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Biology of Neural Cells in Culture: A Review

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= Abstract =Neural tissue grown and maintained *in vitro* provides an opportunity to study some *in situ* properties of neurons and glial cells under relatively simple and carefully controlled conditions. To the extent that a given neural preparation retains properties which approach those exhibited by the intact brain, tissue (cell) culture provides an important model for the nervous system, and thus represents a potentially crucial step in system reduction, bringing us close to the mechanistic explication, at the molecular level, of neurobiological functions.

The term "tissue culture" has been used to denote the *in vitro* cultivation and maintenance of living biological units from multicellular organisms. The techniques which have been developed for this purpose fall into four broad classes:

Organotypic (explant) cultures, which are explanted from specific neuroanatomic loci to substrates as small tissue fragments.

Dissociated cell cultures, which involve the seeding of enzymatically or mechanically dispersed cells on various attachment substrates.

Reaggregate cultures, which require re-association of dissociated cells into small aggregates.

Purified cell populations, which are prepared by the isolation of different cell types by gradient centrifugation or other separation techniques.

Key Words : ***Biology, Neural cells, Culture***

INTRODUCTION

Since Harrison's (1907) success in maintaining *in vitro* embryonic frog spinal cord almost 80 years ago, explant cultures of neural tissue have been the method of choice in the preparation of neural tissue cultures. Until mid 1960's, explant cultures were widely used to study basic neural cytology and to analyze the dynamics of cell movement and nerve fiber growth. Excellent accounts of such studies have been documented by Murray (1965) and Lumsden (1968a).

The attempt, in an *explant* culture system, is to culture small fragments of tissue which will maintain the cytoarchitecture, simulate differentiation and maturation patterns, and preserve the functions of original tissue. Most of the important structural and functional attributes of neurons and glial cells have been found to be maintained in culture that include the formation of synapses, the appearance of normal myelin, the rise in enzyme activity associated with neurotransmitter metabolism, the increase in myelin-specific enzymes and the development of the bioelectric activity characteristic of interneuronal communication. The vast literature dealing with explant cultures of nervous tissue has previously been reviewed by Murray (1971), Nelson (1975) and Crain (1976).

Moscona and Moscona (1952) introduced the enzymatic dissociation technique which made possible preparation of viable single cell suspensions from embryonic neural tissue, and Nakai (1956) provided the earliest descriptions of cultures grown from dissociated chick embryo dorsal root ganglia. Since the introduction of the *dissociated cell culture* method by these authors, studies of mechanically or enzymatically dispersed cells from various regions of the nervous system permitted the careful analysis of interactions between neurons by morphological and neurophysiological techniques.

Several cell type specific markers are now available which make it possible to identify major cell types in dissociated cell cultures of mammalian and avian neural tissues (Weiner and Hauser 1982;

Kim 1985). Galactocerebroside has been shown to be expressed by oligodendrocytes. The glial fibrillary acidic protein (GFAP) is present only in protoplasmic and fibrous astrocytes. The neurofilament triplet proteins are shown to be specific for cultured neurons of both central and peripheral nervous tissues.

Fibroblasts are shown to bind anti-Thyl and anti--fibronectin. These cell type-specific markers have been used effectively in identifying different cell types in dissociated cell cultures of nervous tissue.

Reaggregate cultures, in which neural tissue, dissociated into single cells are allowed to reassemble themselves under controlled conditions, have proved useful in studying the transition from a unicellular to a multicellular system. Histotypic aggregation often occurs and organotypic structures may be recognized even in mixed cultures of various tissues and cell types. There is electron microscopic evidence of synaptogenesis in these aggregate cultures (Seeds and Vatter 1971). In reaggregate cultures cells can not be visualized under direct observation and the morphological examination requires time-consuming and tedious routine histologic methods.

Cultures of *purified cell populations* have recently been recognized as an important new development in the field of neural tissue culture. Recently there have been reports of the cultures of neuron-enriched population (Mains and Patterson 1973a; McCarthy and Patlow 1976), astrocytes (McCarthy and de Vellis 1980; Kim *et al.* 1983a), oligodendrocytes (Szuchet *et al.* 1980; Lisak *et al.* 1981; Gebicke-Harter *et al.* 1981; Kim *et al.* 1983b) and Schwann cells (Wood 1976; Brockes *et al.* 1979; Kreider *et al.* 1981). These culture systems will undoubtedly contribute to a better understanding of morphological, functional and metabolic properties of individual cell types and of neuron-glia interactions.

Each of the culture systems described has some obvious advantages and disadvantages. Therefore, the choice should be made by each investigator with regard to the best fitting model system for one's projected experimental needs.

All of these culture systems are considered to be *primary cultures* because they are started from material obtained directly from an organism, without a subpassage. The inherent inability of normal neurons to divide continually, not only makes the maintenance of neural cultures a true challenge, but also means that these cultures which do survive, will grow, mature, differentiate, reach senescence and die within the course of 3-6 months and certainly survive no longer than a year. While primary cultures cannot divide indefinitely, and will therefore die, *clonal cell lines* are by definition "immortal". Their source is usually neoplastic tissue either explanted from *in situ* neural tumors and subcultured, or from cell lines cloned from chemically, virally or spontaneously transformed cells.

