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Review

Optimization of chemically defined cell culture media – Replacing fetal bovine serum in mammalian *in vitro* methods

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ABSTRACT

Quality assurance is becoming increasingly important. Good laboratory practice (GLP) and good manufacturing practice (GMP) are now established standards. The biomedical field aims at an increasing reliance on the use of *in vitro* methods. Cell and tissue culture methods are generally fast, cheap, reproducible and reduce the use of experimental animals. Good cell culture practice (GCCP) is an attempt to develop a common standard for *in vitro* methods. The implementation of the use of chemically defined media is part of the GCCP. This will decrease the dependence on animal serum, a supplement with an undefined and variable composition. Defined media supplements are commercially available for some cell types. However, information on the formulation by the companies is often limited and such supplements can therefore not be regarded as completely defined. The development of defined media is difficult and often takes place in isolation. A workshop was organised in 2009 in Copenhagen to discuss strategies to improve the development and use of serum-free defined media. In this report, the results from the meeting are discussed and the formulation of a basic serum-free medium is suggested. Furthermore, recommendations are provided to improve information exchange on newly developed serum-free media.

Contents

Abbreviations: ATCC, The American Type Culture Collection; ADCF, animal-derived component-free; BSA, bovine serum albumin; CD, chemically defined; DMEM, Dulbecco minimal essential medium; DSMZ, German Collection of Microorganisms and Cell Cultures; ECACC, European Collection of Animal Cell Cultures; ECOPA, European Concensus Platform for Alternatives; ECVAM, European Centre for the Validation of Alternative Methods; EGF, epidermal growth factor; ESAC, ECVAM Scientific Advisory Committee; ESTIV, European Society of Toxicology *in vitro*; FBS, fetal bovine serum; GCCP, good cell culture practice; GLP, good laboratory practice; GMP, good manufacturing practice; INVITROM, the Dutch-Belgian Society for *in vitro* Methods; ITS, Insulin transferrin and selenium; MEM, minimal essential medium; NGF, nerve growth factor; PET, polyethyleneterephthalate; PL, platelet lysates; SFM, serum-free medium; TEER, trans-epithelial electrical resistance; 3Rs, replacement, refinement reduction of use of experimental animals.

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1. Introduction

In vitro methods are widely used tools to study physiological, biological and pharmacological activities at the cell and tissue level. In addition, *in vitro* methods are also becoming increasingly important in the production of biological components, such as hormones and vaccines. Mammalian cells are generally grown under well-established conditions in incubators, where the temperature is typically kept at 37 °C with a controlled humidified gas mixture of 5% CO_2 and 95% O_2. To achieve good experimental reproducibility, the composition of the cell culture medium is essential. The simplest medium is the classical Ringer's solution (Ringer and Buxton, 1887), which was developed as a solution with optimal concentrations of different salts to preserve frog heart muscle tissue. To maintain cells and tissues for longer periods of time, the medium should also contain components like nutrients and pH buffering substances. This type of medium was formulated by Harry Eagle, who developed Eagle's minimal essential medium (Eagle's MEM or MEM). MEM also contained amino acids, glucose and vitamins (Eagle, 1955). A similar basal medium, MEM modified by Dulbecco (Dulbecco's Modified Eagle's Medium, DMEM), is still used to maintain primary cell cultures and cell lines.

To keep cells alive for longer periods of time and to evaluate proliferation, migration and differentiation a basal medium must be supplemented with several factors. Serum, from animals or humans, is most commonly used to maintain and proliferate cells. Fetal bovine serum (FBS) serves most purposes and is the present standard. FBS is a complex mixture of different factors and contains a large number of components, like growth factors, proteins, vitamins, trace elements, hormones, etc., essential for the growth and maintenance of cells.

However, the use of FBS is controversial for a number of reasons. First of all, the collection of serum causes unnecessary suffering for the unborn calf (van der Valk et al., 2004). Secondly, seasonal and continental variations in the serum composition, produces batch-to-batch variations. This, in turn, causes phenotypical differences in the cell cultures, resulting in variations of the results. Additionally, due to the likelihood of contamination (e.g., BSE), the use of animal products is strongly discouraged for production of new biological medicinal products (Anon, 1993; Schiff, 2005; van der Valk et al., 2004). In fact, as much as 20–50% of commercial FBS is virus-positive (Wessman and Levings, 1999).

Since *in vitro* methods are among the most favoured methods to replace animal methods (Hartung, 2007), there is a demand for reliable and scientifically better defined cell and tissue culture methods including quality assurance (Gupta et al., 2005). Guidelines for good cell culture practice (GCCP), involving recommendations with respect to the use of serum-free media,

have previously been published (Coecke et al., 2005; Hartung et al., 2002). The ECVAM Scientific Advisory Committee (ESAC) has also published a statement that strongly recommends the use of serum-free substitutes for current and new *in vitro* methods (ESAC, 2008). Although, there is no legal basis for applying GCCP, it is recommended that GCCP becomes part of good laboratory practice (GLP) and good manufacturing practice (GMP).

