

Strategies for optimising serum-free media

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Introduction

Serum is neither the *bête noire* of animal cell culture, nor is it a general purpose culture medium additive. An inspection of early cell culture literature demonstrates these points only too clearly – without serum and the plasma clot method, cell culture would have had a long and painful birth. On the other hand, the negative side of serum is seen in its unerring ability to stimulate the growth of unwanted cells in primary cultures! (Paul, 1959; Taub, 1984). In recent years it has become clear that the type of nutrition required for cell multiplication, differentiation or function tends to be specific for the cell concerned, or at least for the cell type concerned, so that optimal conditions cannot possibly be attained by using a general medium containing serum or one of the commercially available single growth promoting supplements that claim to replace, substitute or ‘expand’ the serum otherwise required (Waymouth, 1984). However, the development of a customised serum-free medium for a specific cell type is generally regarded as a long and tedious undertaking. It is nevertheless important to remember that the advantages that serum-free media can offer the cell biologist, more than compensate for the sometimes repetitive and minutely detailed work that is required for their successful development.

The aim of this short review is to summarise the various aspects of the development of serum-free medium and to illustrate these with some

examples from our own laboratories. Before proceeding, a brief consideration of what we are trying to replace – what is serum and what are its functions?

Serum is produced when a plasma clot is formed and thus lacks the plasma components used in the activation of the clotting cascade. Although serum has a very similar composition to that of plasma, one should not regard it as defibrinated plasma (MacLeod, 1988). What remains in the serous fluid after the clot has formed are plasma proteins and polypeptides, lipids and hormones, nutrients and minerals as well as polypeptides actually formed during the clotting process and a variety of other substances that may or may not be advantageous for cell culture. The functions of serum, as far as cell culture is concerned, are summarised in Table 1.

The individual components of serum have certain properties that appear to be essential for the successful culture of cells *in vitro*. For example, albumin will bind toxic components that are present in the culture medium while also acting as a buffer or carrier for nutrients such as fatty acids, amino acids, hormones and trace elements.

Table 1. Functions of serum in cell culture

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1. Source of nutrients, in solution and bound to proteins
 2. Source of protease inhibitors
 3. Source of hormones and growth factors
 4. Facilitates attachment and spreading of cells
 5. Protects non-specifically against mechanical damage
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It also appears to be the component that protects against mechanical damage to cells in agitated culture systems. Transferrin is well-known for its iron-transporting properties but it can also act as detoxifying agent by binding heavy metals; indeed the latter may be its primary role in long-term, continuous cultures. Fatty acids, lipoproteins such as cholesterol, linoleic acid, arachidonic acid and oleic acid appear to be essential for membrane biosynthesis in some cells whereas in others they can also be used as an energy source. Fibronectin and fetuin have properties that support the adhesion of anchorage-dependent cells to the substrate. Alpha-macroglobulin is a protease inhibitor that neutralises the activity of enzymes, such as trypsin, that can be present in cell culture media.

Basic considerations

The cell culture literature is replete with excellent articles on the development of serum-free or defined media ranging from Ham's 'Twenty-one Steps' in 1981 (Ham, 1981) to Bödeker's 'Four Steps' in 1985 (Bödeker *et al.*, 1985). The strategies that will be considered in this article will combine the features of published procedures in order to present a more general impression of methods that can be used to develop serum-free medium for transformed, non-transformed, adherent and non-adherent cells.

At this point we should consider what the term 'serum-free' actually means: although the cellular pharmacologists still discuss the possibility of a truly chemically defined medium, i.e., a medium in which every single component is known, it is generally thought that, while representing an exciting and thought-provoking objective, this is a rarity with limited application from the point of view of mammalian cell technology. It may well be true that the growth requirements of a cultured cell 'can be improved by increasing the extracellular concentration of a rate-limiting nutrient, by increasing the efficiency of transport of that nutrient into the cell, or by increasing the efficiency of that nutrient within the cell' (Ham, 1981) but

until the precise nature of the action of growth factors such as epidermal growth factor or interleukin 2 has been clarified, there is little chance that the cascade of intracellular events that is triggered by the interaction of such molecules with their receptors on the cell surface could be mimicked over a long period of time by increasing the concentration of certain nutrients in the culture medium. The application of serum-free medium, rather than chemically defined medium, has also become more widespread in recent years as recombinant DNA and mammalian cell technologies have become established as effective processes for the production of commercial amounts of biologicals. For these reasons I would like to discuss the development of serum-free media that are considerably more defined than a serum-containing medium although they do not fall into the category of chemically defined media. There are also subclasses of serum-free medium described in the literature – protein-rich and protein-deficient media (Bödeker *et al.*, 1983). Such developments have also arisen from considerations in modern animal cell production processes where down-stream processing requires that the contamination of the product with foreign protein must be as low as possible. As Murakami wrote in a recent review 'the concept for constructing serum-free medium for biotechnology may be obviously different from the counterpart of making serum-free medium to mimic cells *in vivo* physiological conditions' (Murakami, 1990).

