Method for the cultivation of primary cells and for the amplification of viruses under serum free conditions

The present invention relates to a method for the cultivation of primary cells. The primary cells are cultivated in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors. The method for the cultivation of primary cells may be one step in a method for the amplification of viruses, such as poxviruses. According to this latter method the primary cells are cultivated in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors. The cells are then infected with the virus and the infected cells are cultivated in serum free medium until progeny virus is produced.

Background of the invention

Most viral vaccines such as attenuated or recombinant viruses are manufactured from cell culture systems. The cells used for virus/vaccine production may be cell lines, i.e. cells that grow continuously *in vitro*, either as single-cell suspension culture in bioreactors or as a monolayer on a cell-support surface of tissue culture flasks or roller-bottles. Some examples for cell lines used for the production of viruses are: the human foetal lung cell-line MRC-5 used for the manufacture of polio viruses and the human foetal lung cell-line WI-38 used for the manufacture of measles virus, mumps virus and rubella virus (MMR II) (Merk Sharp & Dohme).

Not only cell lines but also primary animal cells are used for the manufacturing of vaccines. An example for primary cells that are used for virus production are chicken embryo fibroblasts (CEF cells). These cells are used for the production of measles and Japanese encephalitis virus (Pasteur Merieux), mumps virus (manufactured by Provaccine), rabies virus (manufactured by Chiron Berhing GmbH & Co.), yellow fever virus (manufacture by Aprilvax), influenza virus

(manufactured by Wyeth Labs and SmithKline & Beecham) and modified Vaccinia virus Ankara (MVA).

CEF cells are often used since many virus vaccines are made by attenuating the virulent disease-causing virus by serially passaging in CEF cells. The attenuated virus does not longer cause the disease but is still capable of stimulating a potent protective immunity against the virulent form of the virus. An example for this type of virus is MVA. This virus is severely replication restricted in humans and in most animals. MVA is being developed as a vaccine vector because it can be used to express antigens derived from a variety of agents causing diseases in humans. Attenuated viruses, such as MVA are preferably not propagated on human cells since there is a concern that the viruses might again become replication competent in cells of human origin. However, viruses that have regained the ability to replicate in human cells could be a health risk if administered to humans, in particular if the individuals are immune compromised. For this reason, some attenuated viruses, such as MVA, are strictly manufactured from CEF cells, if intended for human use.

Moreover CEF cells are used for those viruses that grow only on said cells. Examples for such viruses are in particular avian viruses such as avipox viruses, in particular canary pox virus, ALVAC, Fowl pox virus and NYVAC.

Cell lines and primary cells grown under *in vitro* culturing conditions require a special growth and maintenance medium that can support (I) cell replication in the logarithmic phase and (II) cell maintenance once the cells are no longer dividing, i.e., when the cells are in the stationary phase. The commonly used cell culture media comprise a rich salt solution containing vitamins, amino acids, essential trace elements and sugars. Growth hormones, enzymes and biological active proteins required for supporting cell growth and maintenance, are usually added as a supplement to the medium in the form of an animal blood derived serum product. Examples for animal blood derived serum

products are foetal calf serum, chicken serum, horse serum and porcine serum. These sera are derived from fractionated blood, from which the red blood and the white blood cells have been removed. Primary cells, such as CEF cells are even more dependant on animal serum sources than cell lines. Thus, primary cells are usually cultivated in cell culture media comprising 5 to 10% serum, in most cases fetal calf serum (FCS).

The animal sera do not only comprise factors that are required for the growth of cells but also factors that are required for cells that naturally grow as adherent cells to attach to the cell support surface of the culture vessel. Thus, it is critical for adherent cells that enough serum is added to the medium to enable them to grow and form a monolayer.