A workshop, to discuss the possibilities to reduce the use of FBS in cell and tissue culture was organised in 2003 (van der Valk et al., 2004). The report from this meeting provides clear recommendations to reduce or stop the suffering of live unborn bovine calfs from which blood is drawn for the production of FBS. Ethical, safety and scientific grounds were also given for the replacement of FBS and other animal components in cell and tissue culture methods. In 2009, a follow-up workshop was organised to discuss current *in vitro* methods devoid of FBS, or other animal components. The workshop, held in Copenhagen, Denmark, was organised under auspices of the European Society of Toxicology *In Vitro* (ESTIV), the Dutch-Belgian Society for *In Vitro* Methods (INVITROM) and the Danish *in vitro* Toxicology Network. The results from this workshop clearly demonstrate the possibilities to grow a number of different primary cell and tissue cultures as well as cell lines without the use of animal products. Furthermore, directions were provided on how to develop a serum-free, chemically defined, culture media for mammalian cell and tissue cultures in basic and applied research.

This report aims at discussing the advantages of defined cell culture media and to give directions for the development of a basic defined media for a wider audience.

2. Development of a serum-free medium

The attempts to grow cells date back for at least 50 years (Pumper, 1958; Waymouth, 1955). Early attempts to grow cells in serum-free, hormone-supplemented media were performed to understand the role of serum in cell culture media. The efforts to identify all the serum components that are physiologically relevant to maintain proliferation of cells in culture, and the attempts to replace the serum with its defined components, were not successful (Taub, 1990). Since then, several different serum-free formulations have been developed where the media are supplemented with approximately 10 essential components (Pazos et al., 2004). About 10–20% of these strategies appeared to be successful (Pazos et al., 2004).

The pioneering work by Hayashi and Sato (1976) replacing serum by the addition of selected hormones, promoting growth and stimulating differentiation of specific cells, led to the development of a good chemically defined, serum-free media (see Box 1) (Barnes and Sato, 1980a,b; Bjare, 1992; Grillberger et al., 2009; Gstraunthaler, 2003; Taub, 1990). In the last 10 years, investigations into cell function have led to the identification of a growing number of components which have been useful in the development of modern serum-free cell culture media. Many transformed or newly transfected cell lines can successfully be maintained in these enriched serum-free media without adaptation and the number of cell-specific media is growing steadily. Now, more than 100 different available serum-free media formulations have been developed and can be readily used without great investments in time and money to develop one's own (Zähringer, 2009).

Box 1 Culture media

- **Serum-free media:** serum-free media do not require supplementation with serum, but may contain discrete proteins or bulk protein fractions (e.g., animal tissue or plant extracts) and are thus regarded as chemically undefined (see: chemically defined media).
- **Protein-free media:** protein-free media do not contain high molecular weight proteins or protein fractions, but may contain peptide fractions (protein hydrolysates), and are thus not chemically defined. Protein-free media facilitate the down-stream processing of recombinant proteins and the isolation of cellular products (e.g., monoclonal antibodies), respectively.
- **Animal-derived component-free media:** media containing no components of animal or human origin. These media are not necessarily chemically defined (e.g., when they contain bacterial or yeast hydrolysates, or plant extracts).
- **Chemically defined media:** chemically defined media do not contain proteins, hydrolysates or any other components of unknown composition. Highly purified hormones or growth factors added can be of either animal or plant origin, or are supplemented as recombinant products (see: animal-derived component-free media).

2.1. Basal medium

With time, it has become clear that almost every cell type has its own requirements concerning medium supplements. Therefore, a universal (serum-free) cell and tissue culture medium may not be feasible. Different cell types have different receptors involved in cell survival, growth and differentiation and release different factors to their environment.

The threshold for developing or using a new (defined) medium when the current FBS containing medium works well, is high for obvious reasons. In order to aid in this process, a strategy for the development of new media will be discussed below.

It is recommended to start a new formulation with a 50:50 (v/v) mixture of DMEM and Ham's nutrient mixture F-12 (Ham, 1965). This medium formulation combines the high amino acid content of DMEM with the highly enriched Ham's F-12 (Barnes and Sato, 1980a,b; Jayme et al., 1997). Furthermore, the basal medium must contain an essential, so called, ITS supplement (insulin, transferrin and selenium). Insulin, the first of the components of the ITS supplement, has been known to be essential in cell culture from 1924 and is now the most commonly used hormone in culture media (Gey and Thalhimer, 1924). Transferrin is also an essential protein in culture medium where the main action is to transfer iron into the cells (Bjare, 1992). Selenium is an essential trace element and acts in particular in selenoproteins which protect cells against oxidative stress (Helmy et al., 2000).

2.2. Supplements

Although some cell types can be maintained in the basal medium (Bettger and McKeehan, 1986; Butler and Jenkins, 1989), most cells need additional supplements to survive, proliferate and/or differentiate. The most commonly supplied components will be discussed below.

2.2.1. Hormones

All hormones of mammalian organisms are physiological constituents in blood circulation and are thus also present in serum

in varying amounts (Lindl and Gstraunthaler, 2008; Price and Gregory, 1982). Supplementation with hormones was therefore a first step in the development of serum-free media (Barnes and Sato, 1980a,b; Hayashi and Sato, 1976). Insulin has been shown to be obligatory in all serum-free media formulations. Other hormones most widely used in serum-free cell culture are glucocorticoids (dexamethasone and hydrocortisone), triiodothyronine (T_3), and hormones that cell-specifically act by increasing intracellular cAMP levels (see Section 2.3). Water-soluble complexes of steroids are commercially available.

2.2.2. Growth factors

Growth factors are generally added to the basal medium to increase cell proliferation and to stimulate specific cell functions. Traditionally, growth factors and other supplements are added as bulk in the form of fetal bovine serum (FBS).

Most growth factors are highly cell type specific. Others are of more general use and can also have positive effects on several different cell types. Fibroblast growth factor-2, for example, has a positive effect on the phenotype of chondrocytes cultured in serum-free medium (Mandl et al., 2004). Some cells in culture may release growth factors thereby stimulating their own proliferation and that of other cells (Gospodarowicz and Moran, 1976).