General principles

There are some basic rules that apply to the development of serum-free cell culture media whether or not the final medium is destined for a 5000 L fermenter tank or for a 5 mL culture flask:

1. Use media and supplements that have the lowest possible levels of bacterial endotoxin.
2. Prepare only minimal amounts of basal medium at any one time – a two week supply kept at 4°C is recommended. This avoids decomposition of the various components. Cells

growing in serum-free media seem to be more sensitive to the decomposition products possibly because they are not being protected by the detoxifying characteristics of serum (Rockwell, 1984). The storage of the basal medium should also be in plastic flasks because of the tendency of glass to bind particular components of the basal medium or even to release undesirable elements into the medium during the storage period. Basal medium should be equilibrated to 37°C and the appropriate CO₂-air mixture before adding cells.

3. Store growth factors concentrated at the manufacturer's recommended temperature and add in small volumes, e.g., 20 µl/mL basal medium, immediately after cells have been plated. Fibronectin and EGF should be stored frozen, selenium at +4°C. Use only disposable plastic tubes and, if sterilising by filtration, use filters with a low dead space and minimal protein binding. Avner notes that transferrin and hormone mixtures were not active in fully prepared medium for longer than 48 hr even at +4°C (Avner *et al.*, 1984).
4. Be careful not to expose cells to extremes of pH and temperature. Trypsinisation, centrifugation and resuspension should be carried out as rapidly as possible. If the cells appear to be sensitive to normal trypsinisation procedures, try low temperature trypsinisation (Ham, 1981).
5. Use a trypsin inhibitor like aprotinin or soya bean trypsin inhibitor if required, usually in the wash fluid directly after trypsinisation. Some preparations of soya bean trypsin inhibitor contain cytotoxic components so it may be necessary to screen several batches. If necessary, change the medium the day after setting up the cultures to reduce the levels of proteases in the medium that are released by cells damaged during the sub-cultivation procedure. It is sometimes advisable to include a broad spectrum protease inhibitor like aprotinin in the culture medium itself in order to prevent damage to the cells or medium components by trypsin or related serine proteases (Hewlett, 1989).
6. Avoid seeding, or even washing, the cells in serum-containing medium before culturing the cells under serum-free conditions – this is not a true serum-free culture as traces of serum probably bind to the cell surface or are internalised by the cells during their brief period of contact with the serum.
7. Choose the seeding density carefully to observe supplement dependencies. It might even be necessary to set up clonal growth assays using very low seeding densities (see Ham, 1981).
8. Avoid the use of antibiotics. Some of the more commonly used antibiotics, such as penicillin, streptomycin, and gentamycin can be cytotoxic even in the presence of high concentrations of serum (Metzger *et al.*, 1954). Long-term use of antibiotics can also lead to the selection of resistant microorganisms. Care should be taken, especially in the case of serum-free medium where the cytoprotective role of serum is absent.
9. The quality of water used to make up serum-free medium is most important because serum components that could bind toxic elements in the water are not present in the medium. There are many commercially available systems for purification of water and if a deionised water is used to feed these systems, the resulting water is perfectly adequate for cell culture purposes.
10. Chemicals and biologicals should be of highest purity and equipment and services of reliable quality. Typical equipment would include an analytical balance, an accurate pH meter and an osmometer. Other equipment that is useful, although not absolutely essential, is a glucose and lactate analyser and an amino acid analyser.