Unfortunately, bovine/foetal calf serum as well as sera from other animals may contain adventitious pathogenic agents such as viruses. There is a potential risk that these pathogenic agents are transmitted to the animal/human to be treated or vaccinated with the vaccine or any other pharmaceutical product produced in cell culture. This is of particular relevance if cell culture products are administered to immune-compromised humans. One of the many potential major problems associated with the commonly used bovine serum supplement is the possibility to transmit the agent causing bovine spongiforme encephalopathy (BSE) to the animals/humans that come into contact with the products produced from cell culture.

In view of the possible risk associated with the use of animal sera in cell culture it has become clear that manufacturing processes free from the use of animal products are highly desirable.

To this end specific media that do not have to be supplemented with animal sera have been developed for continuously growing cell lines and for the production of viruses in continuously growing cell lines, respectively. An example for such a serum free medium that can be used to cultivate cell lines

is VP-SFM manufactured by Gibco BRL/ Life Technologies. According to the manufacturers information VP-SFM is designed specifically for the growth of VERO, COS-7, MDBK, Hep2, BHK-21 and other important cell lines (Price, P. and Evege, E. Focus 1997, 19: 67-69) and for virus production in said cell lines. No information is available regarding the cultivation of primary cells in said medium.

US 5,503,582 discloses the cultivation of CEF cells in medium comprising 4% calf serum followed by the infection of the cells with Fowlpoxvirus in serum free medium. Spataro, A.C. et al., J. Cell. Sci. 1976; 21, 407-413 discloses that CEF cells can be maintained in serum free medium for 24 hours once a monolayer has been formed. Thus, according to both publications the media that are used for the cultivation of the cells after seeding comprise serum. Only for the maintenance of cells that already are attached to the surface and that have reached the stationary phase serum free medium was used. If the seeding is done with conventional medium, such as medium 199 or RPMI-1640 lacking serum no monolayer is formed and the cells form non-viable aggregates in the media.

WO 98/15614 refers to a specific serum free medium for the in vitro cultivation of animal cells. Cells which can be cultivated in the medium as disclosed in WO 98/15614 are those of animal origin, including cells obtained from mammals, birds, insects or fish. The mammalian cells may be primary cells from human origin. No reference is made to primary avian cells.

US 5, 405,772 discloses a serum-free medium for the proliferation and development of cells. The cells are preferably hematopoietic cells and bone marrow stromal cells. Primary avian cells are not mentioned.

WO 98/04680 discloses a serum-free medium for growth of anchorage-dependant mammalian cells, such as the cell lines BHK, Vero or MRC-5. WO 98/04680 refers neither to primary cells nor to avian cells.

Object of the invention

It is the object of the present invention to provide a method allowing the cultivation of primary cells, in particular primary avian cells in serum free medium, wherein the method allows (I) the growing of the cells during the logarithmic phase and (II) the maintenance of the cells in the stationary phase. Moreover, if the cells are adherent cells the medium may preferably allow (III) the attachment of the adherent cells to the surface of the cell culture vessel. It is a further object of the present invention to provide a method for the production of virus by using primary cells under serum free conditions.

Detailed description of the invention

The present invention provides a method for the cultivation of primary cells, characterized in that the cells are cultivated in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors.

According to the present invention primary cells that naturally grow as adherent cells attach to the surface of the cell culture vessel after seeding and grow in a logarithmic phase until a monolayer is formed. According to the present invention the resting cells may be maintained in the medium used during the attachment and logarithmic growth of the cells.

The method of the present invention is not restricted to cells that form monolayers. According to an alternative embodiment the method according to the present invention may be used for all other types of primary cells, such as cells naturally growing in suspension culture (e.g. lymphocytes or other types of blood cells) or cells that naturally would grow as adherent cells but that have been adapted to growing in suspension culture.

As shown below the cells can also be used for the serum free amplification of viruses that might be useful as vaccines.

