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Microcarrier Cell Culture

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Introduction

In principle, animal cells are cultured for three reasons. One reason is to use the cells themselves as the product. This is the case when a cell population from a patient is expanded and used for grafting. An already existing application is skin-cell growth, while somewhat more futuristic examples may include growth of artificial pancreases and livers.

The largest application is the use of animal cells as vehicles in which to grow viruses for the production of human and animal vaccines. However, recent developments in the preparation of antigens, or combinations of antigens, from infectious agents by recombinant techniques will probably reduce the importance of this category.

The third reason is the use of animal cells as production systems for pharmaceuticals. If the goal is to produce large quantities of an authentic human protein, there will be no alternative but to use animal-cell culture. It is generally accepted that correct post-translational modifications of proteins, glycosylation, protein cleavage, disulphide-bridge formation, amidation, etc. cannot at present be carried out in genetically engineered bacteria or yeast. However, even if an animal cell is used as a host there is no certainty that these modifications will be correct. It has recently been shown that different glycosylations of human erythropoietin may result if the gene is expressed in different cell lines (Goton *et al.*, 1988). This may indicate that frequently the only way to produce an authentic human protein may be to use cells that normally produce it.

Abbreviations: BHK, baby hamster kidney; CEA, carcinoembryonic antigen; CEF, chick embryo fibroblast; CHO, chinese hamster ovary; DEAE, diethylaminoethylcellulose; EACA, 6-aminohexanoic acid; EDTA, ethylenediaminetetraacetic acid; FS-4, human foreskin fibroblast; HEL, human embryo lung; IL-1, interleukin 1; MDBK, bovine kidney epithelial cells; MDCK, canine kidney epithelial cells; NMR, nuclear magnetic resonance; OTR, oxygen transfer rate; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; tPA, tissue plasminogen activator.

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High-density culture systems

If the goal is to produce 1 kg/day of a pharmaceutical, a number of requirements need to be fulfilled (Katinger, 1987).

CELL-SPECIFIC PRODUCTIVITY

Today the most effective production systems employ hybridoma cells with, typically, a specific productivity of 5×10^3 molecules/cell/sec. Genetically engineered cells normally produce about 10 times less. Thus the production of 1 kg product/day requires about 10^{13} cells.

NECESSARY AREA FOR IMMOBILIZATION CELLS

Most animal cells survive and are most productive when attached to a surface. Even suspension-type cells, for instance hybridomas, tend to give increased product yields when attached.

Anchorage-dependent cells, spread on surfaces, can usually be grown up to 1×10^5 cells/cm^2, while suspension-grown cells with a more spherical morphology may be immobilized at high population densities, up to 2×10^6 cells/cm^2. A surface of between 500 and 10 000 m^2 is thus needed to support the 10^{13} cells required to yield 1 kg protein per day.

SPECIFIC OXYGEN DEMAND

Animal cells usually have a relatively low oxygen demand, 0·15 μmol/10^6 cells × hour for cells in the growth phase. The necessary oxygen input for 10^{13} cells will thus be 1·5 mol/hour.

CULTIVATION METHOD

In general, three different methods are used for animal-cell culture.

1. Simple batch systems. These will yield the lowest cell densities, because formation of waste products and depletions of nutrients result in changes in medium composition and consequent variation in productivity.
2. In the chemostat, fresh medium is added while cells and cell supernatant are simultaneously removed, to maintain controlled constant levels of nutrients and low waste-product concentrations to permit higher cell densities and a more stable specific productivity.
3. In immobilized systems the cells are retained in the fermenter while being continuously perfused with fresh nutrients. This creates the possibility of obtaining extremely high cell densities under stable, optimal conditions.

In practice, the cell densities which can be obtained with the different systems range from about 2×10^6 cells/ml (batch) to 1×10^8 cells/ml (perfused immobilized system).

The corresponding reactor size can thus be calculated to be approximately 20 m^3 (batch, 25%medium changed daily) or 0·1 m^3 (immobilized system). Besides the dramatic decrease in necessary reactor size requirements, high-density immobilized cells also utilize the nutrient media more efficiently and thereby increase product concentration and decrease media costs.

These calculations show that very high cell densities are essential if animal-cell culture systems are to be efficiently employed for the production of pharmaceuticals. Only a limited number of such systems exist today.

In the hollow-fibre membrane system described by Gullino and Knazek (1979) the cells are grown in the extracapillary space while medium is circulated within the fibres. Very high cell densities can be achieved but, because of formation of concentration gradients, increasing fibre dimensions does not lead to corresponding increases in output. Scale-up can only be achieved by increasing the number, rather than the dimensions of the units employed. This same drawback also applies to the ceramic system (Lydersen *et al.*, 1985) whereby cells are grown in a ceramic cylinder with uniform square channels along its length. As the cells are not separated from the growth medium, gradient formation is less pronounced than in the hollow-fibre system. In the packed-bed glass sytem (Whiteside and Spier, 1981) the cells are immobilized on the surface of large glass beads (3 mm diameter) which are packed in a column. Medium is circulated through the bed. This system is easily scaled-up, but as the cells grow and invade the interstitial channels it is very likely that an irregular flow pattern may result due to blocking of the interstitial space. It is also very difficult to harvest the cells for subsequent scale-up.

Another approach is to immobilize the cells within small gel beads which protect the cells from mechanical stress, while nutrients and waste products can freely diffuse throughout the beads (Nilsson *et al.*, 1983). One of the most promising methods is the microcarrier-technique in which the cells are grown on the surface of small beads (0·2 mm) which are suspended in the medium, thus creating a quasi-homogeneous dispersion facilitating control of culture conditions. Basically, this technique is easily scaled-up to the desired production level.

Microcarrier characteristics

An indication that the optimal microcarrier for animal-cell culture still has not been found is the large number of different beads that are used. The general requirement for such a microcarrier has been defined by several groups (Pharmacia, 1982; Reuveny, 1983; van Wezel, 1977). A list of commercially available microcarriers is found in *Table 1*.

ADHESION PROPERTIES

In vivo, cells are attached either to other cells or to a structure termed the extracellular matrix, which may influence metabolism and differentiation as well as providing structural support (Hay, 1981). The components of the extracellular matrix are collagens, proteglycans and a variety of other

Table 1. Commercially available microcarriers

Registered trade name and manufacturer	Characteristics
Cytodex 1 Pharmacia, Sweden	DEAE-dextran
Cytodex 2 Pharmacia, Sweden	Surface-charged quaternary dextran
Superbeads FlowLabs, USA	DEAE-dextran
Microdex Dextran Products, Canada	DEAE-dextran
Dormacell Pfeifer u. Langen, West Germany	DEAE-dextran
Biosilon Nunc, Denmark	negatively charged polystyrene
Cytospheres Lux, USA	negatively charged polystyrene
Acrobeads Galil, Israel	various coatings of agarose-entrapped polyacrolein
Bioglas Solohill Eng., USA	glass-coated plastics
DE 52/52 Whatman, UK	Cylindrical/DEAE-cellulose
Gelibeads KC Biologicals, USA	Gelatin
Ventregel Ventrex, USA	Gelatin
Cytodex 3 Pharmacia, Sweden	Gelatin-coated dextran

substances, including fibronectin, laminin, elastin, chondronectin, etc. Some of these components have been used to coat artificial substrates to mimic the *in vivo* attachment. Collagen-coated surfaces have been used for the culture of primary cells and established cell lines (Bornstein and Sage, 1980). 'Biomatrix', a mixture of the cell fibrous proteins present in an organ, has been used for the culture of differentiated epithelial cells (Reid *et al.*, 1981). For large-scale cultures these components are often uneconomic, hence a number of alternative artificial substrates have been developed.

Ionic microcarriers

Since the growth of anchorage-dependent cells can only occur after adhesion and spreading on a suitable culture surface this interaction is of fundamental importance in the design of microcarriers. All vertebrate cells possess a negative surface-charge at physiological pH (Borysenko and Woods, 1979). But as cells can be cultured on both positive (DEAE-ion exchanger) and negatively charged (tissue-culture plastic) surfaces, it appears that it is not the polarity of the charges that is of importance. It has been shown that one of the basic factors governing cell attachment and spreading is the charge density on the culture surface. Usually there is a critical value, above which cell growth is inhibited and below which adhesion is very poor (van Wezel, 1977; Levine, Wang and Thilly, 1979). This critical charge density is probably related to the interaction between attachment proteins and the charged surface (Grinelli, 1978; Yamada and Olden, 1978).

