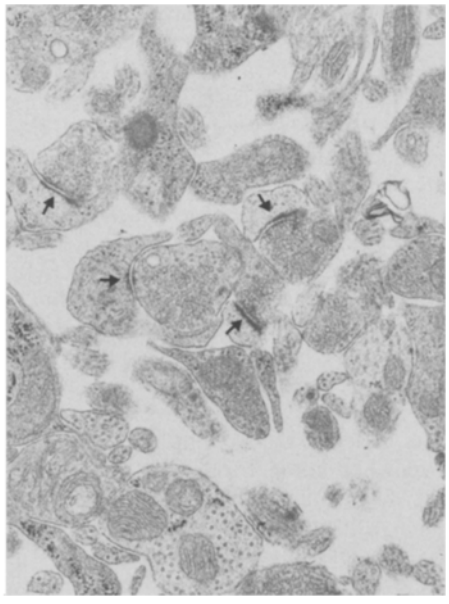
Day 15–day 20: A three-layered structure now appeared in the spheroids (fig. 4). Centrally, neurons and astrocytes were in the majority. A few oligodendrocytes could be observed among them. The layer in the middle mainly consisted of fibers of various thicknesses that surrounded the center of the fragment. By serial sections it could be demonstrated that cellular outgrowths both from the central and peripheral layers intermingled with them. Some cell bodies of neurons and macroglia were also present in this middle layer. In the peripheral layer, macroglial cells, especially oligodendrocytes, dominated. Some scattered neurons were, however, also observed. The structure of this outer zone was looser than the rest of the fragment. A general tendency in the cell-rich areas was an increasing distance between the individual cell nuclei. This seemed to be due to two factors; the increasing amount of cytoplasm and the increasing number of cellular outgrowths. From now on the area without cell bodies, both between the individual cells and between the inner and outer layer, will be referred to as neuropil. Mitoses were present in about half the sections examined, never more than one per 0.5 mm^2 section. They were both peripherally and centrally located. Microglia and macrophages were observed only in a minority of the sections.

Day 20–day 40: The three-layered structure developed between days 15 and 20 lasted all through this period (fig. 4). Some minor changes, however, took place. The number of neurons in the outer layer increased. Large neurons became situated just beneath the surface of the fragments. Macroglial cells were, however, still the most numerous in this area. In the middle layer there were a few cell bodies, both neurons and macroglia. The main constituent was, however, still the neuropil, rich in fibers of various dimensions. The center of the fragments, totally encircled by the middle layer, mainly consisted of neurons and astrocytes. A few mitoses were found at all the different stages in this period. They did not have any specific location. This was also the case with macrophages and microglia, which were present in a few sections. In this period, the number of necrotic cells was reduced compared to earlier stages. Such cells were, however, still observed in all sections examined.

Day 40–day 50: A reorganization of the fragments took place, and separate layers could not be observed any



5a



5b

Figure 5a. Neuron with numerous mitochondria, R.E.R. cisterns and an abundant Golgi apparatus. The nucleus is large with one well defined nucleolus and without chromatin aggregates. 10-day-old fragment, TEM, × 13,200.

Figure 5b. Synapses (arrows) in the center of a 35-day-old fragment. TEM, × 19,200.

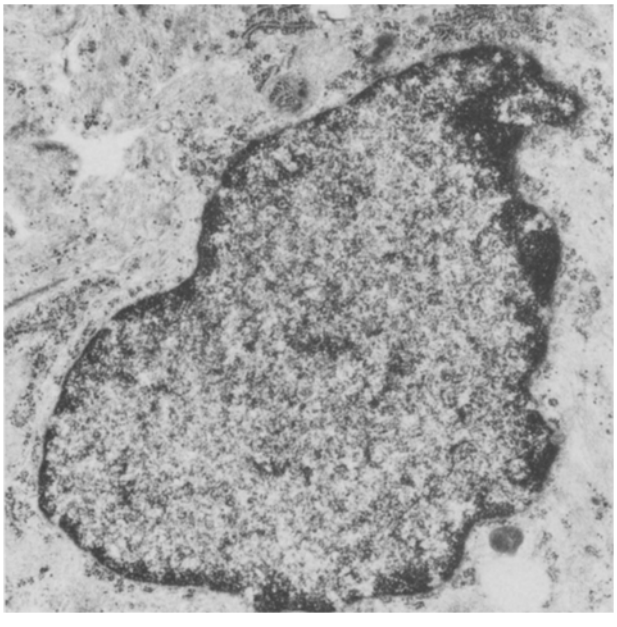


Figure 6. Astrocyte with intermediate filaments and no glycogen granulae in the sparse cytoplasm. Chromatin densities are seen along the nuclear membrane. 45-day-old fragment, TEM, × 21,500.

