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## Chapter 1: Introduction

### BACKGROUND

Tissue culture was first devised at the beginning of this century [Harrison, 1907; Carrel, 1912] as a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment. As the name implies, the technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the migration of cells from the tissue fragment, with occasional mitoses in the outgrowth. Since culture of cells from such primary explants of tissue dominated the field for more than 50 years, it is not surprising that the name "tissue culture" has stuck in spite of the fact that most of the explosive expansion in this area since the 1950s has utilized dispersed cell cultures.

Throughout this book the term *tissue culture* is used as the generic term to include organ culture and cell culture. The term *organ culture* will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue *in vivo*. *Cell culture* refers to cultures derived from dispersed cells taken from the original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation. The term *histotypic culture* will imply that cells have been reassociated in some way to recreate a three-dimensional tissue-like structure, e.g., by perfusion and overgrowth of a monolayer, reaggregation in suspension, or infiltration of a three-dimensional matrix such as collagen gel. *Organotypic* will imply the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined reaggregated culture with dermal fibroblasts.

Harrison chose the frog as his source of tissue presumably because it was a cold-blooded animal, and consequently incubation was not required. Furthermore, since tissue regeneration is more common in lower vertebrates, he perhaps felt that growth was more likely to occur than with mammalian tissue. Although his technique may have sparked off a new wave of interest in cultivation of tissue *in vitro*, few later workers were to follow his example in the selection of species. The stimulus from medical science carried future interest into warm-blooded animals, where normal and pathological development are closer to human. The accessibility of different tissues, many of which grew well in culture, made the embryonated hen's egg a favorite choice; but the development of experimental animal husbandry, particularly with genetically pure strains of rodents, brought mammals to the forefront as favorite material. While chick embryo tissue could provide a diversity of cell types in primary culture, rodent tissue had the advantage of producing continuous cell lines [Earle et al., 1943] and a considerable repertoire of transplantable tumors. The development of transgenic mouse technology [Beddington, 1992; Peat et al., 1992], together with the well-established genetic background of the mouse, has added further impetus to the selection of mouse as a favorite species.

The demonstration that human tumors could also give rise to continuous cell lines [e.g., HeLa: Gey et al., 1952] encouraged interest in human tissue, helped later by Hayflick and Moorhead's [1961] classical studies with normal cells of a finite life-span.

For many years the lower vertebrates and the invertebrates have been largely ignored, though unique aspects of their development (tissue regeneration in amphibian metamorphosis in insects) make them attractive systems for the study of the molecular basis of development. More recently the needs of agriculture and pest control have encouraged toxicity and virological studies in insects, and developments in gene technology have suggested that insect cell lines with baculovirus and other vectors may be useful producer cell lines with less requirement for temperature control. The rapidly developing area of fish farming has required more detailed knowledge of normal development and pathogenesis in fish.

In spite of this resurgence of interest, tissue culture of lower vertebrates and the invertebrates remains a specialized area, and the bulk of interest remains in avian and mammalian tissue. This has naturally influenced the development of the art and science of tissue culture, and much of what is described in the ensuing chapters of this book reflect this, as well as my own personal experience. Hence advice on incubation and the physical and biochemical properties of media refers to homiotherms and guidance on the appropriate modification for poikilothermic animals will require recourse to the literature. This is discussed in a little more detail in a later chapter. Many of the basic techniques of asepsis, preparation and sterilization, primary culture, selection and cell separation, quantitation, and so

on, apply equally to poikilotherms and require only minor modification; on the whole the principles remain the same.

The types of investigation that lend themselves particularly to tissue culture are summarized in Figure 1.1: (1) intracellular activity, e.g., the replication and transcription of deoxyribonucleic acid (DNA), protein synthesis, energy metabolism, drug metabolism; (2) intracellular flux, e.g., RNA, translocation of hormone receptor complexes and resultant signal transduction processes, membrane trafficking; (3) environmental interaction, e.g., nutrition, infection, carcinogenesis, drug action, ligand receptor interactions; (4) cell-cell interaction, e.g., embryonic induction, cell population kinetics, cell-cell adhesion, and invasion; (5) cell products and secretion; and (6) genetics, including analysis, manipulation, transformation and immortalization.