The concept of using small beads (0·2 mm), on which anchorage-dependent cells attach and grow, as a culture system was first reported by van Wezel (1967). In this first experiment a positively charged ion-exchanger was used (DEAE-Sephadex) as microcarrier. The properties making this microcarrier successful were a charged surface, a large surface area/volume ratio, a beaded form, transparency and a relatively low density.

Using the microcarrier at a concentration of 1 g/l, van Wezel demonstrated that cells (HEL, human embryonic lung) were able to grow to high densities and, after inoculation with virus, the titres achieved were comparable with those noted with other growth systems. However, if the concentration of beads exceeded 1–2 g/l toxic effects were encountered in that cell yield was no longer proportional to bead concentration.

Other signs of toxicity were long lag-periods or complete loss of inoculum (van Wezel, 1977). These problems could be reduced by preincubation of the beads with serum proteins (Spier and Whiteside, 1976) or carboxymethyl cellulose (Levine, Wang and Thilly, 1977). Another approach was taken by Levine and associates (Levine, Wang and Thilly, 1979) who found that lowering the ion-exchange capacity from 3·5 meqv/g bead to 1·5 meqv/g bead largely eliminated these adverse effects. This low-charged microcarrier could be used at a concentration of 5 g/l, without any inhibiting effects on the growth of a human diploid cell strain (HEL299).

Only the surface layer of the beads needs to be charged. Microcarriers substituted on the surface with the tertiary amine group, *N,N,N*-trimethyl-2-hydroxyaminopropyl, permitted cell growth at substitution levels of above 0·6 meqv/g, which are substantially lower than the value required for beads substituted throughout the matrix (Gebb *et al.*, 1982). One of the advantages of a lower charge-substitution is a decrease in protein adsorption from the culture medium.

Microgranular DEAE-cellulose preparations of different ion-exchange capacities were tested for their ability to support cell growth (Reuveny *et al.*, 1980; Reuveny *et al.*, 1982a; Reuveny *et al.*, 1982b). As ion-exchange capacity was increased, so rates of cell attachment increased, up to an optimum at 1·0 meqv/g. Below 0·5 meqv/g there was no cell attachment.

Spreading of cells on DEAE-cellulose (1–2 meqv/g) was significantly faster than on DEAE-dextran: 2 hours as compared to 6 hours. Different cells showed large variation in their growth patterns on DEAE-cellulose. Two human diploid cell strains, MRC-5 and FS, failed to grow on DEAE-cellulose, regardless of ion-exchange substitution.

Primary chick embryo fibroblast (CEF) and two established cell lines (BHK and MDCK) showed optimal growth on DEAE-cellulose with an ion-exchange capacity of 2·00 meqv/g. The rate and yield were also higher than on low-charged DEAE-dextran. Contrary to this result, human diploid cell strains (MRC-5 and LUCS) have been successfully cultured on cellulose fibres substituted with DEAE, 2-hydroxypropylaminoethyl or triethylaminoethyl, all at 0·9 meqv/g (Litwin, 1985). The cells were found to grow as aggregates enclosing cellulose fibres within the clumps. Using beads of polyacrylamide as a base, the type and hydrophobicity, as well as the exchange capacity of the

attached group, was investigated (Reuveny *et al.*, 1983a). Cell attachment was significantly faster to beads derivatized with the primary (ethylene diamine) rather than with the tertiary (*N*,*N*-diethylethylendiamine) amine. Cell spreading was also significantly faster on the beads derivatized with the primary amine: 1–2 hours as compared to 5–6 hours for those with tertiary amino groups. The critical charge essential for cell growth was 0·6 meqv/g for primary and 1·8 meqv/g for tertiary amines. Below a certain charge value, no cell attachment, and consequently no spreading or growth, was found on primary amine beads. This threshold phenomenon was not observed with tertiary-amine substitution. Higher yields of cells with epithelial morphology (MDCK, MDBK) were found on beads derivatized with primary rather than tertiary amines. Cells with a fibroblastic morphology grow better on tertiary-amine-derivatized beads. Cell-attachment rate could be increased if the hydrophobicity of the primary amino charged group was increased (Reuveny *et al.*, 1983b). Changes in hydrophobicity were achieved by variation of the hydrocarbon side-chain. Optimal hydrophobicity for cell growth (BHK, MDCK, CEF, MRC-5 and F) was found with hydrocarbon side-chains of 4–6 methyl groups. However, if the hydrophobic element was incorporated into the bead matrix by replacement of the acrylamide monomer with methacrylamide, a drastic decrease in cell yield was obtained. Microcarriers of chitosan (a partially deacetylated product of chitin—poly β-1, 4-*N*-acetyl-D-glucosamine) have also been prepared and found to promote attachment of cells (Nilsson and Mosbach, 1980).

Tissue-culture plastic is polystyrene treated to increase its negative surface charge. When sulphonic acid was used to sulphonate the polystyrene it was found that the maximum number of cells were spread when there were 2–5 negative charges per nm^2 (Maroudas, 1975). Fewer cells per unit surface area were found to adhere to tissue-plastic microcarriers than to sheets of tissue-culture plastic of the same area and bearing the same charge (Nielsen and Johansson, 1980; Reuveny *et al.*, 1985). This may result from repulsion between the negative charges of both cells and beads. Approximately three times as many cells attached under static conditions to a tissue-culture dish as to polystyrene beads with the same charge during continuous stirring conditions (Nielsen and Johansson, 1980). Treatment of sulphonated polystyrene beads with polyethylenimine followed by 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide led to formation of a covalently bound layer of net positive charge (Jacobson and Ryan, 1982). Cells (HeLa-S3) attached, spread and grew to confluence at a much higher rate on this positively charged surface than on the original negatively charged surface. Positively charged beads of polystyrene have also been prepared by co-polymerization of styrene and dimethyl aminopropyl methacrylamide (Reuveny *et al.*, 1985). The surface charge could thus easily be varied by changing the ratio between charged and uncharged monomers. An optimum in cell yields (MRC-5, CEF, MDCK and BHK) was found at 2 mol% of charged monomer. The yield was higher for all of these four cell types than on negatively charged polystyrene. Growth rate and cell yields of BHK, MDCK and CEF were higher on the co-polymer beads than on

DEAE-dextran, but for MRC-5 cells DEAE-dextran beads were superior (Reuveny *et al.* 1985).

A system based on small (0·15 μm) polyacrylein microspheres entrapped in agarose has been used for cell culture. The entrapped microspheres could be derivatized with small ligands (DEAE and diaminohexane) and large ligands (poly-lysine, gelatin and collagen). The derivatized beads supported growth of chick embryo fibroblasts (CEF), human diploid cells (WI-38) and established cells (BHK and MDCK) (Lazar *et al.*, 1985).

The first artificial substrate used was glass. By means of alkali-treatment the negative charge could be varied, and cell attachment and growth were found to be optimal at different charge densities for different cell lines (Rappaport, Poole and Rappaport, 1960). Glass has not often been used in a true microcarrier system, because its high density requires stirring speeds that are not compatible with cell growth. Low density (1.04 g/ml) glass beads have, however, been used for the growth of three established cell lines: human diploid fibroblasts (MRC-5), nasopharyngeal carcinoma (KB) and squamous cell carcinoma (UM-SCC-2). Cell yields were comparable to those obtained on DEAE-dextran microcarriers (Varani *et al.*, 1983). Scanning electron microscopy revealed differences in the appearance of the cells grown on the glass beads and those grown on DEAE-dextran beads. On the glass beads, cells attached to the substratum through long, slender filopodia while on the DEAE-dextran beads, the entire edge of the cell appeared to be in contact with the substratum. Nearly identical growth could be obtained if used beads were washed and reused.

Non-ionic microcarriers

The majority of epithelial cells attach more efficiently to collagen than to other culture surfaces. Different cell types that are routinely cultured on collagen-coated surfaces include hepatocytes, fibroblasts, chondrocytes, epidermal cells, myoblasts and mammary epithelial cells (Bornstein and Sage, 1980).

Fibronectin, which is found in the serum of culture media, has been found necessary for cell attachment, in that cell attachment cannot occur in serum-free media in the absence of fibronectin (Carter, Rauvala and Hakomori, 1981). Spreading of BHK cells (baby hamster kidney) requires the addition of at least 15 ng fibronectin/cm^2 (Hughes, Pena and Clark, 1979). Divalent cations have also been found necessary for attachment (Grinell, 1978). Fibronectin binds to all types of collagens (Gold and Pearlstein, 1980) but with greater affinity for the denatured forms (Bornstein and Sage, 1980; Kleinman, Klebe and Martin, 1981). Dextran beads coated with denatured collagen bind fibronectin more rapidly than dextran beads coated with native collagen (Johansson and Höök, 1980).