It was unexpected that primary cells naturally growing as adherent cells (I) can effectively attach to the surface of the cell culture vessel without forming unacceptable amounts of aggregates and (II) can be grown in the logarithmic phase in the absence of serum since it was generally believed that primary cells are dependant on a multitude of different factors and components comprised in serum. Moreover, it was believed that adherent cells form non-viable aggregates that do not attach to the surface of the cell culture vessel, when cultivated in serum free medium. Thus, it was unexpected that it is sufficient to add to a serum free medium a factor selected from the group consisting of growth factors and attachment factors in order to obtain attachment and growth of adherent primary cells. Moreover, it was also unexpected that primary cells cultivated in suspension culture can be grown with the media used in the method according to the present invention. Furthermore, it was surprising that primary avian cells, such as Chicken Embryo Fibroblasts (CEF) can be cultivated to attach to the surface of a cell culture vessel without forming unacceptable amounts of aggregates in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors since avian cells were known to grow extremely bad in serum free medium not comprising growth factors or attachment factors, i.e. it was unexpected that the poor growth properties of primary avian cells could be improved significantly by adding a factor selected from growth factors and attachment factors to serum free medium.

The term "primary cells" as used in the present description is well known to a person skilled in the art. Without being restricted to the following definition the term "primary cells" refers to cells that have been freshly isolated from an animal or human tissue, organ or organism, wherein the cells are not able to continuously and indefinitely replicate and divide. Usually, primary cells divide in cell culture less than 100 times, often less than 50 times, often less than 25

times. Thus, primary cells have not undergone an immortalizing event. Examples for primary cells are cord blood lymphocytes and human or animal fibroblasts. Preferred examples for animal fibroblasts are avian fibroblasts, most preferably Chicken Embryo Fibroblasts (CEF cells). A preferred example for primary human fibroblasts is human foreskin fibroblasts.

Methods are well known to the person skilled in the art how primary cells can be isolated. Generally, primary cell cultures are directly derived from tissues, organs or embryos. The tissues, organs or embryos are subjected to protease treatment to obtain singles cells. The cells are then cultivated according to the method according to the present invention under *in vitro* culture conditions.

More specifically, CEF cells are obtained from protease digested chicken embryos. According to the present invention CEF cells grow best as adherent cells attached to a solid cell support surface. The cells start replication and establish a monolayer. If CEF cells (after embryo digestion) are cultivated *in vitro* with a standard culturing medium without animal serum the cells will occasionally attach to the solid cell-support surface, but will not replicate to form a confluent monolayer of cells and will with time slowly detach from the solid culturing-support surface. In contrast, if the CEF cells are cultivated according to the method of the present invention, the cells attach to the solid support, grow in the logarithmic phase until a monolayer is formed and can be maintained in the stationary phase for several days.

The term "cultivation of cells" in a serum free medium in the context of adherent primary cells refers to the seeding of the cells into the culture vessel in a serum free medium, to the growing of the cells in a serum free medium in the logarithmic phase until a monolayer is formed and/or to the maintenance of the cells in serum free medium as soon as the monolayer is formed. More preferably the term "cultivation of cells" in a serum free medium refers to a method in which all of the above mentioned steps are performed with serum free medium, so that no animal serum products are present during the whole

cultivation process of the cells. Thus, in a more general meaning the term "cultivation of cells in a serum free medium" refers to the fact that all media leading to the formation of a monolayer are serum free media. The media used in all of the above steps may comprise a factor selected from growth factors and attachment factors. However, it might be sufficient to add such a factor only to the media used for the attachment of the cells and/or the growing of the cells under logarithmic conditions.

The term "cultivation of cells" in a serum free medium in the context of cells growing in suspension culture refers to the seeding of the cells into the culture vessel in a serum free medium, the growing of the cells in a serum free medium in the logarithmic phase and/or the maintenance of the cells in serum free medium as soon as the saturation density at which no further replication occurs is obtained. More preferably the term "cultivation of cells" in a serum free medium refers to a method in which all of the above mentioned steps are performed with serum free medium, so that no animal serum products are present during the whole cultivation of the cells. The media used in all of the above steps may preferably comprise a factor selected from the group of growth factors. However, it might be sufficient to add such a factor only to the media used for the seeding of the cells and/or the growing of the cells under logarithmic conditions. As explained below in more detail it might also be possible to cultivate cells that would normally grow as attached cells also as suspension culture cells if appropriate incubation conditions are chosen (e.g. by applying "wave" incubation). The method according to the present invention also applies for this type of incubation.