insert fig. 1.1

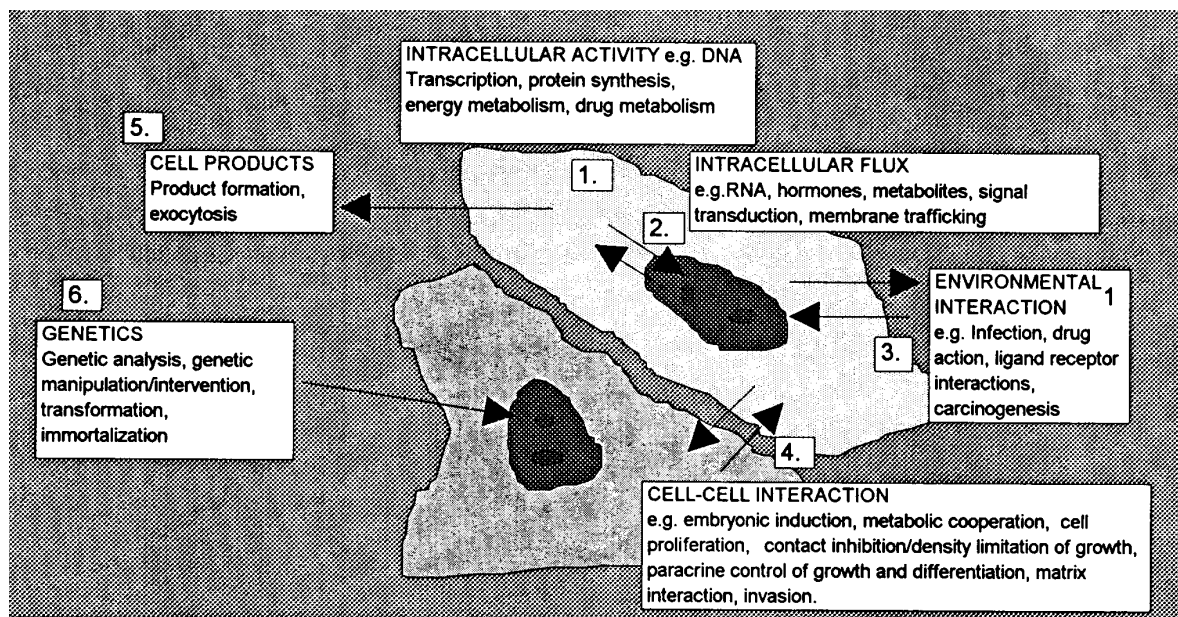


Fig. 1.1. Areas of major interest in tissue culture.

The development of tissue culture as a modern, sophisticated technique owes much to the needs of two major branches of medical research: the production of antiviral vaccines and the understanding of neoplasia. The standardization of conditions and cell lines for the production and assay of viruses undoubtedly provided much impetus to the development of modern tissue culture technology, particularly the production of large numbers of cells suitable for biochemical analysis. This and other technical improvements made possible by the commercial supply of reliable media and sera, and by the greater control of contamination with antibiotics and clean-air equipment, has made tissue culture accessible to a wide range of interests.

An additional force of increasing weight from public opinion has been the expression of concern by many animal-rights groups over the unnecessary use of experimental animals. While most accept that some- requirement for animals will continue for preclinical trials of new pharmaceuticals, there is widespread concern that extensive use of animals for cosmetics development and similar activities may not be morally justifiable. Hence there is an ever-increasing lobby for more *in vitro* assays, the adoption of which only requires their proper validation. While this seemed a distant prospect some years ago, the introduction of more sensitive and more readily performed *in vitro* assays, together with a very real prospect of assaying for inflammation *in vitro*, has promoted an unprecedented expansion in *in vitro* testing.

In addition to cancer research and virology, other areas of research have come to depend heavily on tissue culture techniques. The introduction of cell fusion techniques [Barski, et al., 1961; Sorieul and Ephrussi, 1961; Littlefield, 1964a; Harris and Watkins, 1965] and genetic manipulation [Merril, 1971;

Horst et al., 1975; Maniatis et al., 1978; Sambrook et al., 1989; Frederick et al., 1993 established somatic cell genetics as a major component in the genetic analysis of higher animals, including humans, and contributed greatly, via the monoclonal antibody technique, to the study of immunology, already dependent on cell culture for assay techniques and production of hemopoietic cell lines.