The culture systems described above are often considered *cell cultures,* a classification to denote attempts to grow cells of a homogenous type in contradistinction to organ explants wherein heterotypy is viewed as an opportunity to study organotypic cellular interactions.

One further distinction to be preserved when defining culture systems is based on duration of *in vitro* existence. While short-term incubation methods (24 hours or less) have been commonly used in biochemical experiments and in *in vitro* neurophysiological procedures, these do *not* qualify as culture systems (Fedoroff 1967).

ORGANOTYPIC (EXPLANT) CULTURES

To observe the outgrowth of nerve fibers from individual central neurons, Harrison (1907) developed a technique whereby explants of tadpole spinal cord were embedded in lymph clots on coverslips, inverted and sealed over depression slides (hanging drop position). In demonstrating nerve fiber outgrowth, he contributed significantly to confirmation of his and Cajal's neuronal theory of the structure of the nervous system, and in successfully maintaining explanted embryonic frog spinal cord *in vitro*, Harrison became at once both the father of tissue culture in general and nervous tissue culture in particular.

While a chronicle of developments in tissue culture methodology is beyond the scope of this article, a bit of historical perspective is enlightening. The saga of obtaining successful nerve tissue cultures, one of ardous trial-and-error, focused on a search for a physical and biochemical milieu which optimized the performace in culture of non-deviding cells with finite life spans. The problem was compounded however, by the likelihood that different cell types, or the same cell types at different developmental stages, may require different optimal conditions.

To continue our introduction to neural tissue culture, we will focus in more detail with the methods, advantages and limitations of each of the culture systems.

For the past 80 years, since Harrison's success

in maintaining *in vitro* embryonic frog spinal cord (Harrison 1907), explantation of neural tissue has been the method of choice in the preparation of neural cultures. The years between 1910 to the early 1960's witnessed the extensive use of explant cultures to study basic neural cytology and to analyze the dynamics of cell movement and nerve fiber growth.

The attempt, in explant culture systems, is to cultivate a piece of tissue which will maintain the cytoarchitecture, simulate the differentiation and maturation pattern, and preserve the functions of that tissue. And, indeed, most of the important structural and functional attributes of neurons have been found to be maintained in culture:

Formation of synaptic connections in explant cultures (Kim 1963; Kim 1965; Wolf 1965; Hild 1966; Callas and Hild 1964; Bunge *et al.* 1965; Bunge *et al.* 1967; Guillery *et al.* 1968; James and Tresman 1969; Kim 1970; Seil and Herndon 1970; Pappas *et al.* 1971; Kim and Wenger 1972; Privat *et al.* 1974), the development of the characteristic bioelectric patterns which bespeak interneuronal communication (Crain 1966; Robbins and Yonezawa 1971; Schlapfer *et al.* 1972), the rise in synaptic enzymes (Kim and Johnson 1972; Kim and Tunnicliff 1974), the appearance of normal myelin (Peterson and Murray 1955; Hild 1957; Bornstein and Murray 1958; Ross *et al.* 1962; Field *et al.* 1968; Kim 1971) with a concomitant rise in myelin-associated and myelin-specific enzymes (Silberberg *et al.* 1972; Fry *et al.* 1973; Chida and Shimizu 1973; Pleasure and Kim 1976a,b; Sheffield and Kim 1976), have all been found to mimic the time course of *in vivo* pattern. Extracellular and intracellular microelectrode recording from neurons of central and peripheral neural tissue cultures demonstrated characteristic normal-appearing neuronal electrical excitability in response to stimulation, and electrophysiological studies on co-cultures of muscle and neurons have further demonstrated that functional neuromuscular junctions may form *in vitro* (Crain 1976; Robbins and Yonezawa 1971).

The seemingly increasing ability of explant cultures to reproduce more and more faithfully, the *in vivo* properties of the nervous system has emerged hand-in-hand with the greater facility of experiments to deal with the fastidious requirements of neural tissue. For a detailed discussion of such specific culture lab methods as glassware preparation, sterilization techniques, media preparation, culture incubation, etc., the reader is referred to Willmer (1965), Schneider (1973), Paul (1975), and Schlapfer (1977), Freshney (1983), all virtual handbooks in tissue culture, which should certainly be perused by any one considering employing the method.

A distillation of some of the informative "how-to" data shows some of the requirements of successful explants to be:

1) Optimum age of tissue. Embryonic or new born tissues are most frequently used with the best results obtained when the tissue is explanted before the specific region being studied has completed its maturation (e.g., mouse cerebellum, 1-3 days old; mouse neocortex, 1-5 days old; mouse eyeball and retina, 13-16 days old fetus). Explants of mature tissue frequently lose many of their components and become cell cultures of surviving fibroblasts.

2) Optimum size of explant. The explant must be small enough to allow diffusion of nutrients and oxygen to all parts of the tissue in the absence of a vascular system, yet large enough to leave a maximum number of cells undamaged or with recoverable damage. A total volume of at most $1mm^3$, with one dimension not greater than 1 mm, has been found optimal.