Methods and strategies

We have developed a more or less standard operating procedure in our laboratory for developing serum-free media and this is described briefly in the following paragraphs (for a more

detailed description of our basic methodology see Bödeker *et al.*, 1983):

1. Cultures are set up in 1.0 mL volumes in 24 or 12-well multiplates or in T-25 flasks containing 5 – 10 mL of medium.
2. Seeding densities are usually between 1×10^4 and 1×10^5 /mL or the cells are passaged at a ratio between 1:3 to 1:10. Sometimes it may be necessary to seed at high density to obtain initial growth although the best seeding density is that which will give at least three population doublings before the cells have to be re-passaged. After several passages at high densities, test whether the cells have adapted to the new medium and whether all the required factors are present by examining responses at a lower seeding density.
3. Adherent cells are removed from the plastic surface by treatment with a trypsin/EDTA solution, washing in PBS-A and subsequent resuspension in growth medium. The culture vessels are not treated with any adherence factors before plating unless growth is totally absent.
4. Once the medium has been optimised, growth parameters, such as lag phase, doubling time and maximal cell concentration, are calculated from growth kinetic curves (time versus $\log_{10}$ cell number).

We have found that a standard screening medium consisting of a 50/50 mix of Dulbecco's modification of Eagle's medium (DME) and Ham's nutrient solution F12 (F12) with the addition of bovine insulin, human transferrin, albumin (in our case usually human), bovine lipids bound to lipoproteins and selenium (see Table 2) can promote growth of a number of different cell types, adherent and suspension, from a number of sources and species. Experience has shown that the lipid content of the medium requires the most optimisation.

There are two basic strategies that apply to the development of a serum-free medium for a particular cell, either the cell is adapted slowly to the lack of serum or placed directly in a standard serum-free medium:

Table 2. A basic procedure for developing a serum-free medium for mammalian cells; note order of addition of supplements is as shown; recommended values are shown in square brackets

Set up an assay for cellular behaviour [growth]
Select an attachment factor if required [fibronectin]
Select a basal medium [DME/F12, 50/50]
Add albumin [fatty acid-free; 200 mg/L]
Add insulin [low endotoxin; 10 mg/L]
Add transferrin [90% iron saturation; 10 mg/L]
Add selenous acid [highest grade; 100 nanomolar]
Add lipoprotein/lipids [low endotoxin; 1–30 mg cholesterol/L]
Test medium in selected assay
Titrate supplements as necessary

- 1a. Wean off serum by reducing FCS concentrations slowly from, for example, 10 % to 3 % to 1 % to 0.5 % to 0.1 % to 0 %. What is happening here is a gradual adaptation of the cells to the poorer culture conditions. Eventually the medium is no longer supportive enough for the cells to be able to adapt – this is the crisis point for the culture when serum-free supplements such as albumin, insulin, transferrin, lipoprotein/lipid and selenium should be added. If growth is still not adequate, specific factors such as EGF, FGF, attachment factors, hormones or cytokines should be considered as supplements. If the original cell was specifically chosen for its ability to produce a certain biological activity, this function should be checked at every stage of the adaptation period to ensure that a more rapidly growing, inactive clone is not selected by the procedure.
- 1b. The 'sink-or-swim' (SOS) treatment of transferring the cells directly from full serum-containing medium to a serum-free medium containing the standard supplements albumin, insulin, transferrin, lipoprotein/lipid and selenium. We have had good experience with the SOS method although some of the more fastidious cells, such as HeLa, do seem to adapt better by using the weaning method. If growth is not adequate, consider using the additional supplements noted above.
2. Once the cells have become accustomed to the serum-free conditions, start adjusting the protein content of the medium if this is

required. If the cell is to be used for production of a particular biological activity, a medium with a minimal amount of foreign protein in it might well be a target for the cell technologist; if the cell is to be used to study a particular cellular function, such as motility under serum-free conditions, then a low-protein medium might not be necessary. Some cells do seem capable of growing for extended periods of time in unsupplemented basal DME/F12 alone – we have a clone of A431 cells we developed in our laboratory that does just that, although its rate of growth is slower than the same cell in supplemented medium.

3. The next test at this stage is to examine the behaviour of the cells over a number of passages in the optimised medium. We have chosen 10 passages as a suitable number for determining the efficacy of the medium under development. Other factors may be identified during this stage that are obligatory for long-term culture. Check medium parameters at this stage too, such as glucose consumption, lactate production and amino acid kinetics.
4. If the cell concerned is to be used in a production process, whether for biologicals or for vaccines, it would now be necessary to put the cell and medium into a technical scale version of the final production process. If necessary, compare to the behaviour of the cell in serum-containing medium. It might even be necessary to examine the characteristics of the product itself; a recent report suggests that the composition of the medium can affect the physicochemical properties of the product (Moellering *et al.*, 1990).
5. A final test is to check whether the medium is suitable for freezing the cells down. We have found that our standard protein-rich medium (Table 2), when supplemented with 10% v/v dimethyl sulphoxide, is perfectly adequate for freezing even the most sensitive of cells, such as hybridomas. At all accounts avoid using serum to freeze down cells because this will necessitate readaptation of the cells after

thawing – hardly the most efficient method of working! In addition, if good manufacturing principles are involved – laying down a master cell bank, for example – adding undefined sera to the cells may well be unacceptable to the regulatory authorities.