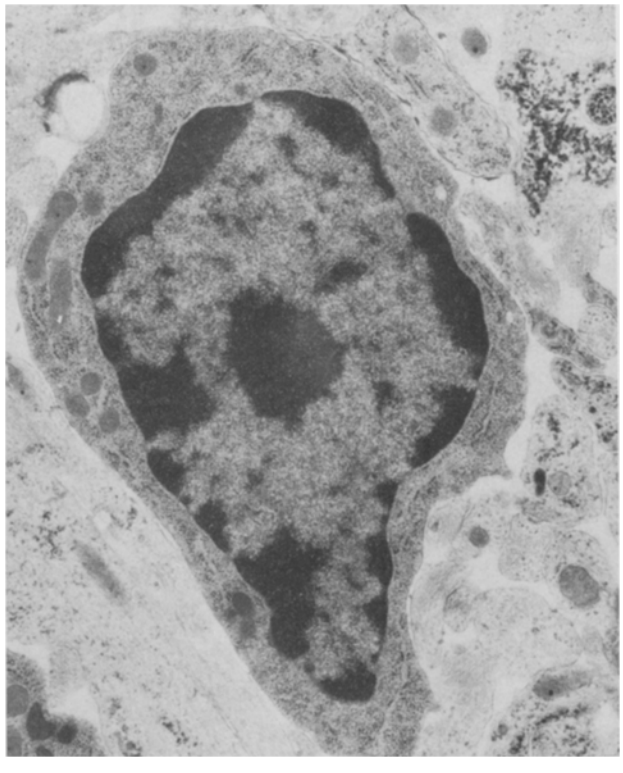


Figure 7. Dark oligodendrocyte in the periphery of a 20-day-old fragment. It is characterized by a nucleus containing large clumps of chromatin and dark cytoplasm. TEM, × 15,500.

more. Neuropil became the main constituent. The cells present, mainly neurons and astrocytes, were spread all over the fragments. Peripherally, astrocytes dominated. The mitotic activity now ceased, and mitoses were not observed at the end of the culture period. No increase in cell death was observed. Examples of ultrastructurally differentiated neural cells and synapses are shown in figures 5–7.

Observations by SEM. During the rounding of the explanted cubes two phenomena occurred: Cells from the pial side of the fragments (fig. 8) moved toward the ependymal surface and eventually covered the structures after 5 days in culture. The fragments curved around a point on the ependymal surface. After two days this gave a convex structure with a 'crater' on one side. After 5 days

this 'crater' was obliterated and a spherical structure appeared. Between day 5 and day 20 the number of pial cells at the surface of the fragments was gradually reduced. During the last 30 days larger cells with extensively branched outgrowths gradually covered the spheroids (fig. 9). Macrophages were seen singly or in clusters, often associated with cell debris.

Observations by immunohistochemistry. GFAP. At the time of explantation, day 0, no GFAP-activity was observed in any of the fragments investigated. After 20 days, numerous GFAP-positive cells were seen in all specimens (fig. 10). In 5 of the 6 fragments most of the labeled cells were peripherally located. In the sixth, these cells were more randomly distributed all over the spheroids. A network of positive processes was seen throughout the fragments. After 40 days the number and position of the labeled cells had not changed.

NSE. At day 0, no NSE-positive cells were observed. After 20 days in culture, several labeled cells were present in all the fragments investigated (fig. 10, inserted). Except for one specimen, in which two positive cells were seen in the periphery, all of them were centrally located.

After 40 days peripherally situated labeled cells were present in all fragments. Most of the peroxidase positive elements were, however, still centrally located.