3) Sufficient oxygenation of all parts of the tissue, insured by allowing only a thin film of nutrient medium to cover the culture.

4) As long an interval between media changes as is feasible, since cultured nervous tissue seems to benefit from "self-conditioning" of its medium.

5) Optimal incubation temperature. Nervous tissue explants do well in the temperature range of 35-37°C.

6) Suitable substrate for cell attachment. Nervous tissue does not adhere well to uncoated glass. Harrison in his initial experiment embedded tissue in lymph clots. Subsequent experiments witnessed the use of plasma clots, ultimately on coverslips, as support substrates (Pomerat and Costero 1956), however, these lasted no longer than 2 weeks. Bornstein (1958) found that coverslips coated with reconstituted rat tail collagen (Ehrmann and Gey 1956) provided a more stable substrate for cultures while eliminating the possibility that the unknown composition of the plasma clot may interfere with experimental procedures. Cells have also been grown on gelatin (denatured collagen)-coated (Luduena 1973) or on polylysine-coated coverslips (Yavin and Yavin 1974).

7) Appropriate nutrient medium. The various

concoctions which have been used to feed cultures of different origins must suggest a bit of witchcraft to new investigators, and indeed, have been based, on an odd combination of empiricism and superstition.

In Harrison's preparation, the embryonic cells probably provided their own nourishment in a drop of lymph fluid during the course of observation. Lewis and Lewis (1912) grew chick intestinal wall sympathetic ganglia embedded in plasma clot for up to 12 days in saline alone. The plasma clot probably provided the needed nutrients. Media was ultimately imporved by including various biological preparations such as serum and embryonic extract. Pomerat and Costero (1956) added chick embryo extract and neoplastic ascitic fluid to their medum. Pomerat and Costero (1956) also supplemented their medium with 3 mg/ml glucose and subsequent workers found that 6 mg/ml glucose or more was necessary for maintenance and optimum differentiation of myelinating mammalian peripheral and central nervous tissues. Glucose concentrations of 5-10 mg/ml are usually used. Balanced salt solution formulations employing bicarbonate buffer systems have been used successfully. Some labs add antibiotics (e.g. penicillin, streptomycin, and gentamicin) while others try scrupulously to avoid the addition of such extraneous material.

Presently, most culture media are composed of balanced salt solutions with mixtures of amino acids, vitamins, high concentrations of glucose and supplemented with heat-inactivated sera (human placental cord, horse, fetal calf or various other fetal, newborn or adult animal sera), or tissue extracts (embryo extracts, brain extracts) in various combinations.

Previously, Sato and his associates have succeeded in growing various cell lines in serum free synthetic media supplemented with several hormones and growth factors (Barnes and Sato 1980). These investigators demonstrated that one of the main functions of serum is to provide a mixture of hormones and/or growth factors which stimulate cell growth. Recently there have been several reports using this approach to develop serum-free chemically defined medium specifically designed for neurons and glial cells in which these cells could be grown in a completely defined microenvironment (Snyder and Kim 1979; Snyder and Kim 1980; Bottenstein *et al.* 1980; Morrison and de Vellis 1981; Messer *et al.* 1981; Romijn *et al.* 1982).

8) Workable vehicle. The method of choice for any explant depends on the demands of the experiment in terms of time consumption and accessibility. Harrison in his landmark 1907 experiment, introduced the hanging drop technique in which embryonic tissue was placed in hanging drop of lymph, however, since feeding medium could not be provided for explants nor could sterility be easily maintained, survival was very limited. With the adaptation of the *Maximow double coverslip assembly* by Murray and Stout (1942), a technique which remains the most popular today in some labs for maintaining differentiating organotypic cultures this problem was overcome and survival was extended far beyond 1-2 weeks. A small ($1 \times 1 \times 1$ mm^3) tissue explant, on a small round coverslip, held by capillarity to the center of a larger coverslip, is fed with a drop of medium and covered by an inverted depression slide, and the unit is sealed by paraffin. The culture is incubated in this lying-drop position with the drop of medium forming a thin film over the explant. The culture on its coverslip is readily available for frequent draining, washing, and re-feeding with transfer each time to a new depression-slide assembly. The cultures obtained are well-suited to allow continuous and repeated observation with relatively high power microscopy without disturbance.

Because the concave well configuration of the depression slide presented some optical limitations for phase contrast microscopy (although explants in Maximow slides can be observed by ordinary light microscopy with a x40 long-working distance objective), several other types of slide chambers were developed which had optical surfaces above and below the living tissue. One example which has been used primarily for micro-cinematographic studies of growth and movement of cells has been the *Rose Chamber,* which utilizes two coverslips separated by a rubber gasket and held together by a set of steel plates (Rose *et al.* 1958).