Cell culture media and supplements: Results and discussion

There are many publications that discuss the properties of the various basal media, nutrients, growth factors and hormones that have been used for serum-free cell culture (see Barnes *et al.*, 1984). In this section we will discuss only those medium components that we and others have consistently found to be useful in initiating the development of serum-free medium (for further examples, see Butler, 1986) and, where appropriate, will present results to illustrate each point.

Basal medium

The basal medium provides many soluble, low molecular weight nutrients to the cell such as ions, carbohydrates, vitamins, amino-acids and nucleosides. Büntemeyer (Büntemeyer, 1988) mentions that most commercially available basal media were originally developed for the clonal growth and that if high density cell growth is required, as might be expected in modern mammalian cell technology devices, the basal medium may also have to be supplemented with essential nutrients such as glucose and amino acids. This in turn implies a careful analysis of the nutritional requirements of the cell concerned by examining the consumption of glucose, lactate and amino acids. The parameters for determining a substrate-limitation should not only include growth of the cell population but also its productivity if appropriate.

As mentioned above, a commonly used basal medium is the 50/50 mix of DME/F12. This is a useful medium for the first steps with serum-free

media and at later stages of the development it can be optimised by altering the ratio of DME to F12 and also by increasing the concentrations of rate-limiting nutrients. Some cells may require unusually high concentrations of a particular metal while others may have defective metabolism or transport systems and require additional supplementation. It is also important to remember that a cell that is secreting high levels of protein, such as a hybridoma cell, will almost certainly require a basal medium that is far richer in amino acids than that required by a quiescent fibroblast.

Albumin

There are many different qualities of bovine serum albumin (BSA) and human serum albumin that can be used in cell culture. For example, we studied the effect of fatty acid-free BSA on the growth of L929 mouse fibroblasts in a DME/F12 medium supplemented with insulin and selenium. The results, shown in Fig. 1, demonstrate the ability of albumin to shorten the lag phase of

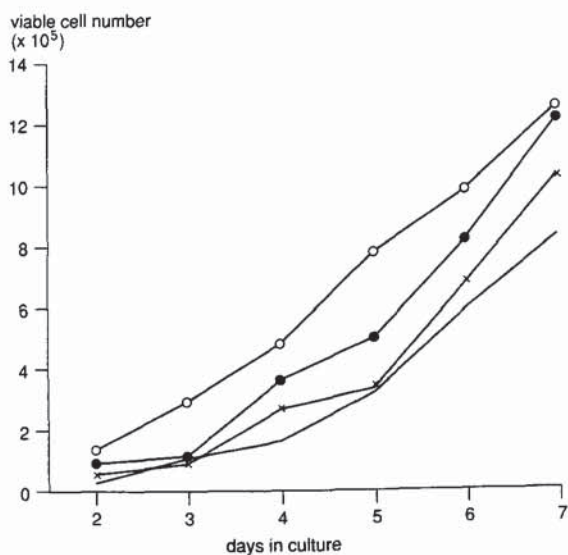


Fig. 1. Bovine serum albumin supports the growth of L929 cells under serum-free culture conditions. L929 were seeded into multiplates containing 1 mL of DME/F12 supplemented with insulin, selenium and various concentrations of BSA as follows: — without BSA; * 16 mg BSA/l; ● 80 mg BSA/l; ○ 400 mg BSA/l. Values are means of daily cell yields.

growth and to increase the maximum cell concentration. Ham describes the effect of 5 mg/mL Fraction V BSA on fibroblast growth and notes that crystalline BSA did not support growth in a comparable manner (Ham, 1984). Clark and colleagues also note that BSA was necessary for growth of MRC-5 and Vero cells in microcarrier cultures but that, while MRC-5 grew poorly when a fatty acid-free BSA was used, Vero were supported equally well by fatty acid-free and by a less pure product. They concluded that the less pure BSA contained factor(s) stimulatory for the growth of MRC-5 cells and that the general requirement for albumin could be attributed to a non-specific protective effect of the biopolymer on the cells (Clark, *et al.*, 1981). The less pure BSA is probably also acting as a lipid carrier for the MRC-5 although it may be supplying other, undefined growth factors to the culture as well. Albumin is often used deliberately as a carrier for fatty acids — albumin and oleic acid, for example, are allowed to react with each other before adding the mixture to the cell culture medium (Jäger *et al.*, 1988).