Viability. At every observation time 2 fragments were transferred to multiwell dishes without agar. Adherence to the bottom and outgrowth of identifiable neural cells were always observed. This was taken as a marker of viability although no quantitation of outgrowth was performed. Otherwise the following features were taken as signs of viability:

- Morphological differentiation into neurons, astrocytes and oligodendrocytes which were not present in the original explants.
- Cell migration and organization in layered structures.
- No overt tissue necrosis, although some vacuolization and death of single cells was observed.
- Production of nervous system specific proteins which were not identifiable at the time of explantation.

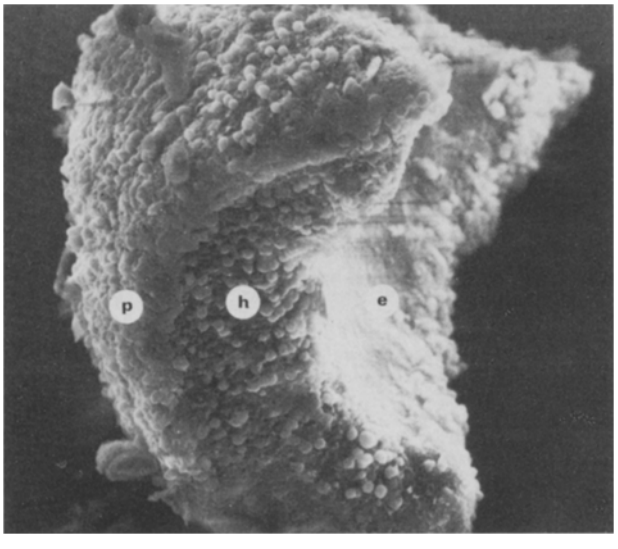


Figure 8. Fragment at day 0 showing the pial and the ependymal surfaces and the hemispheric tissue in between. Abbreviations are: p, pial surface; h, hemispheric tissue; e, ependymal surface. SEM, × 200.

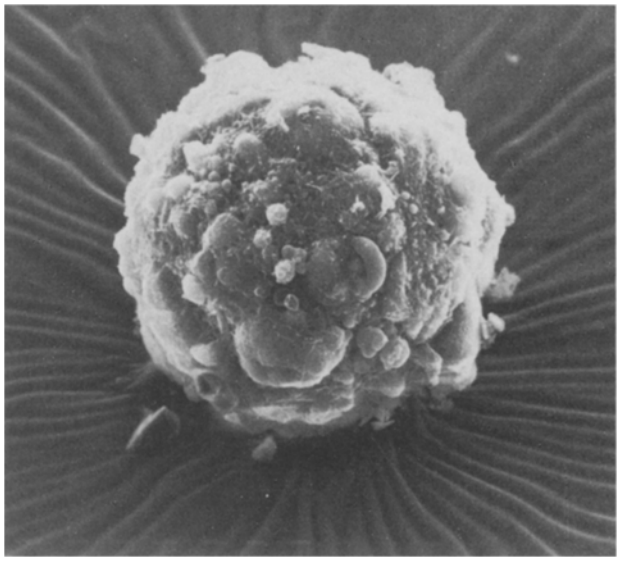


Figure 9. Fragment at day 50 showing a surface dominated by large cells. SEM, × 220.

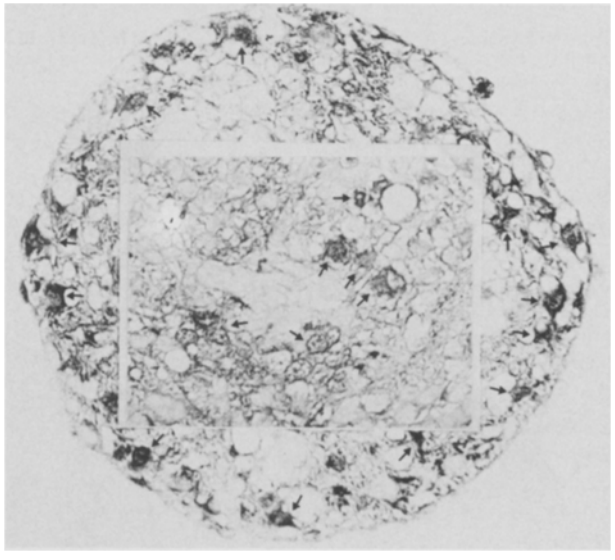


Figure 10. Peroxidase stained sections from a 20-day-old fragment. Main picture: Section incubated with GFAP showing numerous labeled astrocytes (arrows) peripherally situated in the section, × 310. Inserted picture: Central area of the subsequent section incubated with NSE, showing labeled neurons (arrows). × 285.