2.2.3. Protease inhibitors

The protease inhibitors that are introduced by the addition of FBS are α_1-antitrypsin and α_2-macroglobulin (Gstraunthaler, 2003). The inhibitors terminate the trypsination process and act beneficially by inhibiting lysosomal peptidases that may occasionally be released during cell turnover. Protease inhibitors thus have a protective effect on cells, but are not essential. When no protease inhibitors are supplied, one should carefully assess the trypsin concentration.

2.2.4. Protein hydrolysates

Protein hydrolysates are used to deliver amino acids and small peptides. These are not essential in cell culture and the effect is somewhat controversial. In fact, some studies report a beneficial effect in cell cultures (Burteau et al., 2003; Schlaeger, 1996), while other studies demonstrated that protein hydrolysates do not support cell growth and that higher concentrations actually reduce cell growth (Keay, 2004). Protein hydrolysates are chemically not defined (see Box 1) and may cause problems in reproducibility and comparability of experiments.

2.2.5. Shear force protectors

Turbulence in bioreactors and perfusion cultures cause shear stress in cells. Serum protects cells from this shear force (Elias et al., 1995; van der Pol and Tramper, 1998). Pluronic F68 has a similar effect (Zhang et al., 1992), but is not essential for ordinary cell cultures.

2.2.6. Proteins

Proteins are carriers for different low molecular weight components and may facilitate cell adhesion (Taub, 1990). Bovine serum albumin (BSA) is often used as a lipid carrier. However, BSA is derived from animals and may either be contaminated or may contain impurities (Taub, 1990). Nowadays, recombinant proteins, including albumin, are available for animal component-free cell culture (Keenan et al., 2006).

2.2.7. Vitamins

Vitamins are provided by the basal medium. At least seven vitamins were found to be essential for cell growth and proliferation: choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine (Bjare, 1992; Butler and Jenkins, 1989; Taub, 1990). B-vitamins are necessary for cell biochemistry, and are also present in DMEM as well as in Ham-F-12.

2.2.8. Amino acids

All 13 essential amino acids are necessary for culturing mammalian cells (Arg, Cys, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val) and are present in high concentrations (0.5–4 mM) in DMEM. The seven non-essential amino acids (Ala, Asn, Asp, Glu, Gly, Pro, and Ser) are provided by Ham's F-12.

2.2.9. Glutamine

Glutamine is an essential precursor for the synthesis of proteins and ribonucleotides. It is also important respiratory fuel for rapidly dividing cells and cells that use glucose inefficiently (Glacken, 1988; Reitzer et al., 1979; Zielke et al., 1984). However, glutamine also has its drawbacks: it is unstable in solution, and glutamine breakdown and metabolism result in the production and accumulation of ammonia, which is toxic to cells (Schneider et al., 1996), since it is not absorbed by serum proteins in serum-free and/or protein-free media. To overcome these disadvantages, alternatives for the use of glutamine in culture media were developed. Glutamate, for example, can replace glutamine in cell cultures that express sufficient glutamine synthetase activity. A more recent invention is the use of glutamine-containing dipeptides, alanyl-glutamine and glycyl-glutamine, commercially available under the trade name GLUTAMAX™ (Christie and Butler, 1994). These dipeptides are more stable and heat resistant, which even makes it possible to autoclave the media that contain these molecules. The dipeptides are intra- or extracellularly cleaved by peptidases, thereby releasing glutamine and either alanine or glycine. The availability of glutamine is therefore dependent on the peptidase activity, which results in lower rates of glutamine consumption and ammonia production. GLUTAMAX™ can be substituted for glutamine on a 1:1 M basis.

2.2.10. Trace elements

Most trace elements are available in the basal medium since Ham's F-12 is qualitatively rich in necessary trace elements (Ham, 1965).

2.2.11. Lipids

The role of fatty acids and lipids in cell culture has long been neglected. Lipids serve as energy stores, as structural constituents of cellular membranes, and in transport and signalling systems. Some lipids are available in the basal medium. However, essential fatty acids and ethanolamine are recommended as supplements. Water-soluble supplements are commercially available. Serum albumin is a carrier of fatty acids and lipids (see Section 2.2.6).

Essential fatty acids are components of several serum-free media formulations.

2.2.12. Antibiotics

Wherever possible, the use of antibiotics should be avoided (Kuhlmann, 1996). Antibiotic-resistant microorganism may develop, and antibiotics may also have adverse effects on cell growth and function.

2.2.13. Attachment factors

Most mammalian cells need a special culture substratum for cell attachment in order to survive and grow *in vitro*. The plastic culture dish, that is specifically treated to introduce charge and hydrophilicity into the polystyrol surface, e.g., with poly-L(or D)-lysine or ornithine, is the most commonly used substrate for cell attachment. Coating the plastic dishes with other substrates like extracellular matrix components (Kleinman et al., 1987) or