We have found that in high density perfusion cultures of hybridomas, the amount of protein secreted by the cells themselves is often high enough to allow the cells to grow, synthesise and release antibody in the complete absence of albumin. Blasey and colleagues found that albumin could be replaced by polyethylene glycol in a suitably supplemented medium (Blasey and Winzer, 1989). This apparently non-specific function of albumin is also reflected in the reports describing growth promotion by other polymeric substances (Shintani *et al.*, 1988). Similarly, the plasma expander polyvinyl pyrrolidone has been used as a supplement for the culture of rat hepatoma cells (Katsuta *et al.*, 1959). Nonionic surface active polymers, the pluronic polyols, have also been shown to stimulate the growth of a variety of mammalian cell lines, human lymphocytes and insect cells (Swim and Parker, 1960; Mizrahi, 1975; Murhammer and Gooch, 1988). It has been suggested that the pluronics and other polymers protect the cells from mechanical damage, especially in agitated culture systems, by affect-

ing the viscosity and colloidal osmolarity of the medium (Handa-Corrigan *et al.*, 1989; Gürhan and Özdural, 1990).

Lipids

Many cells, especially non-transformed ones, require a source of fatty acids in the medium. Lipids and fatty acids, in a free state or bound to carriers like albumin or lipoproteins, act as precursors for prostaglandin synthesis, represent an alternative energy source and are major components of the cell membrane. In serum, lipids are usually complexed to lipoproteins. So, as serum levels are reduced, levels of lipid in the medium fall accordingly and may even become rate-limiting. If there is no supply of exogenous lipids, some cells are able to synthesise the required lipid materials from glucose but not all cells in culture will multiply in the absence of lipids; in fact, the final test for a serum-free medium, its ability to support clonal growth (see Section III), may require the addition of lipids.

Kan and Yamane found that they needed to supply linoleic and oleic acids, cholesterol and crude lecithin to restore growth of human diploid fibroblasts in serum-free medium with delipidised Fraction V (Kan and Yamane, 1982). Saier showed that LLC-PK1 require cholesterol as well as the other growth factors (Saier, 1984). Until recently, one of the major problems in serum-free culture was that of supplying a satisfactory source of preformed lipids to the cells. If the procedure of adding BSA-complexed fatty acids (see above) is adopted this automatically leads to increased levels of perhaps undesirable protein in the medium. This method also has a certain lack of definition since the level of 'loading' of the BSA is not necessarily reproducible. Alternatively, lipids can be added to the medium dissolved in organic solvents; the disadvantage here is that such solvents are cytotoxic and could present problems when dealing with large volumes. A second problem is that once this organic lipid solution is introduced into the aqueous medium, especially a low-protein medium, the lipids will

precipitate and may be removed during subsequent filtration steps. Thus the use of a water-soluble lipid preparation which contains a range of fatty acids, lipids and lipoproteins normally found in plasma is an ideal way of introducing lipids to the cell culture in an acceptable form. We have carried out extensive studies on the water-soluble mammalian lipid preparations known as Ex-Cyte (Blasey and Winzer, 1989) and their effects in serum-free culture of a variety of cell types. In Fig. 2 the growth and productivity of the A431 human keratinocyte line is depicted as a function of BSA alone and of Ex-Cyte V, a mixture of BSA and bovine lipids. It is clear that while growth of the cells is not altered by the presence of lipids, the production of interleukin 1 by the cells responds positively to the inclusion of lipids in the medium. This is an important result because it demonstrates the pitfalls of using only one parameter as a measure of the cellular response to a particular medium. The result also suggests that lipids in the medium play a role in facilitating transmembrane release of cytokines. In an earlier publication (Hewlett *et al.*, 1989) we