Discussion

A general question when working with tissue culture is to what extent the observed phenomena correspond to events occurring in vivo, rather than being due to the artificial culture conditions.

Cell migration was observed in the brain fragments. During the first days in culture, pial cells gradually covered the explants. Furthermore, neurons moved towards the periphery of the spheroids between day 20 and day 40. The initial expansion of the outer parts of the fragments was similar to that occurring in the normally developing brain cortex. The marginal zone is known to expand considerably faster than the ventricular layer. This is due to cell migration from the ventricular area outwards

along curved pathways and a parallel increase in cell volume[20].

At the time of explantation, the only morphologically differentiated cells were neurons, but neuron specific enolase (NSE) was not yet present. The first macroglial cells appeared after approximately 5 days, and after 20–25 days most of the cells in the fragments could be identified as differentiated neural cells both morphologically and by immunohistochemical methods. This is in accordance with the corresponding time-dependent events in vivo in pre- and postnatal rats[5, 8, 10]. It may therefore seem that the brain tissue fragments grown in the culture system described here mimicked some of the events taking place in the developing rat brain in vivo, including morphological differentiation, production of nervous system specific marker proteins, cell migration and formation of a layered structure. On the other hand, some of the features observed in the cultured brain tissue did not correspond to the in vivo situation. There was a gradual shrinkage of the fragments from a mean diameter of 800 µm to stabilization at a diameter of 300 µm after 20 days. This was accompanied by the observation of scattered pyknotic cells, in conformity with a cell loss. While a mature brain lobe has 6 different zones, only 3 such zones could be recognizied after 20 days of organ culture. Eventually, after 40 days, this layered organization was lost. So far, we cannot explain this.

Another difference was the assembly of large neurons in the periphery of the fragments seen in the later stage of culture. This event may, however, be an in vitro counterpart to the outward migration of neurons from the ventricular zone which is known to occur in vivo.

Another important question in organ culture is to what extent cell viability is preserved, and to what extent there is a real cell proliferation instead of a slowly occurring cell death.

In our set-up, morphological signs of single cell death were observed through most of the culture period. The incidence of dying cells did, however, decrease as the fragments reached their permanent size of about 300 µm. In this connection it is generally known that primary explantation to organ culture is accompanied by increased cell death. It should also be remembered that single cell death is a naturally occurring phenomenon in all developing tissues, and that tissue size reduction will automatically occur if this is not compensated for by a corresponding number of mitoses[30]. In the brain fragments the mitotic activity ceased after 40 days in culture. This also occurs in postnatal rat brains in vivo and at the corresponding time[32].

In the present experiments an average initial explant diameter of 800 µm was used for practical reasons. As exponentially growing spheroids of human glioma cells reach this size[6], there is a proliferation gradient from the periphery to the center. With such diameters the central O_2 tension may be at a minimum[7]. Usually there is an upper limit of 400 µm diameter for oxygenation of tissue in vitro[15]. Furthermore, above this size, static cultures show less oxygen transport as compared to tumor aggregates in spinner flasks[28]. It was therefore interesting that the shrinkage of the brain fragments seemed to be independent of the initial diameter in the range between 1200 and 600 µm, resulting in a stable size of 300 µm. This is well within the limit for sufficient oxygenation.

Many features of the brain tissue fragments described here have also been observed in neural tissue grown in other culture systems. Differentiation of single cells takes place in monolayers as well as in other three-dimensional cultures[2, 12, 18, 23, 26, 33–36, 41, 42]. Cellular organization by segregation of similar cells in groups has also been reported[2, 33, 41]. However, it appears that neural tissue grown in this set-up remains viable and organized for a longer period than other non-adherent cultures[21, 41].

The described brain fragments may then provide the possibility of investigating cellular differentiation, migration and organization in developing brain tissue in a model where the original histiotypic structure is not initially disturbed and where there is no interaction between the tissue and an artificial substratum. In addition this standardized and stable structure ist of particular value in the study of interactions between normal and malignant brain tissue in organ culture[37, 38, 39]. Although invasiveness is known as one of the most critical properties for malignant behavior of primary brain tumors, very little is known about the biology of this process[22]. There is therefore a need for methods where glioma cells can be directly observed during invasion into their natural target tissue outside the body. The culture system described here has already proved valuable for such purposes[37–39].