The term "serum-free" medium refers to any cell culture medium that does not contain sera from animal or human origin. Suitable cell culture media are known to the person skilled in the art. These media comprise salts, vitamins, buffers, energy sources, amino acids and other substances. An example of a medium suitable for the serum free cultivation of CEF cells is medium 199

(Morgan, Morton and Parker; Proc. Soc. Exp. Biol. Med. 1950, 73, 1; obtainable inter alia from LifeTechnologies).

The media used according to the method of the present invention, in particular the media used for adherent cells such as CEF cells contain a factor selected from the group consisting of growth factors and attachment factors. An example for an attachment factor is fibronectin.

For cells that naturally would grow as adherent cells, that, however, are nevertheless cultivated in suspension culture (which is possible e.g. for CEF cells), it is particularly preferred to use a factor selected from the group of growth factors. Examples for growth factors useful for this type of cultivation are recombinant bovine, mouse, chicken or human epidermal growth factor (EGF). Most preferred is recombinant human EGF (rh-EGF) (Chemicon Int., Cataloge number: GF001).

For cells naturally growing in suspension culture the medium may preferably comprise a factor selected from the group of growth factors including EGF. Most preferably the growth factors for this type of cells are factors specific for non-adherent cells. Examples for those growth factors are interleukins, GM-CSF, G-CSF and others. The person skilled in the art may easily determine by routine experimentation, which type of factor is suitable for which type of cells.

If the factor added to the serum free medium is EGF, in particular rh-EGF, it is preferably added to the medium in a concentration of 1 to 50 ng/ml, more preferably 5 to 20 ng/ml. However, the person skilled in the art will be aware of the fact that different cell types may require a somewhat different concentration of EGF in the serum for optimal results.

If the attachment factor added to the serum free medium is fibronectin : (e.g. Chemicon Int.; Human plasma fibronectin; cataloge number FC010), it is preferably added to the medium in an concentration of 1 to 50, more

preferably 1 to 10 µg/cm^2 surface of the cell culture vessel. However, the person skilled in the art will be aware of the fact that different cell types may require a somewhat different concentration of fibronectin in the serum for optimal results.

According to the present invention it is sufficient to add only one factor selected from growth factors and attachment factors to the medium, in particular if the cells are adherent cells. However, it is also possible to add two or more factors selected from growth factors and attachment factors to the medium. The medium may preferably comprise EGF and fibronectin, preferably in the concentration ranges defined above, in particular if the primary cells are adherent cells such as CEF cells.

The medium may further comprise one or more additives selected from microbial extract, plant extract and extract from a non-mammalian animal. The microbial extract is preferably yeast extract or yeastolate ultrafiltrate. The plant extract is preferably rice extract or Soya extract. The extract from non-mammalian animal is preferably fish extract.

According to a preferred embodiment of the present invention Asparagine may be added to the commercially available serum free medium to which a factor selected from growth factors and attachment factors has been added. More preferably asparagine is added to the medium that is used during the infection with virus (see below). Commercial serum free media usually comprise asparagine in a concentration range of 0.3 to 1.0 mM. Preferred amounts of asparagine to supplement the medium are in the range of 0.5 to 1.5 mM. Most preferred is a 1 mM asparagine supplement. The total concentration of asparagine in the medium is preferably less than 2 mM and more preferably in a range of 0.8 to 1.8 mM. The most preferred concentration of asparagine in the medium is 1.3 mM.

Moreover, glutamine may preferably be added to the medium. More preferably glutamine is added to the medium that is used during the infection with virus (see below). Preferred amounts of glutamine to supplement the medium are in a range of 1 to 5 mM, more preferably in a range of 2 to 4 mM. The indicated ranges also correspond to the preferred total concentrations in the medium since most of the commercially available media do not contain glutamine.