While cultures in slide assemblies (particularly Maximow) probably represent the most sophisticated long-term system (Peterson and Murray 1955), the cost and effort involved in producing large numbers of cultures can sometimes be prohibitive: feeding of cultures is such a time consuming task that rapid introduction of experimental solutions is limited, while the risk of contamination during the relatively frequent media changes is enhanced. These considerations have spawned attempts at simplifying culture techniques while still obtaining adequate differentiation, maturation and

survival for meaningful experimentation:

Petri dish methods, in which small quantities of medium form a liquid film over collagen-coated, explant-bearing coverslips, placed at the bottom of a closed petri dish and incubated in a humidified atmosphere with 5% CO_2, are designed to somewhat simplify yet simulate the Maximow technique in terms of serial observation with light microscopy. Cerebellar explants from newborn rat cultured in this manner do indeed develop well-matured neurons and abundant myelin (Bird and Lieberman 1976). Some attempts have been made to grow nervous tissue explants (or dissociated cells) on rat tail collagen coated plastic coverslips (12 or 22 mm diameter) placed in a petri dish, which would make such cultures easily accessible to electrophysiological probing and microscopic observatjion (Kim, unpublished). Recently, Bunge and Wood (1973) have developed small molded fluoroplastic *minidishes.* These dishes are coated with collagen which serves as substrate for explants grown in a small volume of medium and maintained in a CO_2 incubator.

Pomerat and Costero's (1956) use of the *roller tube method* marked the introduction of what is probably today's easiest method for maintaining large quantities of long-term cultures while still yielding a degree of maturation and differentiation comparable to that obtained from the Maximow technique. Explants, on small rectangular (usually 11×22 mm) collagen-coated coverslips, are incubated at 36°C., in test tubes which are tilted at 5 degrees with respect to the horizontal axis confining the culture medium (as much as 0.7 ml) to the bottom of the tubes. For the first 24 hours, culture tubes are maintained in a static position such that the explant is covered with a thin film of medium near a liquid-air interface, allowing the explant to attach itself firmly to the collagen substrate. Subsequently, the tube is transferred for the remainder of its *in vitro* existence to a rotating drum (12 revolutions/hour) whose rolling action affords repeated draining, aeration and refeeding of the cultures. The ease of maintenance is additionally enhanced by the size of the vessel which permits a larger amount of medium to be used and thus allows a relatively longer interval between successive feedings (once weekly), minimizing chances for contamination and sparing cultures from the trauma of frequent disturbances. The feeding procedure itself can be done for many culture tubes simultaneously and involves no transfer of explants-simply the emptying and refilling of tubes. After approximately 2 weeks in culture, the 3-dimensional arrangement of the neurons becomes transformed into a nearly 2-dimensional array of nerve cells embedded in a matrix of dendrites, axons, glial cells and glial processes thin enough to allow good visualization with the bright field microscope. The cultures, while in tubes, are inaccessible, however, to continuous undisturbed high power microscopic observation, and must be removed for this purpose from the culture tubes and sterilely transferred to Maximow slides. Following observation, the coverslips may be returned to roller tubes. This relative inaccessibility constitutes the roller tube methods greatest disadvantage. In some labs, *Leighton tubes* (Hauw *et al.* 1972) have been used as culture chambers to minimize this liability. Leighton tubes are special test tubes with an optically flat area at the bottom. While they may be placed onto an inverted microscope, high power observation with phase contrast optics is still not possible.

While the ideal of each method so far discussed is an attempt to achieve some degree of long-term development and maturation comparable to the *in vivo* situation, there are experimental situations where this goal need not be achieved or where, in fact other properties are more importantly enhanced. For example, in studying the mechanisms of nerve fiber growth and elongation or the formation of neuromuscular junctions, and the effects of drugs, hormones, nutrients, and toxins on these processes, culture systems specifically designed to optimize axon growth are more properly used. Maturation of the culture is not necessary, nor is long-term survival.

Explant culture methods have been found to lend themselves emminently well to the study of the development of neural connections (Crain 1976), the development, by virtue of the direct uptake of proteins by explants (Holtzman and Peterson 1969; Birks *et al.* 1972; Bunge 1973), the incorporation by neural explants of a variety of molecules, especially isotopically labeled metabolites (Mains and Patterson 1973a; Chida and Shimizu 1973; Pleasure and Kim 1976a; Hendelman and Bunge 1969; Hösli and Hösli 1976; Sotelo *et al.* 1972) the action of pharmacological agents (Gähwiler 1975; Crain *et al.* 1978), and experimental neurology (Lumsden 1968b; Raine 1973; Kim *et al.* 1970; Kim 1975a,b; Kim 1983; Sato *et al.* 1985). To elaborate a bit on this last use, explant cultures have shown themselves to be a key in the armamentarium of ex-

perimental neurology because they allow environmental manipulations of neural tissues without mediation by the blood-brain-barrier, or interference from extra-neural systems.

The power of tissue explantation, as stated before, lies in the fact that while some damage is inflicted by dissection (cut edges, severed axons), the explant by and large retains its original cellular composition and organotypic relationship among its component cells. Yet, here too lie its weaknesses and limitations. Because the preparation retains a close similarity to the original tissue, the technique yields cultures which are often many cell layers thick. Single cells are not readily visualized nor selectively approachable. Within the explant some cells die and many others migrate out along the attachment surface until the explant becomes flattened and resolved well enough to permit a better view of the cells remaining within its confines. Additionally, the requirement that explant sizes be small makes them poorly suited for biochemical studies.