The insight into the mechanism of action of antibodies, and the reciprocal information that this provided about the structure of the epitope, derived from monoclonal antibody techniques [Kohler and Milstein, 1975] was, like the technique of cell fusion itself, a prologue to a whole new field of studies in genetic manipulation. This has supplied much basic information on the control of gene transcription and a vast new technology has grown from the ability to insert exploitable genes into prokaryotic cells. Cell products such as human growth hormone, insulin, and interferon have been genetically engineered, but the absence of posttranscriptional modifications, such as glycosylation, in bacteria suggest that mammalian cells may provide more suitable vehicles. The insertion of the appropriate genes into normal human cells (1) to make them continuous cell lines (see Chapter 2) and (2) to make them produce pharmaceutically viable drugs will have profound effects on the drug industry, which can only be overshadowed by radical innovations in organic chemical synthesis that are, as yet, not apparent.

Other areas of major interest include the study of cell interactions and intracellular control mechanisms in cell differentiation and development [Auerbach and Grobstein, 1958; Cunha, 1984; Jessell and Melton, 1992] and attempts to analyze nervous function [Bornstein and Murray, 1958; Minna et al., 1972; Kingsbury et al., 1985; Snyder et al., 1992]. Progress in neurological research has not had the benefit, however, of working with propagated cell lines, as propagation of neurons has not been possible so far *in vitro* without resorting to the use of transformed cells (see Chapter 15).

Tissue culture technology has also been adopted into many routine applications in medicine and industry. Chromosomal analysis of cells derived from the womb by amniocentesis (see Chapter 23) can reveal genetic disorders in the unborn child, viral infections may be assayed qualitatively and quantitatively on monolayers of appropriate host cells (see Chapter 23), and the toxic effects of pharmaceutical compounds and potential environmental pollutants can be measured in colony-forming and other *in vitro* assays (see Chapter 19).

Further developments in the application of tissue culture to medical problems may follow from the demonstration that cultures of epidermal cells form functionally differentiated sheets in culture [Green et al., 1979] and endothelial cells may form capillaries [Folkman and Haudenschild, 1980], suggesting possibilities in homografting and reconstructive surgery using an individual's own cells [Burt et al., 1989; Gallico, 1990; Dennis, 1992]. It has now become accepted clinical practice in some burn units to biopsy a patient's skin, propagate the cells in culture, and graft the cultured cells back onto the areas of most severe burning [Boyce and Hansbrough, 1988].

With the ability to transfect normal genes into genetically deficient cells, it has become possible to graft such "corrected" cells back into the patient. Transfected cultures of rat bronchial epithelium, carrying the  $\beta$ -gal reporter gene, have been shown to become incorporated into the rat's bronchial lining when introduced as an aerosol into the respiratory tract [Rosenfeld et al., 1992]. Similarly, cultured satellite cells have been shown to be incorporated into wounded rat skeletal muscle, with nuclei from grafted cells appearing in mature, syncytial myotubes [Morgan et al., 1992].

The prospects for implantation of normal cells from adult or fetal, tissue-matched donors, or genetically reconstituted cells from the same patient, are now very real. The technical barriers are steadily being overcome, bringing the ethical questions to the fore. The technical feasibility of implanting normal fetal neurons into patients with Parkinson's disease has been demonstrated; society must now decide to what extent fetal material may be used for this purpose.

Where a patient's own cells can be grown and subjected to genetic reconstitution by transfection of the normal gene, e.g., transfecting the normal insulin gene into P-islet cells cultured from diabetics, or even transfecting other cell types, such as skeletal muscle satellite cells [Morgan et al., 1992], it would allow the cells to be incorporated into a low-turnover compartment and, potentially, give a long-lasting physiological benefit. The ethics of this type of approach seem less contentious.

It is clear that the study of cellular activity in tissue culture may have many advantages; but in summarizing these below considerable emphasis must also be placed on its limitations, in order to maintain some sense of perspective.