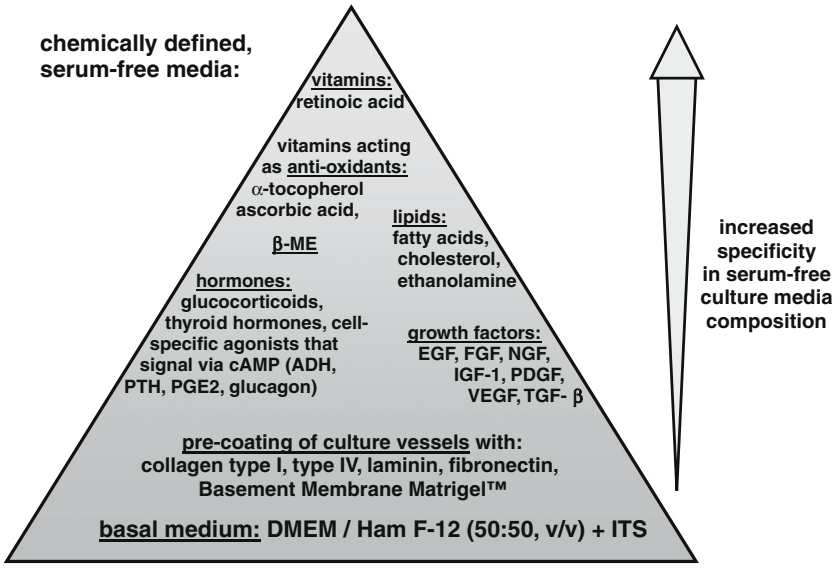

Fig. 1. Media pyramid: a modular approach for the development of serum-free media (for details see Section 2.3). Abbreviations: ADH, antidiuretic hormone; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor 1; ITS, insulin–transferrin–sodium selenite supplement; β-ME, β-mercaptoethanol; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; PTH, parathyroid hormone; TGF-β, transforming growth factor-β; and VEGF, vascular endothelial growth factor.

collagenous matrices (Kleinman et al., 1981) further facilitates the adhesion of anchorage-dependent cells.

2.2.14. Osmolarity

Although mammalian cells express a reasonable wide tolerance to osmolarity, osmolarity should always be carefully checked and compensated for when adapting to a new cell culture formulations.

2.3. "Building" a serum-free medium

As shown above, to exclude FBS from a cell and tissue culture medium, and still maintain cell adhesion, growth and proliferation it is important to include a large number of several components in the cell culture medium. In Fig. 1, a schematic modular approach for the development of serum-free media is shown as a "media pyramid". The bottom of the pyramid contains the basal medium, which includes DMEM/Ham's F-12 (50:50, v/v), supplemented with insulin–transferrin–selenium (ITS). To make adherent cells stick to the bottom of the culture vessel, coating with components of the extracellular matrix should be considered. Cell attachment factors are often required for serum-free culture.

The next step in media formulation development is the addition of specific hormones and growth factors. Epidermal growth factor (EGF) and glucocorticoids (hydrocortisone and dexamethasone), for example, are present in most media. Depending on the cell type, additional cell-specific growth factors may also be needed, like nerve growth factor (NGF) for neurons. It has been demonstrated that cultures of epithelial cells need a supplementation with agonists, that specifically elevates cellular cAMP levels (Gstraunthaler, 2003). In this respect, also forskolin and cholera toxin, although acting as strong pharmacological agents, were used as *in vitro* mitogens.

The tip of the pyramid represents increased specificity in serum-free media composition: the addition of lipids, antioxidants and/or specific vitamins. Retinoic acid (vitamin A) is an additive required in cell culture media for a number of epithelial cell types. Vitamin E (α-tocopherol) and ascorbate (vitamin C) are presumably acting as antioxidants. Other antioxidants found in serum-free media formulations are β-mercaptoethanol (β-ME) and selenium (see above).

2.4. Adaptation of cell lines to serum-free medium

There are several approaches to adapt cultured cells to a serum-free medium (Fig. 2). Typically, a cell culture has to undergo a gradual weaning process which involves progressive adaptation to lower serum concentrations until serum-free conditions are reached. The cultures to be adapted should be in the logarithmic phase of growth and should have viability over 90%. However, one should also keep in mind that an unwanted selection of a change in the population of cells, during the adaptation process, by indirectly selecting cells capable to grow in serum-free media, may occur. Therefore, it is necessary to check the performance of cultures and to monitor cellular morphology and function during weaning.

In order to aid the process of weaning several adaption protocols are listed below (and in Fig. 2):

2.4.1. Reduction of serum content

In this protocol, serum content is reduced at each passage until 0.1% serum is reached. After cultivation in normal medium containing 10% FBS, the consequent serum reduction steps (from 5% to 0.1% FBS) are carried out in serum-free, hormone-supplemented medium.

2.4.2. Sequential adaptation

Similar to protocol 1, cells are passaged into mixtures of serum-containing and serum-free media, until complete serum-free condition is reached. If the last step, changing from 75% to 100% serum-free, is too stressful for the cells, it is recommended to keep the cell culture in a 10% serum-containing and 90% serum-free medium mixture for 2–3 passages, before switching to a complete serum-free medium.

2.4.3. Adaptation with conditioned medium

This adaptation follows protocol 2, however, cells are passaged into decreasing mixtures of conditioned media from the passage before.