Gelatin, a denatured form of collagen, has been used as a microcarrier surface either in homogeneous cross-linked beads or as a surface coating.

Gelatin beads, cross-linked with glutaraldehyde (Nilsson and Mosbach,

1980) promoted the growth of several cell lines. Basically, the same types of beads were later found to support the growth of fibroblasts (L-929), swine aorta endothelial cells, human umbilical endothelial cells and HeLa-S3 cells (Wissemann and Jacobson, 1985). Cell attachment kinetics revealed that the endothelial cells attached to the gelatin beads at the same rate as to tissue-culture plastics, whereas fibroblast cells attached to the beads more slowly. Once attached, the fibroblast cells spread and grow normally.

Dextran beads with a covalently bound surface layer of denatured collagen (Gebb *et al.*, 1982) allowed improved attachments and spreading of cells with epithelial morphology (secondary bovine embryo kidney cells). It was also found that there was a significant decrease in absorption of serum proteins from the culture medium. Covalent application of collagen to beads of cross-linked polystyrene led to decreased absorption of serum proteins from the medium, as well as lower rates of cell attachment and growth of HeLa-S3 cells (Jacobsson and Tyan, 1982).

Gelatin has also been covalently coupled to beads based on the co-polymerization of 2-acryl-amido-2-hydroxymethylpropane-1,3-diol and N,N^1-diallyltartaridiamide (Obrenovitch *et al.*, 1982). Cells (BHK 21 C13) readily attach and spread on the beads. The growth rate was comparable to that found on tissue-culture plastic.

The first-order rate constant for attachment of human embryonic fibroblastic cells (FS-4) was found to be twofold higher for DEAE-dextran beads than for denatured collagen-coated beads (Tao, Ji and Hu, 1987). After attachment no differences in growth kinetics were observed.

Beads coated with a network of lamin and entactin have been prepared by growing mouse endodermal cells (M 1536-B3) on surface-charged dextran and removing the cells with cytochalasin B (Mai and Chung, 1984). The matrix-coated beads were used to study the attachment, spreading and growth of African green monkey (BSC-40), human mammary (MCF-7), mouse fibroblast (L 929), rat liver (CL 9) and rat hepatoma (H-4-II-E) cells in a defined serum-free growth medium. The mechanism of attachment of cells to coated beads differed from that to uncoated beads, in that novel protein synthesis was needed to promote attachment to the uncoated beads. The matrix also influenced the long-term behaviour of MCF-7 cells. These cells remained as separate monolayers on coated beads after 12 days in culture—in contrast, cells cultured on uncoated beads showed a greater tendency to aggregate with adjacent cells. Baby hamster kidney cells, bovine aortal endothelial cells, bovine smooth muscle cells and chick embryo fibroblasts were all observed to attach and grow on agarose beads to which seratonin had been covalently linked (Hannan and McAuslan, 1987). While the growth and morphology of cells on these seratonin-linked beads appeared normal, a change in cell function may have occurred since the pattern of polypeptides expressed by these cells was different from that of cells grown on immobilized fibronectin or tissue-culture plastics. The attachment of cells to immobilized seratonin beads was found to be mediated by a serum component, vitronectin. Heparin covalently coupled to the product obtained by co-polymerization of 2-acryl-amido-2-hydroxymethylpropane-1,3-diol and N,N^1-

diallyltartaridiamide promoted attachment and spreading of BHK 21 C13 cells. No growth characteristics were, however, reported (Obrenovitch *et al.*, 1982).

BEAD DIAMETER

The size distribution should also be as narrow as possible. An uneven size distribution favours cell attachment to the smaller beads, probably because of the higher sedimentation rate of larger sizes. The majority of applied microcarriers have a beaded shape since they are prepared by an emulsion technique. The only non-beaded microcarriers are DEAE-cellulose preparations which are in the form of elongated cylinders. The cells then usually grow in aggregates and form cell bridges that attach these elongated fibers to each other (Kotler *et al.*, 1985; Litwin, 1985).

Minimum inoculation density

A certain minimum cell density is necessary in order to avoid long lag-phases and slow growth. This is mainly due to the need for cell produced factors, proteins as well as small molecular-weight metabolites, essential for growth. The development of more complex media and/or the use of non-growing feeder cells has permitted reductions in inoculum size. In microcarrier cell culture, the cell growth span is also limited by physical parameters.

Cells do not readily migrate from one bead to another, thus a minimum number of cells is required for each bead. This will differ from cell line to cell line and will also depend on the mode of attachment. An often-used method for improving attachment is intermittent stirring (1 minute/h) and a reduced culture volume (one-third of final) in the initial stage of culture (Clark and Hirtenstein, 1981a).

The Poisson distribution can be used to calculate the distribution of cells on microcarriers. This model is well suited for calculation of minimum cell inocula, provided that the medium supports clonal growth. For FS-4 (human foreskin fibroblasts) it was found that a minimum ratio of 6 cells/bead was necessary (Hu, Giard and Wang, 1985).

If 6 is the minimum ratio, the smallest number of cells necessary for inoculation may be calculated, for instance, Cytodex 1 contains $6{\cdot}8 \times 10^6$ microcarriers/g dry weight (data from Pharmacia), hence the minimum cell requirement will be $6 \times 6{\cdot}8 \times 10^6$, i.e. 40×10^6 cells/g dry weight microcarriers. The only way to decrease this figure is to increase the bead size, thus reducing the number of beads. But as the size increases, available surface area per unit bead weight will decrease, thereby reducing the final cell yield/g bead.

Maximum saturation density

Provided correct inoculation procedures have been used, the final cell yield will be directly related to available surface area, if there are no other growth limitations, such as insufficient supply of nutrients, oxygen, etc. The exact yield will depend on the characteristic saturation density of the cell type. At

confluent cell growth there are normally approximately 10^5 cells/cm^2, corresponding to 6×10^8 cells/g dry weight (Cytodex 1, Pharmacia), i.e. 90 cells/bead.

The ratio between maximum saturation density and minimum inoculation density will be the theoretical growth span that can be obtained. For Cytodex 1 this will be approximately 15 times, i.e. 90/6 for final and initial cell number per bead. In an optimized system the microcarrier's physical characteristics will limit each culture step, in this instance to the ratio of 15 : 1 between the starting and final cell concentrations. In practice, a much lower figure is usually found. Normal values are about ¼ – ⅛ of the theoretical value. A dramatic increase in growth span from 4 to 16 times (FS-4, human foreskin fibroblasts) was obtained by simply changing the microcarrier diameter from 180 μm (standard size) to 265 μm (Hu and Wang, 1986).

BEAD DENSITY

To avoid flotation of the beads and minimize the need for agitation, the bead density should be slightly above that of the culture medium. A specific density between 1·02 and 1·04 g/ml is satisfactory in most cases.

OPTICAL PROPERTIES

To allow microscopic observation of the attached cells, a high light transmission is required. Microcarriers which swell in aqueous media are usually sufficiently transparent, e.g. those based on dextran, polyacrylamide or gelatin. Cellulose fibres and polystyrene-based microcarriers usually have inferior optical properties, but cells attached to polystyrene beads can be observed with a special illumination source (Nielsen and Johansson, 1980).

BEAD RIGIDITY

Initially, it was thought that the beads should be smooth and non-rigid, thereby reducing damage to the cells caused by bead collisions. However, no such effects have been seen with, for instance, rigid glass beads (Varani *et al.*, 1983).

Initation of microcarrier cultures

The most critical stage in the culture is the initial phase (Clark, Hirtenstein and Gebb, 1980). Depending on the cells' growth properties, different procedures must be applied. Important parameters affecting initial culture procedures include rate and strength of attachment as well as plating efficiency, i.e. number of cells able to attach and grow on the substrate.

CELL INOCULATION

In small-scale microcarrier culture, the inoculum is usually obtained from standard bottle monolayers. As fermenter sizes increase, this will become an

impractical approach as the inoculum size increases due to the number of batches needed. Inoculation of a 10 l fermenter may sometimes require cells collected from as many as 100–200 roller bottles, thereby significantly increasing the risk of infection. If primary cells are used for production, the number of animals that must be available may set a limit on the production scale. Monkey kidney cells have been used for production of polio vaccine. Two kidneys are needed to obtain the cells to seed one 10 l fermenter (van Wezel, van der Velden-de Groot and van Herwaarden, 1980). Economic and ethical considerations now make the use of primary cells impractical for this purpose.