## ADVANTAGES OF TISSUE CULTURE

### Control of the Environment

The two major advantages, as implied above, are the control of the physiochemical environment (pH, temperature, osmotic pressure,  $O_2$  and  $CO_2$  tension), which may be controlled very precisely, and the physiological conditions, which may be kept relatively constant but cannot always be defined. Most cell lines still require supplementation of the medium with serum or other poorly defined constituents. These supplements are prone to batch variation [Olmsted, 1967; Honn et al., 1975] and contain undefined elements such as hormones and other regulatory substances. Gradually the essential components of serum are being identified, making replacement with defined constituents more practicable [Birch and Pirt, 1971; Ham and McKeehan, 1978; Barnes and Sato, 1980; Barnes et al., 1984a-d; Maurer, 1992] (see also Chapter 7).

### Characterization and Homogeneity of Sample

Tissue samples are invariably heterogeneous. Replicates even from one tissue vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical, and the characteristics of the line may be perpetuated over several generations, or indefinitely if the cell line is stored in liquid  $N_2$ . Since experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

### Economy

Cultures may be exposed directly to a reagent at a lower and defined concentration, and with direct access to the cell. Consequently, less is required than for injection *in vivo* where 90% is lost by excretion and distribution to tissues other than those under study.

Screening tests with many variables and replicates are cheaper, and the legal, moral, and ethical questions of animal experimentation are avoided.

## DISADVANTAGES

### Expertise

Culture techniques must be carried out under strict aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants such as bacteria, molds, and yeasts. Furthermore, unlike micro-organisms, cells from multicellular animals do not exist in isolation and, consequently, are not able to sustain independent existence without the provision of a complex environment, simulating blood plasma or interstitial fluid. This implies a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise. Tissue culture should not be undertaken casually to run one or two experiments.

### Quantity

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (two or three people doing tissue culture) might be 1-10 g of cells. With a little more effort and the facilities of a larger laboratory, 10-100 g is possible; above 100 g implies industrial pilot plant scale, beyond the reach of most laboratories but not impossible if special facilities are provided when kilogram quantities can be generated.

The cost of producing cells in culture is about ten times that of using animal tissue. Consequently, if large amounts of tissue (>10 g) are required, the reasons for providing them by tissue culture must be very compelling. For smaller amounts of tissue ( $\leq$  10 g), the costs are more readily absorbed into routine expenditure, but it is always worth considering whether assays or preparative procedures can be scaled down. Semimicro- or micro-scale assays can often be quicker due to reduced manipulation times,

volumes, centrifuge times, etc., and are often more readily automated (see under Microtitration, Chapter 19).

### **Dedifferentiation and Selection**

When the first major advances in cell line propagation were achieved in the 1950s, many workers observed the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. This was blamed on *dedifferentiation*, a process assumed to be the reversal of differentiation, but was later shown to be largely due to the overgrowth of undifferentiated cells of the same or a different lineage. The development of serum-free selective media (see Chapter 7) has now made the isolation of specific lineages quite possible, and it can be seen that, under the correct culture conditions, many of the differentiated properties of these cells may be restored (see Chapter 14).

### **Origin of Cells**

If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization (see Chapter 13); in addition, the culture conditions may need to be modified so that these markers are expressed (see Chapters 2 and 14).

### **Instability**

Instability is a major problem with many continuous cell lines, resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and capacity to differentiate within the population can produce variability from one passage to the next. This is dealt with in more detail in Chapters 10 and 17.

## **MAJOR DIFFERENCES *IN VITRO***

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and as the cells spread out, become mobile, and, in many cases, start to proliferate, so the growth fraction of the cell population increases. When a cell line forms it may represent only one or two cell types, and many heterotypic interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant *in vitro* than *in vivo* but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media (see Chapter 7) and it seems likely that this trend will continue.

Energy metabolism *in vitro* occurs largely by glycolysis, and although the citric acid cycle is still functional it plays a lesser role.

It is not difficult to find many more differences between the environmental conditions of a cell *in vitro* and *in vivo* (see also Chapter 19) and this has often led to tissue culture being regarded in a rather skeptical light. Although the existence of such differences cannot be denied, it must be emphasized that many specialized functions are expressed in culture, and as long as the limits of the model are appreciated, it can become a very valuable tool.