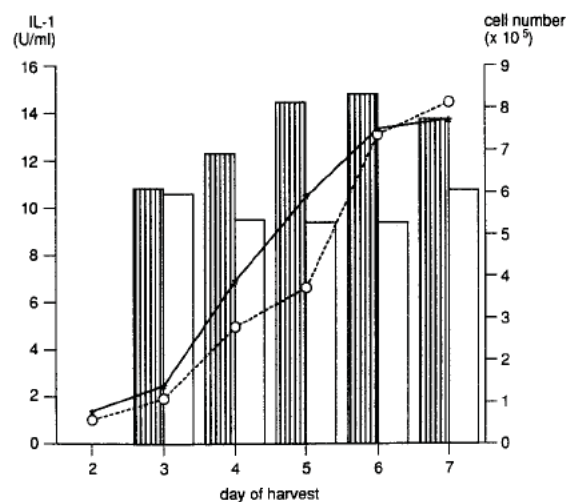


Fig. 2. Ex-Cyte lipoprotein increases the yield of interleukin 1 activity released by A431 keratinocytes under serum-free culture conditions. A431 were seeded into multiplates containing DME/F12 supplemented with insulin, selenium, BSA and Ex-Cyte lipoproteins. Open bars: BSA medium, IL-1; ○ : BSA medium, cell number; hatched bars: Ex-Cyte V medium, IL-1; * : Ex-Cyte V medium, cell number. Values are means of daily harvests.

reported that Ex-Cyte lipids and albumin act synergistically on the growth of L929 mouse fibroblast cells in serum-free medium. We also reported some studies with a hybridoma line, AHT 107, and showed that a serum-free medium supplemented with Ex-Cyte lipoprotein induced a sustained period of antibody production at a higher level than in the serum-containing control medium. Thus, the suggestion that lipids should be regarded as potential growth requirements for cells (Ham, 1981) not only appears to be valid, it also seems that, for some cells, lipids supplied in the correct form can stimulate the release of proteins.

It has been said that oxidation of lipids in the medium can be a problem. We have found that the inclusion of selenium in the medium appears to overcome this problem. Alpha-tocopherol has also been suggested as an anti-oxidant for cell culture medium (Barnes, 1987). Selenium probably functions as a component of systems such as glutathione peroxidase and superoxide dismutase which are designed to eliminate peroxides and oxygen radicals from the cellular environment.

Of the principle lipoproteins, low density lipoprotein (LDL) is a poor mitogen for normal diploid cells and is quite often toxic at >100 mg/L. High density lipoproteins (HDL) are growth stimulatory up to 1000 mg/L – this could be a mitogenic effect, a nutrient effect or even an adhesion effect (Barnes, 1987) – and the action of HDL may also be potentiated by LDL which delivers cholesterol to the cells. There may also be a species specificity for HDL and LDL although there appears to be receptor cross-reactivity between human and bovine LDL and HDL. Human LDL has been reported to react poorly with porcine and canine fibroblasts while porcine and canine LDL bind well to the receptors on human cells. This apparent species specificity shown by lipoproteins obtained from sources other than human and bovine is a factor to be considered when designing a serum-free medium (Gospodarowicz, 1984). Recently, Murakami's group has shown that the very low density lipoprotein (VLDL) in chicken egg yolk promoted the growth of a large number of mammalian cell

lines in serum-free medium (Murakami *et al.*, 1988). An interesting finding reported in the same paper is that the individual protein and lipid fractions of the VLDL are inactive in the growth assay – only the combined fractions are effective. In other words, only lipids in association with lipoproteins are effective growth promoters, a result that supports our findings with Ex-Cyte discussed above.

Insulin

Insulin is a polypeptide of 5700 molecular weight that affects the metabolism of the cell in a number of ways, although it is thought that its principle effect is on glucose metabolism. The fact that the concentration normally used in serum-free medium is approximately 1000-fold higher than the levels found in human serum has stimulated a great deal of discussion amongst cell biologists about the actual nature of its effects on the cell (Butler, 1986). Perhaps the most acceptable explanation, in view of insulin's almost universal use in serum-free media, is that free insulin is unstable in culture medium at incubator temperatures with 90 % being destroyed within thirty minutes of being placed at 37°C. We have found that some cell lines are capable of survival in the absence of insulin but their growth rate is lower than in the presence of the hormone.

More recently it has been found that the growth promoting effects of insulin can also be achieved by adding insulin-like growth factor I (IGF-I) to the culture medium at more or less physiological concentrations (Riss *et al.*, 1988).