The cell seed for inoculation of large-scale fermenters (> 100 l) is preferably obtained from preceeding microcarrier cultures. The physiological status of the cell seed profoundly influences further cultivation, thus optimized procedures for cell harvesting must be used. Cells should be harvested during the logarithmic growth phase because the stationary phase has been found to be less capable of initiating the next culture (Lindner *et al.*, 1987).

CONCENTRATION OF MICROCARRIERS

Provided that there are no limitations of nutrients or gases and no inhibitory effects occur, the cell yield will be directly related to the available surface area and hence to the concentration of microcarriers. The most commonly used concentration of microcarriers is 3 g/l, which corresponds to approximately 2×10^9 cells/l. If higher concentrations are used, frequent media replenishments or perfused systems will be necessary. A concentration of 12 g/l has been employed in a continuously perfused fermenter (Tolbert and Feder, 1984).

CULTURE MEDIA

As the cultures are usually inoculated at relatively low cell densities, the nutritional requirements are at first usually more demanding than for high-density cultures (Ham and McKeehan, 1979). If maximum cell yields are to be obtained from primary and normal diploid cells strains, additional supplementation may be therefore be required. However, when using cells with high plating efficiencies (most established and transformed cells), standard culture media may often be used throughout the culture.

Both attachment and cell yield were found to increase 4–5 times if medium components, such as amino acids, adenine, thymidine, hypoxanthin, inositol, choline chloride, tryptose phosphate broth, HEPES, glucose, glutamine and pyruvate, were added to a standard medium (Hirtenstein and Clark, 1980).

Control of culture conditions

The maintenance of optimal culture conditions involves the monitoring and control of stirring speed, temperature, pH and oxygen, and the measurement of substrates and metabolites, such as ammonia, lactate, glutamine, glucose and the product itself. These are usually measured in discrete samples taken

from the fermenter. A review discussing different analytical sensors has been published recently (Merten, Palfi and Steiner, 1986).

With small-scale spinner vessels, or similar devices, only temperature and stirring control is possible, as these systems generally are not equipped with probes for pH and P_{O_2} measurements.

On this small scale, an atmosphere of 95% air and 5% CO_2 may be used to minimize pH change through the buffering capacity of bicarbonate, which is usually included in the culture medium. Sufficient oxygen, without the need for aeration, may be provided in the head-space of the vessel, provided that relatively low cell concentrations and small culture volumes are used.

Bioreactors for large-scale cultivation processes are generally equipped to control stirring speed, temperature, pH and P_{O_2}.

STIRRING SPEED

It has been suggested that the culture should be initiated in a small volume, either without agitation or with intermittent agitation (Griffiths and Thornton, 1982; Pharmacia, 1982) in order to provide a high attachment efficiency and an even distribution of cells. In larger fermenters lack of agitation can create problems in controlling temperature and oxygen supply to the cells. Reduction of the stirring speed as much as possible permits a good and even attachment of cells during continuous agitation (van Wezel, 1982). The stirring speed should thus be just sufficient to keep all microcarriers in an even suspension and to permit sufficient oxygenation. After the initial attachment phase, stirring speed is usually increased to improve oxygen transfer.

TEMPERATURE

Most bioreactors are fully jacketed, and warm water is used as the heating media. For maximum growth a temperature of 36–37°C is required. Heat transfer may be poor because of the low stirring rate essential in animal-cell bioreactors. The consequent slow rate of heat transfer may create temperature gradients, demanding differential temperature measurements and appropriate control systems.

pH

The main causes of changes in pH are the conversion of glucose to lactate and production of CO_2 by the cells. Lactate can accumulate in the culture, while generated CO_2 may be stripped out of the system by aeration.

Exposure of bicarbonate-containing media to air results in loss of CO_2. This will cause a pH increase, especially in small culture vessels, in which only a few minutes' exposure may result in a pH as high as 8·0. To obtain the highest possible plating efficiency the culture pH should be below 7·6. Different types of cells have different pH optima for their growth, which are not necessarily the same as for the highest plating efficiency (Eagle, 1973). The pH is usually controlled by the addition of acid (HCl) or base (NaOH). When a bicarbonate

medium is used, changes in the gas phase concentration of CO_2 can provide minor correction of medium pH. If base or acid are used, care must be taken not to change significantly the osmolarity of the culture medium. The most effective way of controlling a decline in pH is to minimize lactate formation.

High concentrations of glucose result in high levels of lactate. By daily additions of glucose, to maintain a 25–40 μM level, in the presence of glutamine (50 μM), cultured cells were found to use glutamine as an energy source, with consequent decreased formation of lactate (Zielke, Ozand and Tildon, 1978; Zielke, Sumbilla and Sevdalian, 1980).

OXYGEN

The major problem of large-scale culture is oxygen control. The transfer of oxygen from the head-space is low, due to slow agitation rates. Traditionally, large-scale cell cultures have been oxygenated by surface aeration. When oxygen is supplied through surface aeration, oxygen transfer is greatly affected by stirring rate and by the area of the gas–liquid interface relative to the culture volume.

Experiments have shown that dissolved oxygen can be controlled by surface aeration in reactors up to 200 l. The effect of oxygen limitation has been studied on a 50 l scale (van Wezel, 1982) and on a 140 l scale (Scattergood *et al.*, 1983). By directing the gas mixture (O_2, N_2, CO_2) towards the culture surface through a perforated ring, the rate of oxygen transfer can be increased. With this technique, surface aeration was successfully used on a 350 l scale (van Wezel, 1982). Contrary to these experimental results, mathematical calculations reveal that oxygen limitation will occur at relatively small volumes if oxygen is supplied only through surface aeration.

The oxygen transfer rate (OTR) by diffusion from the head-space to the medium can be expressed by the following equation:

$$\text{OTR} = K_L a \times (C^* - C_L)$$

where

- OTR = oxygen-transfer rate (mmol O_2/l/h);
- $K_L a$ = mass transfer coefficient (mmol O_2/atm/l/h);
- C^* = concentration of oxygen in the gas phase in equilibrium with the saturated concentration of oxygen in the liquid phase (atm);
- C_L = concentration of oxygen in the gas phase in equilibrium with the concentration of oxygen in the liquid phase (atm).

The mass transfer coefficient ($K_L a$) is a function of stirring speed and culture volume. Using the above equation it has been calculated (Glacken, Fleischaker and Sinskey, 1983) that a culture of 1×10^7 HeLa cells/ml (oxygen demand: 0·5 mmol O_2/l/h for 10^6 cells/ml) will become oxygen limited to a volume of less than 1 litre, when the head-space is filled with air ($C^* = 0{\cdot}21$ atm). A maximum volume of 3·5 l can be used if oxygen is used instead of air.

The large discrepancies between theory and practice might be explained by differences in cell concentrations, cellular oxygen demand, fermenter

configuration and mixing system. It is, however, very clear that development of improved systems for oxygenation is of utmost importance if microcarrier systems are to be used on a large scale. Several strategies have been suggested to overcome this barrier to large-scale culture systems. Air sparging, which is commonly used for oxygenation of microbial fermentations, has generally been claimed to be unsuitable for animal cells, due to foaming of the medium and lysis of the cells.

Small amounts of air or oxygen have been sparged directly into large fermenters (van Wezel, 1982; Delzer, Hauser and Lehmann, 1985). The influence of sparging and different antifoams have been studied for recombinant cells of a CHO line (Aunins *et al.*, 1986). By mounting a wire cage on the stirrer shaft and sparging air inside the cage, foaming was avoided (Whiteside, Farmer and Spier, 1985). The wire cage allowed free passage of medium but excluded the passage of microcarriers.

Circulation systems, in which medium is recirculated, while the microcarriers are retained in the reactor, have been used for external oxygenation. The cell-free medium can be oxygenated by surface aeration in another vessel (Griffiths and Thornton, 1982), but more efficient oxygenation sytems could be used. Calculations have shown that using this method for oxygenation of 1000 l HeLa cells, a circulation rate of 30 000 l/h would be needed to maintain 10% saturation in the vessel (Glacken, Fleischaker and Sinskey, 1983). Such a high flowrate is very likely to produce foam and damage the cells. A method for bubble-free aeration that has generated great interest is the diffusion of oxygen through the walls of silicone tubing immersed in the culture medium. However, major disagreement about the effectiveness of this system has been reported. Calculated values for the length of tubing needed for the oxygenation of a 1000 l culture range from 30 m (2·5 cm outer diameter) (Glacken, Fleischaker and Sinskey, 1983) to 1000 m (0·15 cm outer diameter, 0·05 cm wall thickness) (Hirtenstein and Clark, 1983). Oxygen transfer characteristics for different tubing materials, pressure gradients and agitation speeds have been measured for a small fermenter (1·5 l) equipped with silicone tubing wired around the interior of the vessels (Kuhlmann, 1987). Oxygen diffusing through the wall of a hollow fibre has been used to oxygenate an 11 l fermenter for culture of a recombinant mouse fibroblast line. The polypropylene fibre used was wound in the form of a coil and made to slowly move in the culture during the fermentation (Lehmann, Piehl and Schultz, 1987).