We conclude that solid hemisphere fragments grown in a nonadherent stationary organ culture system may be a valuable supplement to existing culture methods for the investigation of phenomena in the developing brain and may be applicable as host tissue in a study of the interaction between normal brain tissue and malignant brain tumors outside the body.

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1 Anderson, T.F., Techniques for preservation of three-dimensional structure in preparing specimens for electron microscopy. Trans. N.Y. Acad. Sci. B (1951) 130–133.
2 Bjerkvig, R., Steinsvåg, S.K., and Laerum, O.D., Reaggregation of fetal rat brain cells in a stationary culture system. In Vitro, in press.
3 Bock, E., and Dissing, J., Demonstration of enolase activity connected to the brain-specific protein 14, 3, 2. Scand. J. Immun. *4*, suppl. 2 (1975) 31–36.
4 Bock, E., Fletcher, L., Rider C.C., and Taylor, C.B., The nature of the two proteins of brain specific antigen 14, 3, 2. J. Neurochem. *30* (1978) 181–185.
5 Caley, D.W., and Maxwell, D.S., Electron microscopic study of neurons during postnatal development of the rat cerebral cortex. J. comp. Neurol. *133* (1968) 17–44.
6 Carlsson, J., and Brunk, U., The fine structure of three-dimensional colonies of human glioma cells in agarose culture. Acta path. microbiol. scand. A *85* (1977) 183–192.
7 Carlsson, J., Stålnacke, C.-G., Acker, H., Hajl-Karim, M., Nilsson, S., and Larsson, B., The influence of oxygen on viability and proliferation in cellular spheroids. Int. J. Radiat. Oncol. Biol. Phys. *6* (1979) 2011–2030.
8 Cicero, T.J., Cowan, W.M., Moore, B.W., and Suntzeff, V., The cellular localization of the brain specific proteins S-100 and 14, 3, 2. Brain Res. *18* (1970) 25–34.
9 Dahl, D., Glial fibrillary acidic protein from bovine rat brain. Degradation in tissue and homogenates. Biochim. biophys. Acta *420* (1976) 142–154.
10 Das, G.D., Gliogenesis and ependymogenesis during embryonic development of the rat. J. neur. Sci. *43* (1979) 193–204.

11 DeArmond, S.J., Eng, L.F., and Rubinstein, L.J., The application of glial fibrillary acidic (GFA) protein immunohistochemistry in neurooncology. A progress report. Path. Res. Pract. *168* (1980) 374–394.
12 DeLong, R.G., Histogenesis of fetal mouse isocortex and hippocampus in reaggregating cell cultures. Devl Biol. *22* (1970) 563–583.
13 Druckrey, H., Genotypes and phenotypes of ten inbred strains of BD-rats. Arzneimittel-Forsch. *21* (1971) 1274–1278.
14 Federoff, S., and Hertz, L., Cell Tissue and Organ Culture in Neurobiology. Academic Press, New York 1978.
15 Folkman, J., and Hochberg, M., Self-regulation of growth in three dimensions. J exp. Med. *138* (1973) 745–753.
16 Gähwiler, B.H. Organotypic monolayer cultures of nervous tissue. J. neurosci. Meth. *4* (1981) 329–342.
17 Giacobini, E., Vernadakis, A., and Shahr, A., Tissue Culture in Neurobiology. Raven Press, New York 1980.
18 Godfrey, E.W., Nelson, P.G., Schreier, B.K., Breuer, A.C., and Randsom, B.R., Neurons from fetal rat brains in a new culture system: A multidisciplinary analysis. Brain Res. *90* (1975) 1–21.
19 Harboe, M., and Ingild, A., Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immun. *2*, suppl. 1 (1973) 161–164.
20 Hicks, S.I., and Damato, C.J., Cell migration to the isocortex in the rat. Anat. Rec. *160* (1968) 619–634.
21 Ling, E.A., Paterson, J.A., Privat, A., Mori, S., and Leblond, D.P., Investigation of glial cells in semithin sections. I. Identification of glial cells in the brain of young rats. J. comp. Neurol. *149* (1973) 43–71.
22 Laerum, O.D., Bjerkvig, R., and Steinsvåg, S., Invasiveness of primary brain tumors. Cancer metastasis Rev. *3* (1984) 223–236.
23 Laerum, O.D., Steinsvåg, S., and Bjerkvig, R., Cell and organ culture of the central nervous system: Recent developments and current applications. Acta neurol. scand., in press (1985).
24 Lumsden, C.E., Nervous tissue in culture, in: The Structure and Function of Nervous Tissue, vol. 1, pp. 67–104. Ed. Bourne. Academic Press, New York 1968.
25 Moore, B.W., Chemistry and biology of two proteins, S-100 and 14, 3, 2, specific to the nervous system. Int. Rev. Neurobiol. *15* (1972) 215–225.
26 Moscona, A., Rotation mediated histogenetic aggregation of dissociated cells. Exp. Cell Res. *22* (1961) 455–475.
27 Møller, M., Ingild, A., and Bock, E., Immunohistochemical demonstration of S-100 protein and GFA protein in inerstitial cells of rat pineal gland. Brain Res. *140* (1978) 1–13.
28 Mueller-Klieser, W.F., and Sutherland, R.M., Influence of convention in the growth medium on oxygen tensions in multicellular tumor spheroids. Cancer Res. *42* (1982) 237–242.
29 Peters, A., Palay, S.L., and Webster, H. de F., The fine structure of the nervous system. The neurons and supporting cells. W.B. Saunders Company. Philadelphia–London–Toronto 1976.
30 Sato, G., Tissue Culture of the Nervous System. Plenum Press, New York 1973.