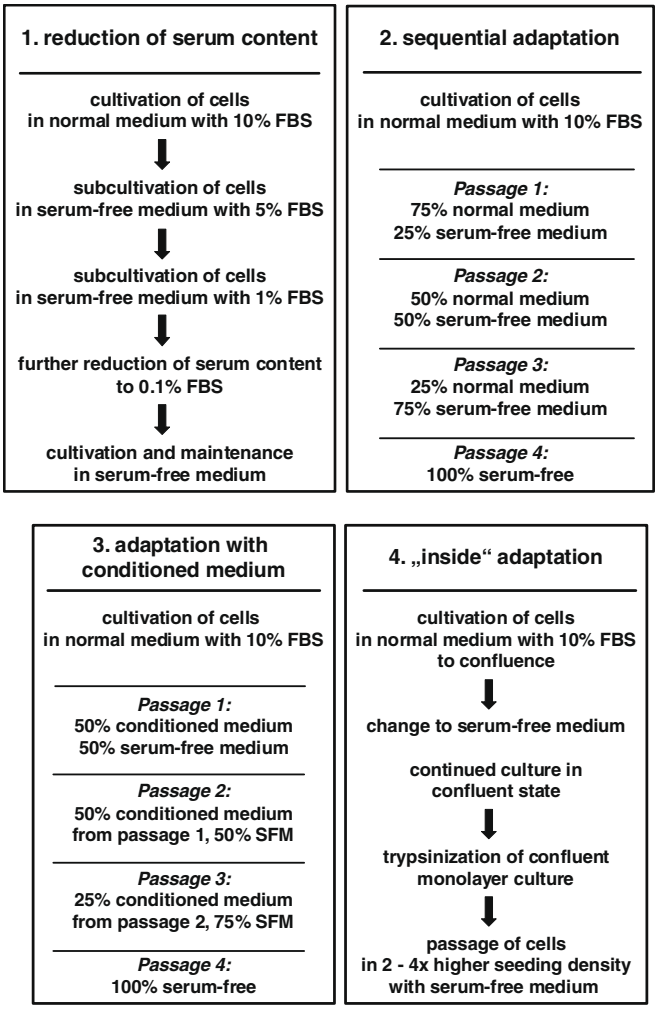

Fig. 2. Adaptation of cultures to serum-free medium. A comparison of the most common adaptation protocols (FBS: fetal bovine serum and SFM: serum-free medium).

2.4.4. Inside adaptation

In this protocol, freshly seeded cells are weaned in serum-free medium, and cultures are grown to confluence. The confluent monolayer is then passaged into serum-free medium.

3. Promoting the development and use of serum-free media

3.1. Information sources

Before using the experimental approach to set up a serum-free medium for a given cell type, cell lines or tissue culture, a search for already existing media formulations should be performed This can be done by a thorough literature survey, or by a search in a recently established serum-free media online database (see Section 3.2).

There are several databases that contain information on commercially available serum-free media formulations and supplements (Anon, 2009a,b,c). Approximately 450 different serum-free cell culture media formulations are now commercially available, but only for a limited number of cell types (Anon, 2009a,b,c). Regrettably, the formulations of specific supplements for the commercially available media are generally not available, and those can therefore not be considered as fully defined media. Such formulations have also been changed without informing the users (Chen et al., 2008; Cressey, 2009), and supplements with the same name may differ in formulation between suppliers.

Today, the information on available serum-free media formulations, particularly when these are not commercially available, is unfortunately scarce. Nevertheless, development of serum-free media and cell adaptation protocols are ongoing processes in several laboratories, often without knowledge about research processes, experiences and results of other laboratories regarding this topic. This is partly due to a lack in communication between labs and particularly the lack of a common forum where such formulations could be posted. It is recommended that these obstacles must be overcome in order to encourage future use and development of serum-free media.

It is further recommended to collect formulations of "in lab developed" media in databases, where access to reliable protocols including detailed formulations, should be free. It is also recommended to publish established protocols in dedicated online databases like Springer Protocols and Nature Protocols. When publishing studies with newly developed serum-free formulations specific keywords should be used in the publication to enable easy retrieval of the publications. Key words like *3R, serum-free media* or *defined media* are recommended.

3.2. The serum-free media interactive online database (D. Brunner)

To make the search for serum-free media easier, a new collection of commercially available serum-free media has been developed in a free accessible unique interactive online database (Brunner et al., 2010; Falkner et al., 2006).

Specifications of serum-free media (i.e., ability to maintain cells of specific organism, organs, tissue, cell type and disease) were collected and systematically arranged with respect to specific standards (ICD 2007 of WHO and ITIS). Additional commercially available cell lines, hybridoma and primary cells from ATCC, ECACC and DSMZ are included in the database to allow a "reverse search" by specifying the used cells to gain a serum-free medium. This search modus is based upon comparison of specifications and can also be used to find most similar serum-free media.

Furthermore, the degree of chemical definition, e.g., serum-free (SFM), animal-derived component-free (ADCF) or chemically defined (CD), and the kind of medium, e.g., basal media, media supplements, or full replacement media can be selected. Presently, 452 serum-free media and 4817 continuous cell lines, hybridoma lines and primary cultures from ATCC, ECACC and DSMZ that are commercially available are included in the database. Despite extensive search for serum-free media and adapted cell lines, there is still a lack of detailed information from companies and suppliers. It is intended to open the database for interactive exchange of information and guidelines from experts in the field in order to continuously improve and extend the serum-free online database. The database is accessible at http://www.goodcellculture.com.

3.3. Validating new media and adapted cells

In a statement on the use of FBS and other animal-derived supplements (ESAC, 2008), the ECVAM Scientific Advisory Committee strongly argues for the development of new serum-free *in vitro* culture methods. Furthermore, when an *in vitro* method using serum-containing media is presented to ECVAM for validation, a justification for using serum must be provided. To promote the use of serum-free media, ECVAM (European Centre for the Validation of Alternative Methods) will encourage the submitters of new tests systems for validation studies to make their protocols public if their model is designed under serum-free conditions. Existing culture methods where animal components are being replaced, should be validated against serum-containing media to ensure that

the original endpoints are not affected. When serum is being replaced in an already validated *in vitro* method, the new method should be validated in a catch-up study (Hartung et al., 2004).