Harvesting and subculturing

ENZYMIC TREATMENTS

For many biochemical studies, or in the isolation of membrane proteins, it may not be necessary to remove the cells from the microcarriers. However, when subculturing and scaling-up, removal of the cells is required. In scaling-up to the final production fermentation the volumetric size ratios of one fermenter to the next normally ranges from 1 : 20 to 1 : 4. To ensure an even distribution of

cells on the microcarriers in the new fermenter, the harvested cells should be obtained as a disaggregated single-cell suspension with a high viability. Cell release is usually accomplished by treatment with proteolytic enzymes, trypsin (EC 3.4.21.4), pronase (EC 3.4.24.4) or Dispase, alone or in combination with a chelating agent, EDTA, which complexes divalent ions required for cell attachment.

Originally, standard methods used for the release of cells from traditional surfaces were applied to microcarriers, i.e. cell layers were washed with phosphate-buffered saline (PBS) containing EDTA and incubated with a mixture of trypsin and EDTA (van Wezel, van der Velden-de Groot and van Herwaarden, 1980; Billing *et al.*, 1984). This method is unsatisfactory for a number of cell types, especially fibroblasts. By adjusting the pH to 8·2, cell release was found to be more efficient and it was also possible to reattach the cells to both new and already used microcarriers. The use of standardized trypsin under optimal conditions for enzyme activity (presence of calcium ions and pH 8·0) resulted in 95–100% detachment of cells with a viability greater than 95% following a 10 min exposure to trypsin (Lindner *et al.*, 1987). Such cells could reattach to both new and used microcarriers (Hu, Giard and Wang, 1985). Harvesting cells by using trypsin and chelating agents is known to alter cell viability and to remove large amounts of surface-associated molecules (Angihiteri and Dermietzel, 1976). Studies requiring intact cell membranes or the maximization of the number of viable cells required for scaling-up have focused research on the need for alternate methods for cell harvesting. An extract of plant origin, RDB, has been found to disperse monolayer cell cultures with a higher viability than trypsin, 98% viability versus 85% (Ben-Nathan *et al.*, 1985). The matrix of some microcarriers is susceptible to proteases.

Cells growing on homogeneous cross-linked gelatin beads can be released through enzymic digestion of the matrix by collagenases (EC 3.4.24.3) or Dispase. Collagenase and Dispase give rise to a higher population of viable cells (Nilsson and Mosbach, 1980). When trypsin, collagenase and Dispase were compared for release of cells growing on denatured collagen-coated microcarriers, differences in the extent and rate of detachment of different cell types were found (Gebb *et al.*, 1984). Collagenase was found to result in the highest recovery and viability, especially after pre-washing with EDTA. The requirement for a pre-wash with EDTA may indicate that the morphology of a cell is an important factor, because washing with EDTA results in cells with a more rounded morphology thereby exposing sites of attachment between the cells and exposing the cell surface to the enzyme.

Treatment of confluent dextran-based microcarriers with dextranase (EC 3.2.1.11) detached the cells with a high viability and completely dissolved the matrix. Use of dextranase alone resulted in the release of sheets of cells, but if used in combination with trypsin, a single-cell suspension of harvested cells was obtained (Lindskog *et al.*, 1987).

Microcarriers based on a completely digestible matrix eliminate the need for separation of released cells from the matrix. Using conventional matrices it is usually necessary to separate released cells from microcarriers, because the

used microcarriers usually retain a variable number of viable cells. If these microcarriers are transferred to the next step they will start with a larger number of cells and thus become confluent earlier. Protein residues remaining on the beads after harvesting may also impair bead function.

Released cells can be separated from microcarriers by gravity sedimentation, filtration or density gradient centrifugation. Gravity sedimentation makes use of the fact that cells and microcarriers sediment at different rates. After harvesting, culture medium is added and the microcarriers are allowed to settle. After separation of the cell-containing supernatant the procedure may be repeated if higher recovery is necessary. Microcarriers can be retained on a sterilizable filter, approximately 100 μm pore size, while cells will pass through. Cells and microcarriers have also been separated in a discontinuous density gradient (Billig *et al.*, 1984). Of these three methods only filtration has been tested on a large scale (van Wezel, van der Velden-de Groot and van Herwaarden, 1980).

SUBCULTIVATION BY BEAD-TO-BEAD TRANSFER

The most desirable approach for scale-up should be to add new microcarriers to an already growing system. This approach has most commonly failed because of the inability of cells to move from one bead to another.

Transfer of cells to fresh microcarriers has been achieved at low calcium concentrations on addition of a mature, almost confluent (70–80%) culture on microcarriers to a fresh, low calcium culture containing new microcarriers. Cells were released and these reattached to the fresh microcarriers and entered the required experimental growth phase on restoration of the appropriate calcium concentration (Crespi and Thilly, 1981).

An increase of 10^4 in the relative cell number could be obtained with two cell lines (CHO-Kl and LLC-MK_2). Traditional one-step microcarrier culture was found to give a relative cell number increase of approximately 15 for each cell type. Although useful for typical epithelial cells, this method has not been successfully applied to human diploid fibroblasts (Hu, Giard and Wang, 1985). Serial propagation of human foreskin fibroblasts and Vero cells has used a pH-trypsination method, with a 200-fold increase in the relative cell number. A confluent microcarrier culture was treated with trypsin at high pH (8·2–8·8) to detach the cells. The detached cells, along with the cells remaining on the microcarriers, were then used to inoculate a four-times larger vessel containing new microcarriers (Hu, Giard and Wang, 1985).

Microcarrier culture vessels

Microcarrier culture is a very flexible technique, almost any type of culture vessel can be used. Addition of microcarriers (5 mg/ml) to Petri dishes will improve the cell yields at least twofold. Optimal yields of microcarrier cultures are obtained if equipment that provides an even suspension of microcarriers with gentle stirring is employed. The choice of vessel will depend on the purpose of the culture.

SMALL-SCALE CULTURE VESSELS

Cultures ranging from 5 ml to 1 litre are usually performed in a closed system without monitoring or control of pH, dissolved oxygen or metabolites. The most commonly used vessel is the spinner, which consists of a hanging Teflon-coated magnetic bar, suspended in the medium, driven by a base unit. This system was devised during the late 1950s for microbial fermentations employing high stirring speeds (30–60 rpm). The equipment has been developed in several ways for use in microcarrier cell culture. The position of the magnetic bar is adjusted to a few millimetres above the bottom, to avoid sedimentation of microcarriers under the axis of rotation. Traditional spinner vessels have been modified for improvement of stirring conditions. These modifications include vessels with round bases (to avoid slack points where microcarriers may accumulate) and impeller modifications. The necessary stirring speed for keeping microcarriers in suspension can be reduced from 50–60 rpm for traditional spinner vessels to 20–30 rpm for paddle impeller and further down to 15–30 rpm for a plough-type impeller (Pharmacia, 1982).

A new principle for keeping microcarriers in suspension has been developed by Techne (Cambridge) Ltd. A bulb-shaped rod with one end fixed above the culture moves with a circular motion in a culture vessel having a rounded and indented base. This system eliminates sedimentation of microcarriers, and results in a gentle and even circulation of microcarriers. Increased yields have been achieved as compared to traditional spinner vessels, especially with cells having low plating efficiency (Pharmacia, 1982).

LARGE-SCALE EQUIPMENT

The large-scale equipment used for culture of micro-organisms or animal cells in suspension fermentation is usually not suitable for microcarrier culture because of the changes occurring during the course of fermentation. In the initial stage, there is a heterogeneous suspension, consisting of small cells (10–20 μm) and larger beads (100–200 μm). The agitation system must bring these two in close contact with each other to promote cell attachment. After attachment, conditions are needed to permit growth of a confluent monolayer on each bead. During this period agitation must be carefully controlled. Too high an agitation rate may dislocate cells from beads or mechanically damage beads or cells. Too low an agitation can result in aggregation of beads through cell bridging or sedimentation at the bottom of the fermenter.