According to a further embodiment the invention concerns a method for the amplification of a virus comprising the following steps: in the first step primary cells are cultivated according to the method described above, i.e. primary cells are cultivated in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors, depending on the cell type. All conditions, definitions, preferred embodiments and also the order of preferred to most preferred embodiments given for the description of the method for the cultivation of primary cells above, also apply for the definition of the first step of the method for the amplification of virus according to this embodiment of the present invention. In a second step the primary cells are infected with the virus. In the third step the infected cells are incubated in serum free medium until progeny virus is produced.

The term "amplification of a virus" is used to make clear that the method according to the present invention is preferably used to increase the amount of virus due to a productive viral replication of the virus in the infected cells. In other words the ratio of output virus to input virus should preferably be above 1. Thus, according to the present invention those primary cells are chosen for a specific virus, in which the virus is able to productively replicate. The term "reproductive replication" refers to the fact that the specific virus replicates in the specific primary cell to such an extent that infectious progeny virus is produced, wherein the ratio of output virus to input virus is above 1.

The person skilled in the art knows, which viruses can be productively replicated in which type of primary cells. By way of example the primary cells

may be human foreskin fibroblasts if the virus to be amplified is the human Cytomegalovirus; the primary cells may be CEF cells if the virus to be amplified is measels virus, mumps virus, rabies virus, Japanese encephalitis virus, yellow fever virus, influenza virus or a poxvirus such as vaccinia virus, in particular the modified vaccinia virus Ankara (MVA).

Methods to infect primary cells according to the second step of the present embodiment are known to the person skilled in the art. By way of example the virus may simply be added to the medium. Alternatively, the medium may be removed and the virus may be added to fresh medium, which in turn is added to the cells. To obtain an efficient infection the amount of the virus/medium suspension should be as low as possible to have a high virus concentration. After the attachment of the virus additional medium may be added.

In the third step the cells are inoculated in serum free medium until progeny virus is produced.

The serum free medium that is used in the second and third step of the method for the amplification of a virus may be the same medium that has already been used before, i.e. a serum free medium comprising a factor selected from growth factors and attachment factors, depending on the cell type. However, to save cost it also possible to use at one or both of the second and third step a serum free medium that does not contain a factor selected from growth factor and attachment factors.

During all stages the medium may be supplemented with asparagine and/or glutamine as outlined above, wherein the total concentration of asparagine in the medium is preferably as defined above.

The progeny virus may be concentrated and purified according to methods known to the person skilled in the art.

According to a preferred embodiment the method for the amplification of viruses is used for the amplification of poxviruses. Thus, according to this preferred embodiment the invention relates to a method for the amplification of a poxvirus comprising the following steps: (I) cultivation of primary cells according to a method as described above, i.e. a method in which the primary cells are cultivated in serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors, depending on the cell type, (II) infection of the primary cells with the poxvirus and (III) cultivation of the infected cells in serum free medium until progeny virus is produced.

It was unexpected that poxviruses can be amplified on cells cultured under serum free conditions since cells grow very badly with the known serum free medium. Thus, it was expected that the additional stress associated with a poxvirus infection would kill the already stressed cells before a significant amplification of the poxvirus occurred.

The poxvirus is preferably an orthopoxvirus. Examples for orthopox viruses are avipoxviruses and vaccinia viruses.

The term "avipoxvirus" refers to any avipoxvirus, such as Fowlpoxvirus, Canarypoxvirus, Uncopoxvirus, Mynahpoxvirus, Pigeonpoxvirus, Psittacinepoxvirus, Quailpoxvirus, Peacockpoxvirus, Penguinpoxvirus, Sparrowpoxvirus, Starlingpoxvirus and Turkeypoxvirus. Preferred avipoxviruses are Canarypoxvirus and Fowlpoxvirus.