Transferrin

Transferrin is an iron-transport protein and almost every cell line shows a positive response to its presence in the medium. One important aspect of transferrin is its ability to chelate toxic metals and it is this ability which could make it obligatory for long-term, large-scale continuous cultures in serum-free medium especially if albu-

min, also recognised for its chelating properties, is not present in the medium (Glassy *et al.*, 1988). Transferrin has also been the subject of discussion on two fronts – whether to use the human or the animal protein and whether to substitute transferrin with a simple iron salt such as ferrous sulphate. It was recently shown, for example, that hybridomas respond differently to transferrins from different species (Ill *et al.*, 1988) and that human transferrin appeared to be the most effective for all types of hybridoma. However, this conclusion does not necessarily apply to all hybridomas or even to other cell types. The second discussion is somewhat older (Walthall and Ham, 1981) and is based on the fact that only oxidised iron requires transferrin for transport into the cell; therefore, if the ferrous ion is added to the medium immediately before the cells it should be taken up independently of transferrin. However, the iron remaining in the medium will be oxidised with time so the transferrin molecule will be needed anyway to prevent an iron deficiency in the multiplying cells. Ill and colleagues have also shown that hybridomas can only respond to lipo-

philic iron chelates, such as ferric citrate, when the human genome is present in the hybrid (Ill *et al.*, 1988). We have carried out a series of tests with Madin-Darby canine kidney (MDCK) cells with respect to their requirement for transferrin in serum-free medium. One notable feature is that MDCK seeded at a relatively high density in DME/F12-based medium do not require transferrin at all and can be passaged quite independently of the transport molecule. However, a transferrin dependence is shown by the same cells when they are seeded at low density in the same medium (Fig. 3). In this situation the cells often die during the first passage in the absence of transferrin. The reason for this seeding density dependence is not at all clear but it may be more related to the detoxifying property of transferrin than to its iron transporting function.

Trace elements

Although selenium is the most prominent trace element used in serum-free media, several other

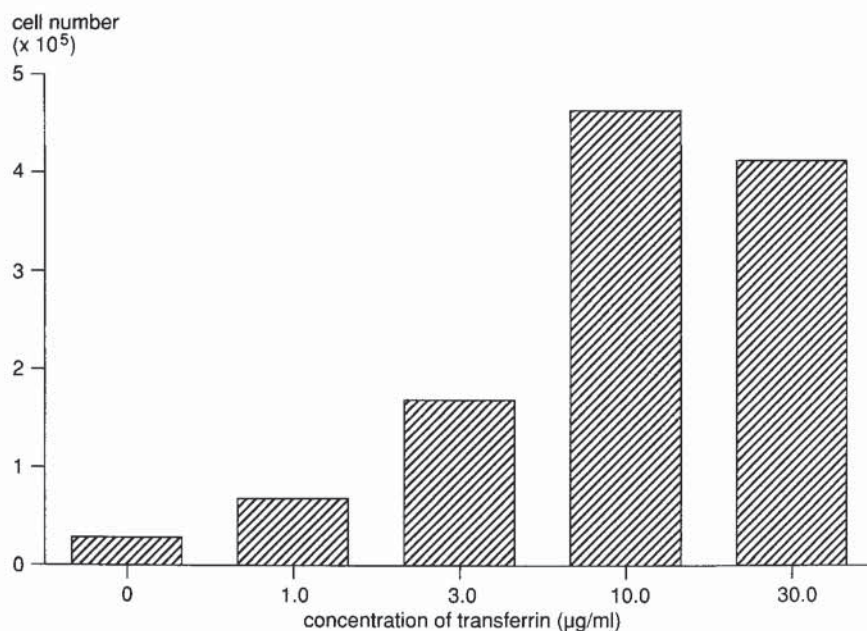


Fig. 3. MDCK respond to transferrin in a dose-dependent manner under serum-free culture conditions. MDCK were seeded into DME/F12 supplemented with insulin, selenium, human serum albumin and transferrin as shown. Values are means of daily harvests.

Table 3. Growth of HeLa and A431 cells in the presence or absence of 100 nM selenium. The serum-free medium is as described in Table 2

Cell type	Medium	Growth
Hela	0.5% FCS + Ex-Cyte + Selenium	++++
	0.5% FCS + Ex-Cyte	+
	0.5% FCS + Selenium	—
A431	Serum-free + Selenium	++++
	Serum-free	+

elements are regarded as requirements for cell growth (Ham, 1981; Maurer, 1986). Although basal medium is probably contaminated with sufficient amounts of these elements, as purity of water and quality of nutrients used for manufacturing medium improves so might it become necessary to provide the more complex mixture of trace elements (Ham, 1981).