## **DEFINITION OF TYPES OF TISSUE CULTURE**

There are three main methods of initiating a culture [Schaeffer, 19901 (see Glossary and Fig. 1.2): (1) *Organ culture* implies that the architecture characteristic of the tissue *in vivo* is retained, at least in part, in the culture (see Chapter 22). Toward this end, the tissue is cultured at the liquid-gas interface (on a raft, grid, or gel), which favors retention of a spherical or three-dimensional shape. (2) In *primary explant culture* a fragment of tissue is placed at a glass (or plastic)-liquid interface where, following attachment, migration is promoted in the plane of the solid substrate (see Chapter 9). (3) *Cell culture* implies that the tissue, or outgrowth from the primary explant, is dispersed (mechanically or enzymatically) into a cell

suspension, which may then be cultured as an adherent monolayer on a solid substrate, or as a suspension in the culture medium (see Chapters 9 and 10).

insert fig. 1.1

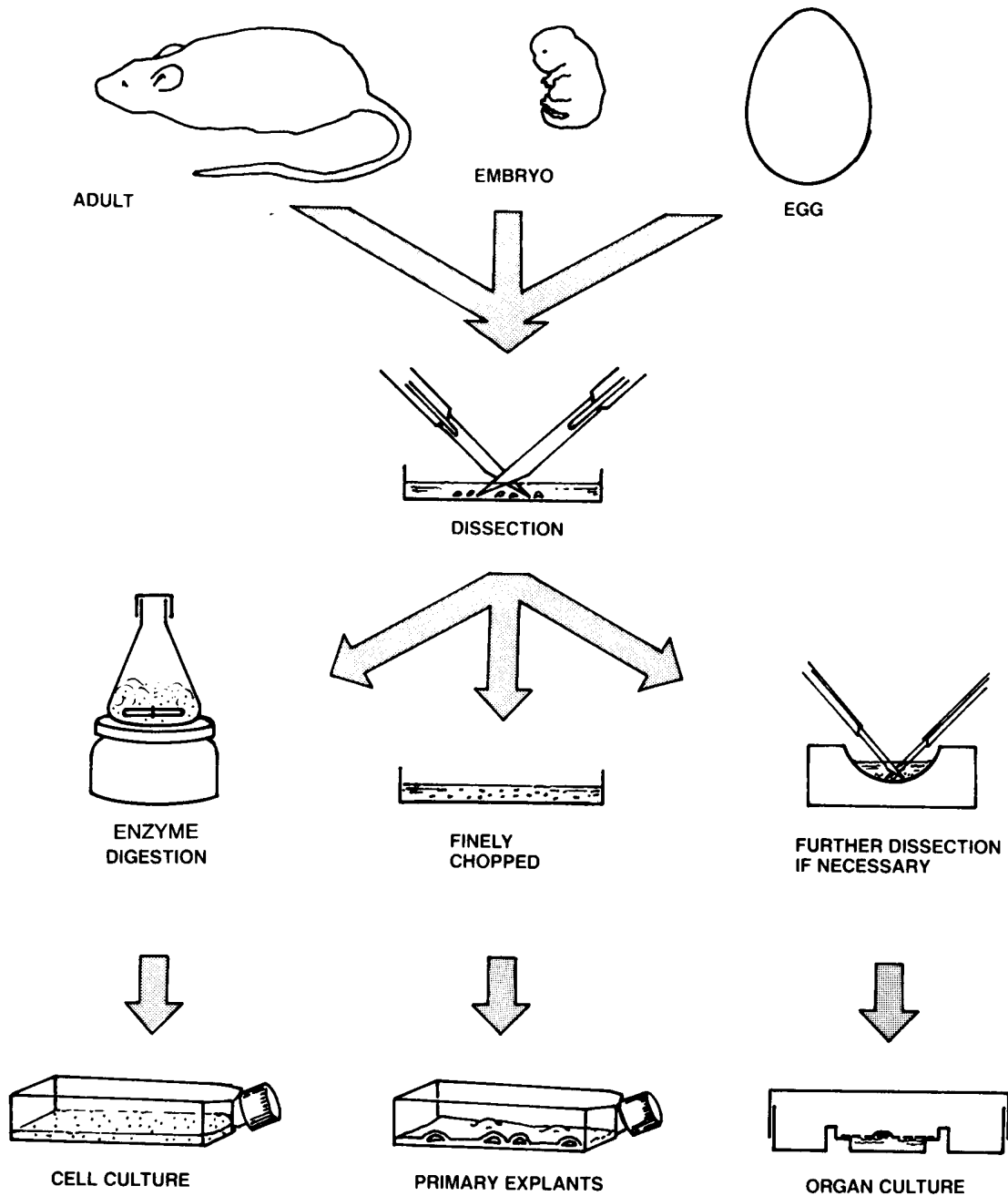


Fig. 1.2. Types of tissue culture.