An example for a canarypox virus is strain Rentschler. A plaque purified Canarypox strain termed ALVAC (US 5,766,598) was deposited under the terms of the Budapest treaty with the American Type Culture Collection (ATCC), accession number VR-2547. Another Canarypox strain is the commercial canarypox vaccine strain designated LF2 CEP 524 24 10 75, available from Institute Merieux, Inc.

Examples of a Fowlpox virus are strains FP-1, FP-5 and TROVAC (US 5,766,598). FP-1 is a Duvette strain modified to be used as a vaccine in one-day old chickens. The strain is a commercial fowlpox virus vaccine strain designated O DCEP 25/CEP67/2309 October 1980 and is available from Institute Merieux, Inc. FP-5 is a commercial fowlpox virus vaccine strain of chicken embryo origin available from American Scientific Laboratories (Division of Schering Corp.) Madison, Wisconsin, United States Veterinary License No. 165, serial No. 30321.

Examples for vaccinia virus strains are the strains Temple of Heaven, Copenhagen, Paris, Budapest, Dairen, Gam, MRIVP, Per, Tashkent, TBK, Tom, Bern, Patwadangar, BIEM, B-15, Lister, EM-63, New York City Board of Health, Elstree, Ikeda and WR. The invention is preferably carried out with modified vaccinia virus Ankara (MVA) (Sutter, G. *et al.* [1994], Vaccine 12: 1032-40). A typical MVA strain is MVA 575 that has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC V00120707. Most preferred is MVA-BN or a derivative thereof, which has been described in the PCT application PCT/EP01/13628 filed at the European Patent Office on November 22, 2001, entitled „Modified Vaccinia Ankara Virus Variant". MVA-BN has been deposited at the European Collection of Animal Cell Cultures with the deposition number ECACC V00083008. The features of MVA-BN, the description of biological assays allowing to evaluate whether a MVA is MVA-BN or a derivative thereof and methods allowing to obtain MVA-BN or a derivative thereof are disclosed in the above referenced PCT application which is herewith incorporated by reference.

The virus to be amplified according to the method of the present invention may be a wild-type virus, an attenuated virus or a recombinant virus.

The term "recombinant virus" refers to any virus having inserted into the viral genome a heterologous gene that is not naturally part of the viral genome. A

heterologous gene can be a therapeutic gene, a gene coding for a peptide comprising at least one epitope to induce an immune response, an antisense expression cassette or a ribozyme gene. Methods to construct recombinant viruses are known to a person skilled in the art. The most preferred poxvirus vector is MVA, in particular MVA 575 and MVA-BN (see above).

An "attenuated virus" is a virus originating from a pathogenic virus but that upon infection of the host organism leads to a lower mortality and/or morbidity compared to the non-attenuated parent virus. Examples of attenuated poxviruses are known to the person skilled in the art. An example for an attenuated Vaccinia virus is strain MVA, in particular the strain that has been deposited at ECACC with the deposition number V00083008 (see above).

As pointed out above, for poxviruses the primary cells may preferably be a primary avian cells such as CEF cells or primary duck embryo fibroblasts. Again, the person skilled in the art is aware of which primary cells are suitable for the amplification of which poxvirus. CEF cells are particularly preferred for the amplification of MVA. For MVA amplification in CEF cells it is a preferred embodiment to select one or two of the factors selected from EGF and fibronectin.

If the method according to the present invention is used for the amplification of MVA in CEF cells it is preferred that the starting pH of the medium is in a range of about 7.0 to about 8.5. Particularly preferred is a starting pH of 7.0.

The invention further refers to viruses, in particular poxviruses obtained by the above-described method. According to a preferred embodiment the poxvirus is a vaccinia virus, most preferably a MVA strain such as MVA-BN.

The invention further concerns a composition comprising a virus, in particular a poxvirus produced by the method according to the present invention. As pointed out above the poxvirus is preferably a vaccinia virus, most preferably a

MVA strain such as MVA-BN. Due to the method for amplification of the virus the composition is free of any products and/or infectious agents comprised in animal sera. In contrast, compositions comprising viruses produced according to conventional methods comprise residual compounds derived from animal serum. This is especially the case for compositions comprising poxviruses such as vaccinia virus strains, in particular MVA strains such as MVA-BN.