For these reasons, many investigators have turned to *dissociated cell cultures* where single cells may be better visualized. Most of the general requirements for satisfactory growth and maintenance detailed in this section on explant cultures (e.g. medium, substrate, incubation, temperature, etc.) may be assumed to be applicable to all culture systems except where otherwise stated in the foregoing sections.

DISSOCIATED MONOLAYER CELL CULTURES

In *dissociated cell cultures,* the emphasis is on the *single* cell rather than on a complex system of cells. Moscona and Moscona (1952) introduced the enzymatic dissociation techniques which made possible preparation of viable single cell suspensions from embryonic neural tissue, and Nakai (1956) provided the earliest descriptions of cultures grown from dissociated neural structures (in this case, embryonic chick dorsal root ganglia)

Dissected tissues from central and peripheral nervous system are first cut into small pieces and then dissociated into single cells by enzymatic and mechanical techniques, alone or in combination. The most commonly used enzyme for tissue dissociation is trypsin dissolved in calcium and magnesium-free balanced salt solution. Other enzymes such as pronase and collagenase may also be used. A cell suspension is then obtained by mechanical disruption by gently passing tissue through one or several pore systems including nylon bolting cloth, stainless steel screen or narrow bore pipettes. The dispersed cell suspension is then seeded on an attachment surface where the cells with settle and attach individually. Flat bottomed culture vessels such as petri dishes or flasks have been used, ordinarily not coated with collagen. Sealed bottles may be grown in non-humidified incubators; open vessels (e.g. petri dishes) ordinarily are maintained in humidified 5-10% CO_2 incubators. The cells will begin to undergo shape changes and move along the surface, making contact with other cells and establishing population patterns. A two-dimensional surface arrangement of cells is sought with a minimum of reaggregation allowing for maximal visual resolution of cell morphological details and accessiblility to single cells. The original tissues organization no longer exists and cell-to-cell contacts as they existed *in vivo* are abolished. Intercellular distances are increased. The numerical balances among the different cell types are changed while individual cell types are altered to varying extents depending on the type, source and procedure. Positive identification of isolated cells in culture is very difficult by morphological criteria alone. The cells upon isolation from their source tissues, tend to round up and lose their tissue-characteristic morphology and their context references. Recognition of different cell classes can only be made by immunocytochemical techniques utilizing cell type-specific markers (Weiner and Hauser 1982; Kim 1985). Galactocerebroside and myelin basic protein have been shown to be expressed by oligodendrocytes. The glial fibrillary acidic protein (GFAP) is present only in astrocytes, and the neurofilament triplet proteins and tetanus toxin receptors are shown to be specific for neurons. Fibronectin is also used as a specific marker for fibroblasts. Viability can be evaluated directly by making use of the ability of live cells to exclude certain dyes and differentially counting dye-positive and dye-negative cells in a sample of the dissociate.

Losses in tissue-like attributes may be offset by gains in resolution and independent analysis of different properties. Cells are always accessible to observation. The total accessibility of each cell permits direct, uniform, and immediate interactions between cell and experimental medium constituents. Dynamic changes (Shapes, positions, collective patterns) can be followed by time-lapse

cinematography and bioelectric properties can be examined under visual control.

Non-neural cell elements characteristically cover the surface of the culture dish with a fairly thick, poorly cellular connective tissue layer, neurons then distributing themselves over this sheet with their axons criss-crossing over the connective tissue. Cinematographic time-lapse sequences of the vigorous movementse of the neuronal growth cones were recorded by Nakai (1964) as the neuronal cell processed branched into their extensive networks. Dissociated cell cultures have in fact proven valuable in exploring the mechanisms of neurite extension (Yamada *et al.* 1971; Bray 1973; Bunge 1973).

Scott *et al.* (1969), Okun (1972), Varon and Raiborn (1971) have found good yields of morphologically and electrophysiologically differentiated dorsal root ganglion cells. No spontaneous electrical activity in the form of either action potentials or synaptic potentials was found, but resting membrane potentials on the order of 40-50 mV with complex action potential response patterns to extracellular stimulation were recorded. Chalozonitis *et al.* (1974) reported qualitatively similar findings in sympathetic ganglia. Using intracellular recording techniques, Peacock *et al.* (1973) presented data suggestive of substantial synaptic coupling between co-cultured dorsal root ganglion and spinal cord cells, and between pairs of spinal cord cells.