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Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*

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Summary. The predominant host-selective toxin from *Cochliobolus victoriae*, victorin C, is a peptide with an apparent mol. wt of 796, representing a cyclic array of the subunits **1**–**6**. The structure of the toxin has now been established as in **16** through analysis of the degradation products generated by enzymic and non-enzymic partial hydrolysis. The presence of a hydrated aldehydo group requires for victorin C the composition $C_{31}H_{45}O_{13}N_6Cl_3$ with an amended mol. wt of 814, for which independent experimental support has been secured.
Key words. Blight of oats; *Helminthosporium victoriae;* phytotoxins; NMR-spectra; structural elucidation; unusual oligopeptides.

The causal agent of victoria blight of oats, *Cochliobolus victoriae* Nelson produces a host-specific toxin called victorin[2]. Victoria blight of oats became a major disease about 40 years ago as a consequence of the introduction of commercial varieties of oats carrying the Pc gene for resistance to crown rust, *Puccinia coronata*[3,4]. It was later discovered that rust resistance and susceptibility to *C. victoriae* and its toxin were either closely linked or controlled by the same genetic locus. Subsequent studies on the inheritance of susceptibility to *C. victoriae* have shown that sensitivity to the fungus and its toxin, victorin, is controlled by a single dominant gene with the homozygous dominant genotype conferring sensitivity to the toxin and the homozygous recessive genotype conditioning insensitivity[5]. Thus, victorin appears to be a gene-specific toxin affecting only those genotypes of oats that carry the dominant allele for susceptibility to *C. victoriae*. In a previous communication[6] we reported on the isolation of several homogeneous host-selective toxins from the culture filtrate of *Cochliobolus victoriae* Nelson (*Helminthosporium victoriae* Meehan and Murphy)[7] and, in addition, have shown that the most abundant compound, victorin C, (apparent mol. wt 796, corresponding to $C_{31}H_{43}O_{12}N_6Cl_3$) must represent a cyclic combination of the components glyoxylic acid, **1**, 5,5-dichloroleucine (Cl_2leu), **2**, *erythro*-β-hydroxyleucine (OHleu), **3**, victalanine, **4**, *threo*-β-hydroxylysine (OHlys), **5**, and α-amino-β-chloroacrylic acid (aClaa), **6**. We now outline new evidence which has led to the derivation of a complete structure for victorin C, thus establishing its correct composition as $C_{31}H_{45}O_{13}N_6Cl_3$ (mol. wt 814).

Materials and methods

Acid hydrolyses. a) 40 mg of victorin C were incubated in 10 ml of 6N HCl at r.t. for 21.5 h. The sample was dried in vacuo and redissolved in water, a small portion was injected onto an analytical reverse phase C_{18} column (Wa-