*Organ cultures*, because of the retention of cell interactions as found in the tissue from which the culture was derived, tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue) and hence cannot be propagated; each experiment requires fresh explantations and this implies greater effort and poorer sample reproducibility than with cell culture. Quantitation is, therefore, more difficult and the amount of material that may be cultured is limited by the dimensions of the explant ( $\sim 1 \text{ MM}^3$ ) and the effort required for dissection and setting up the culture. However, it must be emphasized that organ cultures do

retain specific histological interactions without which it may be difficult to reproduce the characteristics of the tissue.

*Cell cultures* may be derived from primary explants or dispersed cell suspensions. Because cell proliferation is often found in such cultures, propagation of cell lines becomes feasible. A monolayer or cell suspension, with a significant growth fraction (see Chapter 18), may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a *passage* and the daughter cultures so formed are the beginnings of a *cell line*.

The formation of a cell line from a primary culture implies (1) an increase in total cell number over several generations and (2) that cells or cell lineages with similar high growth capacity will predominate, resulting in (3) a degree of uniformity in the cell population. The line may be characterized, and those characteristics will apply for most of its finite life-span. The derivation of *continuous* (or "established," as they were once known) cell lines usually implies a phenotypic change or *transformation* and is dealt with in Chapters 2 and 1S.

When cells are selected from a culture, by cloning or by some other method, the subline is known as a *cell strain*. Detailed characterization is then implied. Cell lines, or cell strains, may be propagated as an adherent monolayer or in suspension. *Monolayer* culture signifies that the cells will attach to the substrate given the opportunity and that normally the cells will be propagated in this mode. *Anchorag dependence* means that attachment to (and usually some degree of spreading on) the substrate is a prerequisite for cell proliferation. Monolayer culture is the mode of culture common to most normal cells with the exception of hemopoietic *cells*. *Suspension* cultures are derived from cells that can survive and proliferate without attachment (*anchorage independent*); this ability is restricted to hemopoietic cells, transformed cell lines, or cells from malignant tumors. It can be shown, however, that a small proportion of cells that are capable of proliferation in suspension exists in many normal tissues (see Chapter 15). The identity of these cells remains unclear, but a relationship to the stem cell or uncommitted precursor cell compartment has been postulated. This concept implies that some cultured cells represent precursor pools within the tissue of origin; the generality of this observation is discussed more fully in the next chapter. Cultured cell lines are more representative of precursor cell compartments *in vivo* than of fully differentiated cells, as most differentiated cells do not normally divide.

Because they may be propagated as a uniform cell suspension or monolayer, cell cultures have many advantages in quantitation, characterization, and replicate sampling, but lack the potential for cell-cell interaction and cell-matrix interaction afforded by organ cultures. For this reason many workers have attempted to reconstitute three-dimensional cellular structures using aggregated cell suspension (*spheroids*) or perfused high-density cultures on microcapillary bundles or membranes (see Chapter 22). Such developments have required the introduction, or at least redefinition, of certain terms. *Histotypic* or *histiotypic* culture, or *histoculture* (I use *histotypic culture* here), has come to mean the high-density, or "tissue-like," culture of one cell type, while *organotypic* culture implies the presence of more than one cell type interacting as they might in the organ of origin or a simulation of it. This has given new prospects for the study of *cell* interaction among discrete, defined populations of homogeneous, and potentially genetically and phenotypically defined, cells.

In many ways some of the most exciting developments in tissue culture arise from recognizing the necessity of specific cell interaction in homogeneous or heterogeneous cell populations in culture. This may mark the transition from an era of fundamental molecular biology, where many of the regulatory processes have been worked out at the cellular level, to an era of cell or tissue biology, where this understanding is applied to integrated populations of cells, and to the ultimate definition of the signals transmitted among cells.