On the ECVAM web site (http://ecvam.jrc.ec.europa.eu) a special discussion forum will be opened in the near future for the sharing of information which is relevant to serum-free culturing, particularly in the context of validation studies.

3.4. Other activities

Other incentives to promote the substitution of FBS could be provided by funding institutions requesting a priori use of serum-free media. Workshops and conferences on *in vitro* culturing and processing should also be encouraged to host specific seminars and poster sections with emphasis on exchange of experiences related to serum-free media. Seminars could be prepared by working groups within the scientific cell and tissue cultures societies as e.g., ESTIV. In addition, the value of serum-free cell and tissue culture should be educated and be part of basic culture techniques education and training (Coecke et al., 2005; Hartung et al., 2002; Lindl and Gstraunthaler, 2008).

Support for coordinated actions, promoting exchange of information related to specific cell and tissue cultures should be encouraged at national, Europeans well as international, levels by the announcement of project calls, etc.

4. Examples of serum-free studies

In the following sections, participants of the workshop describe ways in which serum substitutes are developed and how specific cells are cultured in serum-free media.

4.1. Human platelet lysates as a serum substitute in cell culture media (G. Gstraunthaler)

Below, the use of human platelet lysates (PL) as a serum replacement is reported. PL was prepared from outdated human donor thrombocyte concentrates. Maximum activation of thrombocytes was achieved by freeze–thawing in hypo-osmolar saline. The extent of the release of platelet granule growth factors was determined by ELISA and Western blotting (Rauch et al., 2008; Rauch et al., 2009). The growth promoting and mitogenic capacity of PL was tested on a broad selection of continuous cell lines, for which growth characteristics, phenotypes, and differentiation endpoints are well-established (Gstraunthaler, 1988). PL in DMEM support growth, proliferation and differentiation, as assessed by dome formation of proximal tubule-like LLC-PK_1 (porcine kidney) and HK-2 (human kidney) cells, whereas distal tubule-like MDCK (dog kidney) cells grow well in serum-free DMEM/Ham-F-12 supplemented with platelet extracts (Gstraunthaler et al., 1985). In addition to adherent epithelial cell lines, anchorage-independent Raji human lymphoma cells were investigated. PL fully supported growth and proliferation of Raji cells in suspension. Proliferation was monitored in all cell lines by determination of cell density of epithelial cultures (cell number per growth area) (Pfaller et al., 1990), and by resazurin or WST-8 assays. Rates of growth and proliferation were comparable between media conditions with 10% FBS or 5% PL, respectively. Serum-free media served as negative controls. In order to biochemically determine the proliferative potential of PL, the stimulation of extracellular signal-regulated MAP kinase (ERK1/2) was determined (Feifel et al., 2002). Activation of the MAP kinase signalling pathway by GR leads to specific phosphorylation of downstream kinases, like ERK1/2. Addition of PL to quiescent LLC-PK_1 cultures resulted in specific phosphorylation, and thus activation, of ERK1/2 within minutes. The time course is identical with ERK1/2 activation upon addition of FBS.

The data show the high potential of PL as a valuable substitute for FBS in mammalian cell and tissue culture.

4.2. Serum-free aggregating brain cell cultures (P. Honegger)

Thirty years ago, the original method for the preparation and maintenance of aggregating brain cell cultures in serum-free chemically defined medium was published (Honegger et al., 1979). The cultures derived from embryonic rat brain and maintained as suspension culture under continuous gyratory agitation in a chemically defined medium, reproduced critical morphogenic events such as migration, proliferation, differentiation, synaptogenesis, and myelination. This enabled reconstitution of highly differentiated histotypic brain structures and functions (Honegger and Monnet-Tschudi, 2001). This approach also enabled studies into the role of growth factors and hormones in brain development and offered a suitable model for neurotoxicological investigations, including the study of developmental neurotoxicity and long-term (chronic) toxicity of chemicals (Forsby et al., 2009; Monnet-Tschudi et al., 2000; Zurich et al., 2003). The advantages of this 3D cell culture system include a high yield, robustness, easy handling, and excellent reproducibility. Nevertheless, careful analyses showed that despite their excellent features, serum-free aggregating brain cell cultures did not reach the same level of maturation as their counterparts grown in the presence of FBS. The most significant differences were found in the level of myelination and in the frequency and intensity of spontaneous electrical activity. None of the various commercialized serum substitutes could further improve the maturation of aggregating brain cell cultures. Similar observations were reported by others working with cell lines. Although it was possible to adapt various cell lines to serum-free medium, they showed increased fragility and altered growth characteristics in comparison with the original cell lines grown in serum-containing medium. New attempts were undertaken to identify the factors, presumably present in serum, that were able to further enhance brain cell maturation. The current working hypothesis is that the missing factors are lipid-soluble constituents such as cholesterol, which is difficult to handle in aqueous solutions and in isolated form. Interestingly, it was observed that the dialyzed serum was as effective as complete serum, and it was thus assumed that one or several macromolecular factor(s) have beneficial effects. The different lipoprotein fractions are currently isolated from serum (i.e., fetal calf serum, newborn calf serum, and human plasma), and their activity in aggregating brain cell cultures derived from 16-day embryonic rat brain is investigated. The isolated fractions are examined for their beneficial effects on neuronal and glial cell maturation in these cultures. If essential macromolecular serum component(s) are identified which reproduce the developmental effects of serum, these will be extensively tested in brain cultures and in different cell lines. When they are proven successful, synthetic production will be explored, e.g., by animal-free recombinant DNA technology.