The main difference between fermenters used for microcarrier cell culture and microbial culture relates to the agitation system. In general, the standard turbine impeller is replaced by low speed, large diameter impellers to reduce shear. The bioreactors are made of glass (smaller sizes) or polished stainless steel (larger sizes). A variety of different configurations have been used successfully (van Hemert, Kilburn and van Wezel, 1969; Meigner, 1979; Griffiths, Thornton and McEntee, 1980). Modification of the 'Bilthoven Unit' (van Hermet, 1964), originally developed for bacterial fermentation, has been used for culture volumes up to several hundred litres (van Wezel, van der Velden-de Groot and van Herwaarden, 1980; Montagnon, Fanget and Nicolas,

1981). An agitation system consisting of four flexible sheets held vertically and spanning the depth of the culture fluid has been found to produce homogeneous suspensions of microcarriers at very low stirring speeds.

Application of microcarrier culture

Application of microcarrier culture falls mainly into four categories: production of cells, viruses, cell products (homologous or heterologous proteins) and *in vitro* studies of cells.

PRODUCTION OF CELLS

As the use of microcarriers increases the available surface area/volume ratio (approximately 20 cm^2/ml versus 4 cm^2/ml in Petri dishes), it is possible to achieve high cell-densities. These high cell-densities lead to conditioning of the culture medium and also to a stimulation of cell growth, which is very important when only small amounts of cells are available for inoculation, e.g. in prenatal diagnostics (Bernstein, 1980). If very large numbers of cells are necessary, e.g. in the isolation of cell components, the high cell-densities that are possible permit a reduction in reactor sizes. Plasma membranes have been isolated from cells by a procedure in which the attached cells were lysed. The plasma membranes were retained on the beads while the internal components were easily removed. The enzyme markers from the plasma membranes of mouse fibroblast cells were purified 10–20-fold (Gotlib, 1982).

As anchorage-dependent cells become round during mitosis (Terasima and Tolmach, 1963) it is possible to dislodge and then isolate large numbers of mitotic cells by increasing the shear forces.

By treating an exponentially growing culture with a mitotic inhibitor and selecting the appropriate stirring speed, cells with a mitotic index of up to 95% can be collected (Mitchell and Wray, 1979; Ng, Crespi and Thilly, 1980). A list of cell types cultured on microcarriers is found in *Table 2*.

Table 2. Cell types grown on microcarriers

Cell	References
Bovine	
endothelial	Boiadjieva, Hallberg and Högström (1984)
	Busch, Cancilla and De Bault (1982)
	Busch and Owen (1982)
	Davies (1981)
	Davies (1982)
	Davies and Kerr (1982)
	Davies, Ganz and Diehl (1985)
	Hannan and McAuslan (1987)
	Jacobson and Ryan (1982)
	Killackey, Johnston and Movat (1986)
	Ryan, Mortara and Whitaker (1980)
kidney	Gebb *et al.* (1982)
muscle	Davies and Kerr (1982)
	Hannan and McAuslan (1987)

Table 2 (*continued*)

Cell	References
Canine	
MDCK	Crespi *et al.* (1981)
	Kotler *et al.* (1985)
	Lazar *et al.* (1985)
	Reuveny *et al.* (1982a)
	Reuveny *et al.* (1982b)
	Reuveny *et al.* (1983a)
	Reuveny *et al.* (1983b)
	Reuveny *et al.* (1985)
	Sayer, Butler and MacLeod (1987)
Chicken	
embryo	Mered, Albrecht and Hopps (1980)
	Nielsen and Johansson (1980)
fibroblast	Clark and Hirtenstein (1981a)
	Fiorentine, Shahar and Mizrahi (1985)
	Griffiths, Thornton and McEntee (1980)
	Hannan and McAuslan (1987)
	Hirtenstein and Clark (1980)
	Kotler *et al.* (1985)
	Lazar *et al.* (1985)
	Norrgren, Ebendal and Gebb (1984)
	Reuveny *et al.* (1982a)
	Reuveny *et al.* (1982b)
	Reuveny *et al.* (1983a)
	Reuveny *et al.* (1985)
	Scattergood *et al.* (1983)
muscle	Pawłowski *et al.* (1979)
myoblasts	Shahar *et al.* (1985)
nerve	Shahar *et al.* (1985)
Fish	
RTG-2	Nicholson (1980)
AS	Nicholson (1980)
CHSE-214	Nicholson (1980)
Guinea pig	
GPK	Griffiths *et al.* (1984)
	Griffiths *et al.* (1985)
	Griffiths, Cameron and Looby (1987)
Hamster	
BHK	Butler (1985)
	Griffiths (1984)
	Hannan and McAuslan (1987)
	Kotler *et al.* (1985)
	Reuveny *et al.* (1982a, b)
	Reuveny *et al.* (1983a)
	Reuveny *et al.* (1983b)
	Reuveny *et al.* (1985)
	Spier and Whiteside (1984)
	Whiteside, Farmer and Spier (1985)
BHK 21	Duda (1982)
	Marchev and Marcheva (1981)
	Meigner, Mougeot and Favre (1980)
BHK 21 C13	Obrenovitch *et al.* (1982)
	Reuveny *et al.* (1982a, b)
CHO	Himes and Hu (1987)
	Hirtenstein and Clark (1980)
	Lai, Hopwood and Schwartz (1980)
	Mitchell and Wray (1979)
CHO-K1	Crespi and Thilly (1981)
	Crespi *et al.* (1981)
CHO (recombinant)	Aunins *et al.* (1986)
	Nilsson, Birnbaum and Mosbach (1988)

Table 2 (*continued*)

Cell	References
Human	
adenocarcinoma	Kotler *et al.* (1985)
	Lazar *et al.* (1987)
	Reuveny *et al.* (1987)
amniotic	Bernstein (1980)
bladder cancer	Lundgren *et al.* (1987)
breast cancer	Neeman *et al.* (1987)
endothelial	Busch *et al.* (1982)
	Davies (1982)
	Griffiths and Electricwala (1987)
	Killackey, Johnston and Movat (1986)
	Wissemann and Jacobson (1985)
fibroblast	Hoeg, Osborne and Brewer (1982)
	Larsson and Litwin (1987)
	Litwin (1985)
	Morandi, Stranghellini and Valeri (1985)
	Sando and Rosenbaum (1985)
Flow 4000/clone 2	Gebb *et al.* (1982)
	Gebb *et al.* (1984)
FS	Reuveny *et al.* (1983b)
	Reuveny *et al.* (1985)
FS-4	Crespi *et al.* (1981)
	Giard and Fleischaker (1980)
	Hu, Giard and Wang (1985)
	Levine, Wang and Thilly (1979)
HeLa	Billig *et al.* (1984)
	Carter and Ewell (1982)
	Duda (1982)
	Ewell and Carter (1982)
	Jacobson and Ryan (1982)
	Kotler *et al.* (1985)
	Miller-Faurés *et al.* (1985)
	Mitchell and Wray (1979)
	Nilsson and Mosbach (1980)
	Thebline *et al.* (1987)
	Wissemann and Jacobson (1985)
HEL 299	Levine, Wang and Thilly (1979)
	von Seefried and Chun (1981)
IMR-32	Duda (1982)
IMR-90	von Seefried and Chun (1981)
K 562	Nilsson and Mosbach (1980)
KB	Hirtenstein and Clark (1980)
	Varani *et al.* (1983)
kidney	Hirtenstein and Clark (1980)
lymphoblastoid	Hirtenstein and Clark (1983)
lymphocyte	Engström, Wanger and Sundqvist (1984)
	Sundqvist and Wanger (1980)
	Sundqvist and Wanger (1981)
McCoy	Mai and Chung (1984)
MCF-7	Hassel *et al.* (1987)
	Nielsen and Johansson (1980)
	Miller, Henrotte and Miller (1986)
monocyte	de Vries, Vyth-Dreese and Figdor (1983)
MRC-5	Billig *et al.* (1984)
	Clark and Hirtenstein (1981a)
	Clark and Hirtenstein (1981b)
	Clark, Gebb and Hirtenstein (1980)
	Clark, Gebb and Hirtenstein (1982)
	Gebb, Clark and Hessle (1980)
	Gebb *et al.* (1982)

Table 2 (*continued*)