Our experience to date has shown that selenium is the only trace element with any obvious effect on cell growth, especially of human cells. This can be seen in Table 3 which summarises the effects of selenium on the growth of HeLa and A431 cells in media with and without serum. It is obvious that selenium supports growth both in low serum and serum-free medium indicating that this trace element is maintaining a fundamental survival-related function.

Attachment and spreading factors

The requirement for attachment and spreading factors such as fibronectin or collagen is, of course, peculiar to anchorage-dependent cells, although some cell lines appear to be capable of attaching and spreading in their absence. For example, we have found that A431 epithelial cells, Vero and MDCK cells are quite capable of attachment, spreading and growth in a protein-free medium, although their principle growth parameters are down-modulated in such a simple medium (data not shown). It appears that the role of fibronectin may be to stimulate the synthesis of extracellular matrix so that the attachment-dependent cellular processes can be initiated be-

fore the cell is irreversibly damaged. Some factors appear to stimulate attachment more than spreading, or vice versa; for example, poly-D-lysine is not suitable for the clonal growth of fibroblasts because the cells are more motile and the resulting colonies are poorly defined (Greenwood *et al.*, 1989). Some anchorage-dependent cells seem to have a facultative requirement for attachment factors – for instance, we have found that when CHO-P cells are placed in a serum-free medium, they no longer attach to the plastic substrate but grow in a suspension or aggregate form with normal growth characteristics. When the medium is supplemented with serum the cells grow in an anchorage-dependent manner again, whereby the number of cells attaching to the substrate within the first 24 hr of culture is dependent on the level of serum (Fig. 4). Similarly, pretreatment of the substrate with fibronectin permits anchorage-dependent growth in the serum-free medium. This simple system also allows us to examine other components in the extracellular matrix, such as laminin and elastin, and to determine their role in the attachment process.

Conclusion

Two of the main reasons for developing serum-free medium are to improve the growth and to increase the productivity of a cell. This review has attempted to show that these goals can be achieved far more easily than generally realised by utilising the methods that have been developed during the past decade and by using a limited number of standard supplements. Serum-free me-

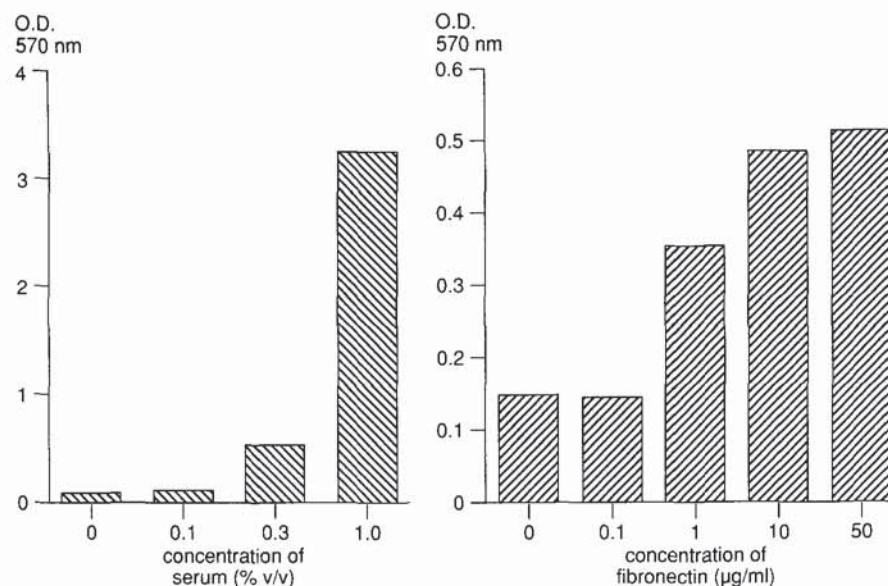


Fig. 4. CHO-P cells attach to the substrate only when attachment factors are present in the serum-free medium. CHO-P cells were seeded into DME/F12 supplemented with insulin, transferrin, selenium and human serum albumin. Various concentrations of foetal calf serum were added to the medium (left panel) or the substrate was pretreated with different amounts of fibronectin (right panel). Attachment was measured after 24–48 hours of culture by staining the adherent cells and measuring absorbance with an ELISA reader.

dia have contributed greatly to the advances made in recent years in the fields of cell biology and mammalian cell technology and it is clear that they will continue to play a major role in the clarification of cellular regulatory processes and in the refinement of processes in modern cytotechnology.

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