4.3. Organotypic brain slice cultures and defined serum-free medium Neurobasal with B27 (J. Noraberg)

Organotypic brain slice cultures can be grown for weeks and months, preserving their basal cellular structure, composition and connections, and have been increasingly used as models to study mechanisms and treatment for neurodegenerative disorders (Noraberg, 2009; Noraberg et al., 2005). Organotypic brain slice cultures have been grown since the early 1980s, first as roller tube cultures, with each culture embedded in a plasma clot on a coverslip in a test tube placed in a roller drum (Gahwiler, 1981),

and later, in the early 90s mostly as interface cultures, where the slices grow on a porous membrane in the interface between medium and air (Stoppini et al., 1991). In the mid 1990s, the Serum Optimem with 25% horse serum culture medium was replaced by the defined serum-free Neurobasal medium with B27 supplements (Brewer et al., 1993). This medium is changed 2 days after culture start up in Serum Optimem (Noraberg et al., 1999). The change to a serum-free medium was made to avoid the effects of unknown growth factors and the variability in the different batches of serum. Neurobasal with B27 supplement worked well and produced excellent cultures until the beginning of 2000 (Bonde et al., 2003), when necrotic holes in the cultures were found. After a thoroughly evaluation it was decided to grow the cultures in Serum Optimem and at the lower temperature of 33 °C (compared to the earlier 36 °C) for the first 2–3 weeks. After that, the cultures were changed to Neurobasal with B27 and 36 °C 1 day before experiments were performed. This has worked well until recently (Montero et al., 2009), when it was discovered that just changing to Neurobasal with B27, 24 h before studies of oxygen–glucose deprivation (OGD), induced a high and significant amount of cell death compared to Serum Optimem in cultures just submersed in control medium for the standard time of 30 min. Now, the cultures are kept in Serum Optimem for the entire time of culture, though good replacement for this serum-containing medium is preferred. Other groups have experienced similar difficulties using B27 (Cressey, 2009), and an American group has recently published the recipe for a new supplement, N21, a re-defined and modified formulation of B27 (Chen et al., 2008). Sigma–Aldrich has just announced that they, in the beginning of 2010, will launch a series of complete media for neuronal and stem cell cultures, Stemline™, where all necessary supplements are included. Using Neurobasal + N21 and the complete media from Sigma–Aldrich, cell viability will be tested and compared with Serum Optimem in two types of cell cultures present in our laboratory: organotypic brain slice cultures and primary neuronal cultures (see 4.4).

4.4. Defined medium and serum-containing medium occasionally induce cells to use different signal transduction pathways to proliferate (Å. Fex Svenningsen)

Dissociated cultures made from different parts of the rat or mice fetal central and peripheral nervous system are useful and perhaps necessary tools when investigating cell–cell interactions or responses to toxic agents. Such cell culture systems have been developed and used to study neuronal development as well as toxic effects on neuronal development (Svenningsen et al., 2003). Some of these cell cultures can be grown for up to 3 months, developing synapses, robust myelination and astrocytic networks in *defined* medium. The different cell cultures were developed with the aim to keep all cells normally present in the nervous system in the culture, to make the environment as "*in vivo*-like" as possible and to use a defined cell culture medium to be able to control the cellular environment and avoid variations from various batches of serum.

Neurobasal medium supplemented with B27 (Brewer et al., 1993) was chosen because, apart from being completely defined, it enables long-term culture without fibroblast or astrocyte overgrowth. However, using a defined medium instead of one containing serum to grow primary cultures sometimes produces disparate, inconsistent results (Svenningsen and Kanje, 1998). e.g., our preliminary data show that some glial cells grown in defined media, together with neurons, use other signal transduction pathways to proliferate than cells grown in serum-containing media. Which signal transduction pathways these cells use for proliferation "*in vivo*" is thus not yet clear.

In order for primary cell culture to be an useful tool, where cell interactions *in vitro* can be investigated and results can be relied on, it is necessary to compare *in vitro* with *in vivo* results, and to adjust media and cells to what is most *in vivo*-like.

4.5. Optimization of culture conditions for human intestinal Caco-2 cells to improve functional differentiation in serum-free media (M.L. Scarino)

Although representing the best and most extensively used cell culture model of absorptive enterocytes, the human Caco-2 cell line displays a high degree of heterogeneity in the expression of differentiated functions that is largely due to differences in culture procedures (Sambuy et al., 2005). Fetal bovine serum (FBS) in the culture medium is an important source of variability in the performance of this differentiated cell model and several attempts have been made over the years to cultivate these cells using serum-free media.