Cell	References
	Gebb *et al.* (1984)
	Griffiths (1984)
	Griffiths and Thornton (1982)
	Griffiths, Cameron and Looby (1987)
	Griffiths, Thornton and McEntee (1980)
	Griffiths, Thornton and McEntee (1982)
	Hirtenstein and Clark (1980)
	Hirtenstein, Clark and Gebb (1982)
	Lindner *et al.* (1987)
	Lindskog *et al.* (1987)
	Litwin (1985)
	Morandi and Valeri (1982)
	Morandi, Bandinelli and Valeri (1982)
	Nielsen and Johansson (1980)
	Reuveny *et al.* (1982a, b)
	Reuveny *et al.* (1983a)
	Reuveny *et al.* (1985)
	Varani *et al.* (1983)
	von Seefried and Chun (1981)
osteosarcoma	Hirteinstein and Clark (1983)
pancreas	Kelly and Grant (1980)
	Kelly and Grant (1982)
S 156 N	Nilsson and Mosbach (1980)
S 158 A	Nilsson and Mosbach (1980)
WI-38	Lazar *et al.* (1985)
	von Seefried and Chun (1981)
Insect	
embryonic	Lazar *et al.* (1987a)
Monkey	
BSC-1	Billig *et al.* (1984)
	Gebb *et al.* (1982)
BSC-1-a	Kotler *et al.* (1985)
BSC-40	Mai and Chung (1984)
CV-1	Mered, Albrecht and Hopps (1980)
CV-1-2c	Duda (1982)
Frhk-4	Widell, Hansson and Nordenfelt (1984)
kidney	Crespi and Thilly (1981)
	Crespi *et al.* (1981)
	Hirtenstein and Clark (1980)
LLC-MK_2	Hirtenstein and Clark (1983)
	von Seefried and Chun (1981)
	Mered, Albrecht and Hopps (1980)
Vero	Billig *et al.* (1984)
	Clark, Gebb and Hirtenstein (1982)
	Clark, Hirteinstein and Gebb (1980)
	Ewell and Carter (1982)
	Gebb, Clark and Hessle (1980)
	Gebb *et al.* (1982)
	Gebb *et al.* (1984)
	Griffiths, Cameron and Looby (1987)
	Griffiths, Thornton and McEntee (1982)
	Hassel *et al.* (1987)
	Hirtenstein and Clark (1980)
	Hirtenstein, Clark and Gebb (1982)
	Lindner *et al.* (1987)
	Lindskog *et al.* (1987)
	Mered, Albrecht and Hopps (1980)
	Montagnon, Fanget and Nicolas (1981)
	Montagnon, Vincent-Falquet and Fanget (1984)
	Polastri, Friesen and Mauler (1984)

Table 2 (*continued*)

Cell	References
	van Oss, Charny and Absolom (1983)
Mouse	
fibroblast	Horst, Kern and Ulmer (1980)
F9	Gotlib (1982)
J-129	Hirtenstein and Clark (1983)
J 82/18	Hirtenstein and Clark (1983)
L 929	Mai and Chung (1984)
	Reuveny *et al.* (1982a, b)
	Wissemann and Jacobson (1985)
L 929 (recombinant)	Delzer, Hauser and Lehmann (1985)
	Lehmann, Piehl and Schultz (1987)
	Schultz *et al.* (1987)
LM	Gotlib (1982)
	Gotlib and Searls (1980)
macrophage	Östlund, Clark and Kruse (1983)
	Ren (1982)
mesenchyme	Wright, Elmer and Dunlop (1982)
M-109	Yefenof, Schwartz and Katz-Gross (1983)
M1536-B3	Mai and Chung (1984)
NCTC	Duda (1982)
P388D	Prestidge *et al.* (1981)
PYS-2	Gotlib (1982)
RL6-1	Yefenof, Schwartz and Katz-Gross (1983)
3T3	Gebb *et al.* (1984)
3T3 NIH	Thebline *et al.* (1987)
Terc	Gotlib (1982)
TerJ	Gotlib (1982)
Y-1	Dhainaut, Gerbert-Gaillard and Maume (1987)
Pig	
endothelial	Wisseman and Jacobson (1985)
IBR-32	Hirtenstein and Clark (1983)
NLST	Baijot, Duchene and Stephenne (1987)
testicular	Frame and Hu (1985)
thyroid	Fayet and Hovsepian (1979)
Quail	
QT-6	Fiorentine, Shahar and Mizrahi (1985)
Rat	
CL9	Mai and Chung (1984)
DMH W49	Nilsson and Mosbach (1980)
DWH W1073	Nilsson and Mosbach (1980)
epithelium	Chessebeuf, Mignot and Padieu (1983)
H-4-II-E	Mai and Chung (1984)
myoblasts	Shahar *et al.* (1985)
NRK	Hirtenstein and Clark (1983)
Pancreas	Bone and Hellerström (1980)
	Bone and Swenne (1982)
	Spiess, Smith and Vale (1982)
pituitary	O'Conner, Clary and Kellom (1988)
	Smith and Vale (1980)
	Smith and Vale (1981)
Turkey	
pituitary	Proudman and Opel (1981)

PRODUCTION OF VIRUS

Vaccines constitute the largest market outlet for biological agents produced by animal-cell culture, with foot-and-mouth disease vaccine as the principal

Table 3. Examples of virus grown in cell culture using the microcarrier technique

Virus	Selected reference
Aujeszky virus	Baijot, Duchene and Stephenne (1987)
Cytomegalovirus	Griffiths *et al.* (1984)
Fish viruses	Nicholson (1980)
Foot-and-mouth disease virus	Meigner, Mougeot and Favre (1980)
Hepatitis A virus	Widell, Hansson and Nordenfelt (1984)
Herpes simplex virus	Griffiths *et al.* (1984)
Influenza virus	Reuveny *et al.* (1982a)
Polio virus	Larsson and Litwin (1987)
Sindbis virus	Reuveny *et al.* (1982a)

product. Human vaccines produced in this way include polio, rabies, measles and rubella. The cells serve as the host for attenuated strains of the various viruses.

The microcarrier systems permit cultivation of large quantities of virus in compact culture units and, when compared to other cell-culture methods, usually confer increased productivity, reduced costs and reduced contamination. A list of viruses that have been grown in cultures using the microcarrier technique is found in *Table 3*.

PRODUCTION OF CELL PRODUCTS

Animal cells can be used as production systems for proteins in two main ways. Homologous proteins, which are proteins normally produced by the cell (either spontaneously or after induction), can be produced after selection, and large-scale culture of a specific cell line can be achieved. During recent years animal cells have also been employed as an expression vector for recombinant proteins. In this case, a heterologous gene is inserted into a suitable animal-cell host.

Production of homologous proteins

Human foreskin fibroblast has been used for the preparation of human β-interferon on a relatively large scale (Morandi, Stanghellini and Valeri, 1985). As these cells have a very low specific productivity, large amounts of cells were needed in order to obtain sufficient product for clinical trials. In the production cycle, different culture systems were used. The first phase employed roller bottles to provide inoculum for four-litre microcarrier cultures. After four days of culture the microcarriers were transferred to 50-litre fermenters, in which the cells were cultured to confluency. To initiate production of β-interferon the culture was superinduced with Poly I : Poly C and cycloheximide followed by actinomycin D. The production phase lasted for 40 hours, after which the crude β-interferon was harvested.

Plasminogen activators (EC 3.4.21.31) catalyse the conversion of plasminogen to the active fibrolytic enzyme, plasmin (EC 3.4.21.7). Small amounts of this enzyme have been isolated from cultured cells, but it was not until the discovery of a human melanoma cell line secreting higher levels, that procedures for large-scale production became feasible (Rijken and Collen,

1981). An extensive review concerning the production of tissue plasminogen activator has recently been published (Griffiths and Electricwala, 1987).

Carcinoembryonic antigen (CEA) is a tumour-associated glycoprotein. CEA serum levels are used for monitoring cancer patients both for response to therapy and for detection of recurrences (Mackay *et al.*, 1974). CEA can be isolated from liver metastases of colon adenocarcinoma. The drawbacks encountered with this source, limited availability and non-reproducible nature, can be overcome by use of a CEA-secreting human colon adenocarcinoma cell line which has been isolated and cultured on a large scale using the microcarrier technique (Lazar *et al.*, 1987b).

Prourokinase, a precursor of urokinase (EC 3.4.21.31) and a component of the fibrinolytic system, is produced by a human kidney cell line (TCL). Different culture systems, ceramics, roller bottles and microcarriers have been investigated for their capacity to sustain cell growth and productivity (Tao *et al.*, 1987). Two different microcarriers, one collagen-coated and one low-charge ion-exchanger, were tested. It was found that the surface nature had a profound influence on cell behaviour. Collagen-coated microcarriers were found to be superior for long-term production.