Nelson and Peacock (1973) offered similar electrophysiological evidence in cerebellar cell cultures for the formation of small circuits of functionally related cells. Anatomical evidence for synapse formation has been found in dissociated cultures of spinal cord (Kim 1972; Meller *et al.* 1969), medulla and cerebrum (Bornstein and Model 1972). Enzymes involved in neurotransmitter metabolism have been demonstrated in dissociated cell cultures by microchemical assay techniques (Werner *et al.* 1971; Schrier 1973). Sympathetic granglion cells, though not dorsal root ganglia, selectively take-up catecholamines *in vitro* (Burdman 1968; England and Goldstein 1969) and the biosynthetic activities leading to catecholamine synthesis have been found to be preserved in dissociated cell cultures of these cells (Mains and Patterson 1973b). In an autoradiographic study using ^{3}H-GABA, an inhibitory transmitter, Lasher (1974) has shown that several neuronal groups in dissociated rat cerebellum culture selectively take up the labelled amino acid. Thus, dissociated cell cultures may represent an excellent model for the study of the control of neurotransmitter biosynthesis.

Shimada and Fischnan (1973) demonstrated that, when cells from dissociated chick embryo spinal cord were co-cultured with dissociated myogenic cells, morphologically differentiated and functional (indicated by synchronous contractions of groups of muscle cells) neuromuscular junctions developed. Electrophysiological evidence of spontaneous and neurally evoked end-plate potentials have corroborated the functional nature of these *in vitro* formed neuromuscular junctions (Fischbach 1972).

Recently, dissociated neuron cultures have been used to study the localization and dynamics of neuronal plasma membrane receptors. The probes used have included lectins (ricin, wheat germ agglutinin) (Gonatas *et al.* 1977), cholera toxin (Joseph *et al.* 1978) and nerve growth factor (Kim *et al.* 1979).

Worthy of note is the development of new methods for the preparation of *pure neuronal cultures.* By incubating dissociated cells in a medium lacking biocarbonate and CO_2, Mains and Patterson (1973a) prepared 90% pure sympathetic neurons. Ninety-nine per cent pure sympathetic neuronal populations were isolated by McCarthy and Partlow (1976) utilizing the differences in cell-to-substrate adhesiveness between neurons and non-neuronal cells. Similarly Dvorak *et al.* (1978) have isolated and cultured neuron enriched population of chick sympathetic ganglion by selective binding of neurons to latex beads coated with α-bungarotoxin. Such pure cell preparations will undoubtedly aid biochemists immensely in their studies.

REAGGREGATE CULTURES

Reaggregate cultures, in which neural tissue, dissociated into its cellular components (by the methods described in the previous section) are permitted to "reassemble" themselves under controlled conditions. These cells have proved useful in studying the transition from a unicellular to a multicellular condition. They provide a means for analyzing the genetic developmental, organotypic and environmental factors which affect the self-assembly phenomenon and the subsequent formation of histotypic differentiated structures. Such study may help unravel the secrets of complex morphogenesis and its disorders.

Moscona's method (1961) is more or less the technique generally adopted today. A cellular suspension (prepared as one might for a monolayer culture) is submitted to gentle rotation in a gyratory shaker, the resultant vortex enhancing the number of cell collisions. Faster rotations would increase the shearing forces and limit the size, though increase the number, of aggregates. The presence of calcium, serum proteins and protein factors, and a temperature of 36-37°C facilitates bond formation following these mechanically induced collisions. The aggregation can be totally disrupted by blocking protein synthesis or by lowering the temperature to 15°C. Once the aggregates are optimally formed (usually in less than 24 hours), the mixture can be transferred to a stationary system and maintained as an *aggregate culture* in chosen medium for several weeks to months. Early on, cells will be seen to migrate within the aggregate (a universal property of animal cells) to assume selective positions and orientations. Histotypic aggregation often occurs and organotypic structures may come to be recognized even in mixed cultures of various tissues and cell types, for, as the different classes sort themselves out, the histotypic reorganization will largely mimic the original tissue architecture. Sorting out reflects differences in the adhesive properties of different cells. By using various combinations of species, organs and developments stages, the biochemistry of morphogenesis and cellular adhesion can be explored, providing a valuable model for studying the developing nervous system *in vitro*.

Indeed, there is electron microscopic evidence of synaptogenesis and myelinogenesis in aggregate cultures (Stefanelli *et al.* 1970; Sheffield and Moscona 1970) with associated time-appropriate increases in enzymes specific to neurotransmitter metabolism (Honegger and Richelson 1977). Trypsin-dissociated hippocampus and isocortex from fetal mice less than 18 days *in utero* and cultured in rotating Erlenmeyer flasks reaggregated into forms resembling the *in vivo* architecture (DeLong 1970). Aggregate cultures from other neural sources which have been studied include retina (Stefanelli *et al.* 1970; Sheffield and Moscona 1970) and cerebellum (DeLong and Sidman 1970).

Admittedly, in aggregate cultures, cells are no more accessible to direct observation and approach than are early explant cultures. Their morphological examination requires the same histological techniques as required by normal tissue. However, by allowing us to examine the histogenetic process itself-its patterns, sequences, and influences-and the cell surface properties relevant to that process, we are permitted to appraise the degree to which such histogenesis depends on the intrinsic properties of the cells themselves. Having thus "re-constructed" an analogue of the original tissue, one may then continue to observe the further development and function of the reorganized neural cells.