Halleux and Schneider developed, in 1991, a serum-free medium for Caco-2 cells that was adapted from a nutritive medium studied for hepatocytes (Basal Defined Medium – Gibco) supplemented with insulin, epidermal growth factor, albumin–linolenic acid, hydrocortisone and triiodothyronine (T3) (Halleux and Schneider, 1991). Cells were grown on polyethyleneterephthalate (PET) filters coated with collagen, as a model of the intestinal barrier, and showed after 15 days in culture many differentiated functions of enterocytes, correct polarization and a decrease of paracellular permeability, indicating the establishment of functional tight junctions. Shortly after, Jumarie and Malo obtained differentiated Caco-2 seeded on plastic substrate using DMEM supplemented with ITS (Jumarie and Malo, 1991). This defined medium allowed for normal differentiation of the cells after 3 weeks, as shown by morphological and functional characteristics. Only sucrase activity was lower than in cells cultivated with serum, but was increased by addition of T3. A serum-free medium containing ITS and lipids (modified from Jumarie and Malo (1991)) was used to differentiate the parental Caco-2 line and three clonal lines cultivated on PET filter inserts and the permeability characteristics were investigated. Medium composition did not affect the establishment of trans-epithelial electrical resistance (TEER) while an increase in paracellular permeability to the extracellular marker mannitol was observed in cells grown in serum-free conditions as compared to cells grown in serum supplemented medium (Ranaldi et al., 2003). Increased paracellular permeability was also observed by Gangloff et al. that used Caco-2 cells differentiated in serum-free medium to investigate iron uptake (Gangloff et al., 1996).

In the attempt to improve the performance of the ITS-lipid medium, an investigation is in progress using Caco-2 cells grown for up to 21 days on permeable polycarbonate cell culture inserts. Cells were allowed to differentiate in media containing different supplements: (A) insulin, transferrin, selenium (ITS) and lipid mixture (oleate, palmitate, cholesterol and BSA as carrier); (B) a defined mixture of growth factors and hormones derived from ITS (MITO + serum extender), and; (C) MITO + serum extender and lipid mixture. Since the supplements were added only to the basolateral compartment, the control conditions included medium supplemented with 10% FBS in both compartments (symmetrical control) or in the basolateral compartment (asymmetrical control). The effects of the different serum substitutes on cell differentiation were assessed by permeability assays, gene expression studies, apical enzyme assays (alkaline phosphatase and sucrase) and transport activity of P-glycoprotein. Preliminary results indicate that Caco-2 differentiation occurred under all tested conditions, although distinct endpoints responded differently to the various serum substitutes (Ferruzza et al., 2009). Thus, a single recipe for optimal Caco-2 differentiation in defined medium cannot yet be

recommended and further studies are required to define the role of single supplements in this process.

5. Conclusions

The use of serum to enable cell and tissue cultures is problematic, both for ethical and scientific reasons. It is therefore recommended to change from serum supplemented cell and tissue culture media to serum-free media, with, preferably, animal component-free and chemically defined supplements, by using already existing formulations and developing formulations for cells and tissues where SF media do not yet exist.

It is therefore recommended to apply the "No, unless..." principle: no supplementing with serum, unless a SF medium has not yet developed. In which case, all efforts should be undertaken to develop a SF medium for the particular cell or tissue culture. Information on existing SF media formulations is readily available in databases, which should be continuously fed with newly developed formulations.

Several components are essential, and others useful, when developing SF media. Furthermore, several approaches are available to adapt cells to the new SF medium. Special care should be taken to the validation of using the new medium so that the original studied biomarkers are still expressed by the cells and tissues. Successful approaches to develop SF media, also for specific cell types and cultures, are described. The described models show that the development of SF media is not an easy task and that several hurdles, which may be dependent on the cell type and the culture system used, have to be taken. Extensive information exchange, through databases, meetings/workshops and electronic networks will facilitate and stimulate the development of new SF media formulations.

6. General recommendations

(1) When considering supplementing cell and tissue culture media with animal serum the "No, unless..." principle should be applied. Preferentially, the medium should not contain any animal-derived component, unless it was proved to be an absolute requirement.
(2) For scientific arguments, cell and tissue culture medium should be chemically defined.
(3) In particular *in vitro* methods that are used in a regulatory testing context should be based on a chemically defined culture medium.
(4) To use defined media is a strong recommendation from ESAC and the GCCP. Since GCCP has no legal basis, it is strongly recommended to make it part of GLP and/or GMP.
(5) The use of (fetal bovine) serum as supplement in *in vitro* studies has to be justified.
(6) Many media supplements are commercially available, but the formulation of these should be chemically defined.
(7) For *ethical* reasons, FBS should be replaced by supplementing with defined chemicals (e.g., recombinant components) or animal or plant extracts.
(8) For *scientific* reasons, FBS should be replaced by defined supplements, which should have a non-animal origin when human safety is at stake.
(9) SOP's of established SF and, preferably, chemically defined media should be made readily available through publications and databases.
(10) It is recommended that a platform is established for serum-free media developers and users to discuss experiences, to facilitate further progress and to organise meetings on this subject.

7. Recommendations for developing serum-free cell culture media

(1) When developing FBS-free media, start with an appropriate basal medium. A 50:50 (v/v) mixture of DMEM and Ham's-F-12, supplemented with ITS has been successfully used in numerous studies.
(2) When glutamine is used, it should be added at a concentration of 2–4 mM. Also Glutamax I™ (L-Ala–L-Gln) can be supplemented for some cell lines.
(3) Supplement with cell type specific growth factors, hormones, vitamins, trace elements and lipids wherever necessary.
(4) Pay attention to osmolarity.
(5) For some studies or cell types, specific proteins may be added.
(6) Some essential fatty acids, not present in the basal medium, may have to be added.
(7) Preferably, no antibiotics should be used.
(8) For some cell types and primary cultures a substrate for attachment may be used. Many serum-free formulations require a pre-coating of culture dishes.
(9) For bioreactors and perfusion cultures a shear force protector (Pluronics F68) may be added.
(10) Carefully adapt the cells to the new medium.
(11) Always check whether the performance of the cells has changed and whether the endpoints of the study are affected.
(12) When successful, share your formulation with colleagues, and through existing cell culture databases.

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