A murine macrophage-derived cell line ($P388D_1$) is commonly used for the production of interleukin 1 (IL 1) and other macrophage products. This cell line, normally adherent, can readily adapt to suspension culture. Cells adapted to suspension culture have the disadvantage that they undergo a selection to a subpopulation of cells not producing IL 1. Cells grown on microcarriers do not undergo this selection. The cells are grown to high densities, and, after induction with lipopolysaccharide, secrete IL 1 into the medium, from which it is isolated (Prestidge *et al.*, 1981).

Production of heterologous proteins

As noted in the foregoing section, the production of homologous proteins by animal-cell culture has several disadvantages. Usually the production has to be induced and the desired proteins are only produced during a short period and in low yield.

By using genetic-engineering techniques, animal cells can be constructed which are consistently high producers for very long periods. This is a very recent development and only a few reports have appeared on the use of microcarriers for their culture.

A mouse fibroblast cell (L 929) has been engineered which expresses glycosylated human β-interferon consistently. The cells were grown on microcarriers to high cell-densities ($2{\cdot}2 \times 10^6$ cells/ml) and produced the protein for more than one month (Schultz *et al.*, 1987).

Transfected mouse C 127 cells, expressing the recombinant gene for kappa-nuclease (EC 3.1.26.1), have been cultured on collagen-coated microcarriers (Lundgren and Nilsson, 1987). Serial subcultivations of nearly confluent microcarrier cultures were used to simulate a scale-up process. After the final subcultivation step the cells were maintained at stationary phase for production in the presence or absence of a serum supplement. The gene

expression rate was found to remain constant during the production phase in the presence of serum but decreased in its absence.

Recombinant chinese hamster ovary (CHO) cells have been cultured on microcarriers on a small scale. Depending on the type of microcarrier, gelatin or low-charge ion-exchanger, differences in the specific productivity were found (Nilsson, Birnbaum and Mosbach, 1988). When grown on a gelatin matrix, the specific productivity of cells producing tissue plasminogen activator (tPA) (EC 3.4.21.31) was almost three times higher than when grown on a low-charge ion-exchanger matrix. Human gamma-interferon, on the other hand, was found to be produced at approximately the same rate on both types of beads. Cells producing tPA could only be grown to low densities on either carrier, because of cell detachment. The detachment effect was probably caused by plasmin (EC 3.4.21.7), which has been produced from serum plasminogen through the action of tPA. Plasmin, a broad-spectrum protease, induced detachment by degradation of proteins necessary for cell attachment.

In the case of low-charged ion-exchange microcarriers, this effect could be prevented by 6-aminohexanoic acid (EACA) which reacts with the lysine-binding site of plasmin and inhibits its action.

STUDIES ON CELL DIFFERENTIATION, METABOLISM AND STRUCTURE/FUNCTION

When compared to traditional monolayer techniques, microcarriers provide new opportunities in many studies of cell biology.

Cell differentiation

The differentiation of chick embryo skeletal muscle cells grown on microcarriers could clearly be followed by microscopy. After culture, myotubules with extensive myofibril formation were found (Pawlowski *et al.*, 1979).

Macrophages cultured on microcarriers retain their ability to ingest latex beads (Östlund, Clark and Kruse, 1983).

Pancreatic cells sustained synthesis and release of insulin during 7 days of growth on microcarriers. Furthermore, insulin release could be modulated by glucose and also stimulated with theophylline (Bone and Swenne, 1982). A number of other differentiated cells have been studied using microcarriers: bovine pulmonary endothelial cells (Ryan, Mortara and Whitaker, 1980), pulmonary artery endothedial cells (Busch and Owen, 1982), porcine thyroid cells (Fayet and Hovsepian, 1979), pituitary cells (Smith and Vale, 1981) and neuronal cells (Shahar *et al.*, 1985).

It has been proposed that cylindrical microcarriers might be better than beaded microcarriers for the study of cell differentiation because the growth on cylindrical microcarriers would more closely resemble the *in vivo* growth than the growth on beaded microcarriers (Shahar *et al.*, 1985). As cells grow in multilayered form, a higher degree of cell differentiation is achieved. This has been demonstrated for neuronal cultures where a high specific activity of

acetylcholinesterase (EC 3.1.1.7) and myelination was observed. Also, in muscular cultures high specific activities of specific proteins, cross-striation, spontaneous contraction and parallel fibre orientation supported this belief.

Metabolic studies

Microcarriers, with attached cells packed in columns, provide an opportunity to create systems with close resemblance to *in vivo* relationships. The environment (cells at very high density, in close contact with each other) is very suitable for pharmacological studies. A continuous flow through the column can create a perfused system in which the influence of chemicals injected into the medium can be observed. Rat anterior pituitaries were disaggregated and cultured in the presence of microcarriers for 4–5 days. The beads with firmly attached cells were packed into a column and perfused. The cells were found to respond in a normal physiological manner to hypothalamic releasing and inhibitory peptides (Smith and Vale, 1980). The effects of culture conditions, media composition, duration of cell attachment time and perfusion rate have been evaluated for cultures of anterior pituitary cells (O'Connor, Clary and Kellon, 1988).

The release of insulin and other hormones from perfused pancreatic cells, in response to glucose and other stimuli has also been investigated (Bone and Swenne, 1982).

Perfused endothelial cells were used as models for the microcirculation of blood (Busch and Owen, 1982). This perfusion technique has permitted the study of the metabolism of mammalian cancer cells under controlled experimental conditions. Microcarriers with attached human colon adenocarcinoma cells (HT 29) were continuously perfused in a specially designed NMR chamber. The ^{31}P NMR spectra displayed a series of resonances assigned to nucleoside triphosphates, inorganic phosphate and various phosphomonoesters (Fantini *et al.*, 1987). A fluorescent probe has been used to monitor changes in cytosolic Ca^{2+} associated with von Willenbrand factor release on exposure to histamine of umbilical vein and endothelial cells cultured on microcarriers (Hamilton and Sims, 1987).

Cell structure and interactions

The preparation of monolayer cells for transmission electron microscopy (TEM) usually involves the detachment of cells from their substrate. The resulting preparation may thus be damaged and the relationship between the cells destroyed.

Cells (HEp2) have been grown on polystyrene microcarriers, embedded in agar and, after fixation and staining, dehydrated with ethanol. Polystyrene is not affected by dehydration, thus the monolayer was not distorted. After dehydration, 1:2-epoxypropane was added to dissolve polystyrene so that the embedded sphere of cells could be cut into thin sections and studied by TEM (Sargent, Sims and McNeish, 1981).

The permeability response of endothelial monolayers on microcarriers to mediators of vasopermeability has been studied (Killackey, Johnston and Movat, 1986). Permeability was measured by the incorporation of a stain, Evans blue dye. Treatment with histamine or thrombin (EC 3.4.21.5) caused an increased uptake of the dye. The thrombin- and histamine-mediated permeability changes could partially be inhibited by prostaglandin E, and isoproterenol.

Electron spin resonance has been used for the study of membrane fluidity of cells (CHO) growing on microcarriers. The effect of different treatments on intercellular adhesion has been investigated by studying the binding of ^{32}P-labelled cells to confluent monolayers on microcarriers (Vosbeck and Roth, 1976).

Junctional communication between cultured monolayers of aortic endothelial and smooth muscle cells has been established in an *in vitro* model of vessel-wall interactions. Confluent monolayers of endothelial cells on microcarriers placed on the surface of a smooth muscle cell monolayer became attached within one hour. After 3 hours of contact co-culture, a small portion of each monolayer was involved in heterocellular attachment. Heterocellular gap junctional transfer of ^{3}H-uridine was found to be a rapid process (Davies, Ganz and Diehl, 1985).

New trends in microcarrier technology

Only the surface area of microcarriers is utilized for cell growth. This results in several drawbacks. First, the cells are subjected to mechanical stress both by the mixing system in the reactor and by the motion of the beads in the medium. Secondly, the physical characteristics of solid beads limit the number of cell doublings that can be obtained in each culture step. These problems may be reduced by using macroporous beads in which the cells may utilize the interior pore surfaces.

A double emulsion procedure has been employed for the preparation of macroporous gelatin beads. In comparison with solid gelatin beads, cell yields were approximately doubled for the tested cell lines (Vero and BHK) (Nilsson, Buzsaky and Mosbach, 1986).

A freeze-thaw process has been used to manufacture macroporous collagen beads.

Incorporation of dense particles into the matrix can increase specific bead density to 1·5 g/ml thereby allowing their use in fluidized bed reactors. Very high densities of cells have been reported in such cultures: 3×10^8 cells/ml beads for hybridomas and 5×10^8 cells/ml beads for CHO cells (Young and Dean, 1987).

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