PURIFIED CELL POPULATION CULTURES

According to Herschman (1973), a culture of dissociated CNS tissue without prior separation of cell types does not provide much additional information beyond that gained from explant culture studies. The products of density gradient centrifugation and other separation procedures have been viewed as a possible source for bulk amounts of homotypic normal cells for culture. Indeed, purified cell populations that have retained viability may allow one to study important distinctive properties of large numbers of a single cell type *in vitro*, e.g. their survival requirements, morphological and morphodynamic performances, metabolic activities, and the regulatory influences to which individual cell-specific traits may be subjected. Recombined with selected partner populations in deliberately varied proportions, and co-cultured as reaggregates or as monolayers, one might then glean information on heterotypic cell influences and interactions. Previously, Poduslo and McKhann (1977) and Pleasure *et al.* (1977) have isolated oligodendrocytes in bulk from the subcortical white matter of lamb or calf brain and have grown them *in vitro* for up to 24-48 hours. These authors used sucrose density gradient which did not yield viable cell population capable of surviling long-term culture conditons. By using similar procedures but more physiological isolation and gradient procedures, several groups of investgatiors have recently succeeded in obtaining enriched populations of oligodendrocytes from lamb, cow, pig and human brains which could be grown an extended period of time in culture for up to several months (Szuchet *et al.* 1980; Lisak *et al.* 1981; Gebicke-Harter *et al.* 1981; Kim *et al.* 1983). By use of a unit sedimentation procedure, Currie *et al.* (1976) and Campbell *et al.* (1977) have reported the establishment of monolayer cultures of neuron-enriched or glia-enriched populations from 6-12 days old rat or 6 day-old mouse cerebellum.

While cell purification by density gradient procedures is gradually assuming greater importance in

the studies of biochemical and immunological characterization of homotypic cells in culture, other approaches also are available that include the use of differential adhesion and immuno selection procedures. Taking advantage of the differential adhesion properties of viable cells (glia and fibroblasts attach more readily to appropriately selected surfaces than neurons), and of the differential sensitivity of cells to medium composition and gas phase, enriched population of a single cell type can be obtained.

The choice of a meidum and a thorough elimination of any contaminating cell debris has been found essential for minimizing cell breakage. Recently, two groups of investigators have reported long-term cultures of a neuron-enriched population. Mains and Patterson (1973a) succeeded in preparing 90% pure rat sympathetic neurons by incubating dissociated cells in a medium lacking bicarbonate and CO_2. McCarthy and Partlow (1976) cultured 99% pure chick sympathetic neurons utilizing the differences in cell-to-substrate adhesiveness between neurons and non-neuronal cells. Using this principle of differential cell adhesion, McCarthy and DeVellis (1980) have obtained astrocyte or oligodendrocyte-enriched populations from newborn rat brain.

A recent study by Meyer *et al.* (1982) has utilized immune selection procedure by which purified newborn mouse oligodendrocytes were obtained through the binding of these cells to magnetic spheres coated with oligo-specific antibody.

More recently Morrison and De Vellis (1981) and Kim *et al.* (1983a) have obtained astrocyte-enriched populations from newborn rat brain using mitogen supplemented serum-free chemically defined media.

For the isolation of Schwann cells, several methods have been available which include prolonged treatment with mitotic inhibitor (Wood 1976), antibody-mediated cell lysis (Brockes *et al.* 1979), differential cell adhesion (Kreider *et al.* 1981) and culturing in serum-free medium (Manthorpe *et al.* 1980). The common factor in these procedures is to eliminate undesirable fibroblast population so that pure populations of Schwann cells can be obtained. Once pure population of Schwann cells is established, then one can stimulate the cells to propagate by use of growth factors as described by Brockes *et al.* (1979).

CULTURE OF CLONAL CELL LINES

One may turn to the continuously dividing populations of transformed or neoplastic cells (naturally occurring or experimentally induced) which provide a rich source of repetitively reproducing neurons for nutritional, metabolic, morphological, and biochemical studies. The latter method has emerged as the most popular and successful source for clonal cell lines.

Culture methods of human and experimentally induced animal CNS and PNS tumors have included explantation of solid tumors onto plasma or collagen-coated coverslips as well as dissociated culture techniques. Clonal cell cultures offer the same technical advantages as monolayer cultures from normal material in providing single cells of a homotypic nature which are easily accessible to observation and manipulation. The homotypic composition is, in fact, inherent in their very derivation. Additionally, cultures from clonal cell lines seem to overcome the problem of de-differentiation which ultimately plagues many growing monolayer cultures of normal neurons both because the cell division of normal neurons is limited and because such cultures in time often become overgrown by non-functional connective tissue. Not only can tumor cells be readily grown in bulk amounts (glial cell tumors are capable apparently of indefinite propagation whereas normal glia undergo usually no more than 10-17 doublings in culture), but several tumors have been shown to retain at least some differential characteristics of their normal counterparts even when adapted to *in vitro* living.

Clonal cell populations which continue to carry out brain-specific functions in culture have been isolated from functional astrocytomas (Benda *et al.* 1970; Lightbody *et al.* 1970) and neuroblastoma (Augusti-Tocco and Sato 1969; Schubert *et al.* 1969). However, while neuroblastoma in culture could be shown to respond to electrical stimulation and to evidence of functional synaptic inter-relationships between such cells in culture, normal synape has never been demonstrated between mouse neuroblastoma and muscle cells.

Good reviews of the biochemically and electrophysiologically elicited properties of clonal cells in culture are available (Harris and Dennis 1970; Nelson *et al.* 1971).

The successful use of clonal tumor cell cultures, especially for studying the genetic control of specified phenotypes, presupposes (Harrison 1907) the ability to select or induce tumors with the chosen traits and the ability of the tumor to adapt to a cell culture where such traits are retained, and the

availability of adequate biochemical or immunochemical assays for organ-specific functions expressed at the cellular level (Murray 1965). Of course, in appraising any data obtained from such cultures one must always remain cognizant of the aberrant nature of the source material, related, of course, to its neoplastic origin. Detailed findings, therefore, should be compared with whatever related information is available from normal cells in order to verify the extent to which they are compatible and directly applicable to normal expression of a differentiated trait. The substantially abnormal genetic content of the culture material actually constitutes this culture system's major limitation.

Other disadvantages include the considerable instability of the karyotype. To help insure genetic, and hence biochemical, homogeneity, early subcultured cells of a given clonal line are routinely frozen in growth medium (containing 5-10% DMSO) and stored in liquid nitrogen at temperatures that ensure viability for many years. Since after many generations, cells may change genetically by losing chromosomes, after working with a particular clone for 1-2 months, cells of high passage are discarded and early passage cells are removed from the freezer, thawed, and plated.

A final disadvantage in planning to use clonal cell cultures is the very limited choice of available cell lines. Most studies carried out have involved predominantly two neoplastic neural sources: mouse neuroblastoma C1300 and C6 cell strain from rat astrocytoma.

A good review of data obtained from clonal cell cultures is offered by McMorris *et al.* (1973) and Richelson (1976).

CONCLUSIONS

The range of biological phenomena expressed in the above listed preparations parallels the degree of normal tissue organization. The less organized preparations are less reproducible in their behaviour, however, they are often the more accessible to manipulation, observation and assay. Thus, as in any reductionist approach to bilogical systems, neural tissue culture represents a philosophical choice point on the costs-benefit continum. The price of accessibility and manipulability is obvious considerable disruption of the normal cellular environment, with distortion of the *in vivo* topographic and physiological relationships of tissue. The benefits bought, however, constitute a list impressive enough to assure those employing this methodology that the "system" may ultimately be reconstructed much more accurately for having attacked certain phenomena initially in this way.

Tissue culture systems allow:

1) Control of the physical and chemical environment (nutrients, ions, substrates, cofactors, temperature and gas phases);
2) Isolation from the controlling and modifying influences (neural, hormonal, humoral) of other tissues in the body;
3) Direct accessibility of the cells to labelled precursors, metabolic inhibitors, tracers without the interposition of selective barriers (blood-brain barrier) or confounding by non-uniform diffusions;
4) Continuous and direct visual observation of the cells in their living state under a suitable microscope (phase contrast, time lapse, polarizing, interference and fluorescence) permitting study of morphology and cell dynamics;
5) Accessibility of individual cells to exploration with microelectrodes, microbeams and other precision instruments;
6) Recovery of the incubation medium for biochemical analysis; and
7) Rapid and excellent tissue preparation for electron microscopy and other morphological techniques and for histochemical, immunocytochemical and biochemical analysis.

All of these factors are of special significance in the study of nervous tissue, because the nervous system is a complex organ consisting of various functionally and structurally different cell types. Simplification, coupled with the controlled environment, visualization, and accessibility made possible by the techniques of tissue and cell culture, therefore have been and will be one of the productive roads to the study of the function and structure of the nervous system.

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= 국문초록 =

배양신경세포의 생물학적 특성

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신경조직의 시험관적 성장과 유지는 비교적 단순하고 주의깊게 조절된 상태하에서 원위치의 뉴론 또는 신경교세포의 성질을 알아보는 연구에 이용된다. 주어진 신경조직 표본이 정상뇌의 기능에 가까운 성질을 보지할 정도로 조직(또는 세포) 배양은 신경계에서 중요한 연구모델이 되는 것이며 생체계의 축소와 신경생물학적 기능의 분자 생물학적 수준의 기전 규명에도 접근할 수 있게한다.

조직배양이라 함은 다종세포 유기체로부터 살아있는 생물학적 단위를 시험관적으로 배양 유지하는 것이라 하겠다. 이같은 목적으로 개발된 배양기술은 크게 다음의 네가지로 분류될 수 있다.

*Organotypic (explant) cultures*는 특정 신경해부학적 부위의 작은 조직편을 배양하는 것이다.

*Dissociated cell cultures*는 여러가지 기질을 이용하여 효소 또는 기계적으로 분리시킨 세포를 배양하는 것이다.

*Reaggregated cultues*은 해리된 세포들을 작은 응집괴로 재결합시켜 배양하는 것이다.

*Purified cell populations*는 농도 구배 원심 분리법등의 분리기술에 의하여 각기 다른 세포형을 단리하는 것이다.