The invention further relates to a virus, in particular to the viruses as defined above as a medicament or vaccine. If the virus is a wild-type virus or an attenuated virus the virus can be used for vaccination against the virus as such. For this purpose attenuated viruses are particularly preferred. If the virus is a recombinant virus expressing proteins expressed from genes heterologous to the viral genome, it is possible to vaccinate against the virus as such and/or against the expressed heterologous protein. If the recombinant virus expresses a therapeutic gene such as an antisense RNA or a ribozyme the virus may be used as a medicament.

The invention further relates to the use of a virus as defined above or a composition as defined above for the manufacture of a vaccine.

The invention further concerns a method for the treatment or vaccination of an animal, including a human, in need thereof, comprising the administration of a virus as defined above or a composition as defined above to the animal or human body.

Examples

If not indicated otherwise in the following examples the serum free medium is medium 199 (LifeTechnologies). The added EGF is usually recombinant human EGF obtained from Chemicon. Fibronectin (FN) was obtained from Chemicon.

Example 1: Preparation of Chicken Embryo fibroblast (CEF) cells

Specific pathogen free (SPF) fertilized eggs were stored not longer than 12 days at 4°C. The eggs were put into an incubator and incubated for 10-12 days at 37.8°C ± 8°C. One petri dish per maximum 11 eggs was prepared with 10-20ml PBS. The eggs were put in a dedicated egg carton and treated extensively with Bacillol to sterilize the outside of the egg shell. After drying, a hole was made into the eggs and the shell was removed carefully. The chorioallantoic membrane was put aside. The embryos were lifted up by the feet and then their heads were cut off. The embryos were then transferred into the prepared petri dishes. After removing the feet the trunks were washed again with PBS. 11 trunks maximum were put into a 20ml plastic syringe and squeezed into an Erlenmeyer flask. 5ml of prewarmed (37°C) Trypsin/EDTA-solution per trunk were added and the solution was stirred for 15 minutes with serum free medium at room temperature using a magnetic stirrer. Trypsinized cells were poured through a layer of mesh into a beaker. All cells were transferred to one 225ml-centrifuge tube and centrifuged down at 20°C, 470xg for 7 minutes in a bench top centrifuge. After discarding the supernatant, the pellet was resuspended in 1ml fresh pre-warmed (37°C) serum free growth medium comprising 10 ng/ml EGF per trunk by pipetting up and down thoroughly. Fresh prewarmed (37°C) serum free growth medium comprising 10 ng/ml EGF was added to a total volume of 150ml. The centrifugation step was repeated. The supernatant was removed and the pellet was resuspended as described above. Fresh prewarmed (37°C) serum free growth medium comprising 10 ng/ml EGF was added to a total volume of 100ml. Cells were counted as described in the following section. The required amounts of cells were seeded in roller bottles with serum free growth medium comprising 10 ng/ml EGF and incubated at 37°C. Cells were ready for virus infection at day four after seeding.

Example 2: Counting Cell Density

A sample of the cell suspension (see section CEF preparation) was taken and mixed with one volume of Trypan blue, resulting in a final cell count of 20 to 100 cells per 16 small squares of a hemocytometer supplied by Fuchs-Rosenthal under the name of Hemocytometer Fast Read 102 (1:2 – 1:10 dilution). The sample was taken immediately after resuspending the cells in order to prevent reaggregation/sedimentation of the cells. After a few minutes of incubation time with Trypan blue in order to get the dye properly into dead cells, 10µl of the cell suspension was added to the hemocytometer. Only white, living cells were counted under a light microscope using a 10x objective. In total, 3 representative big squares consisting of 3x16 small ones were counted. From every big square only two borders in L-Form were included in the counting. The average of counted cells was taken and the final cell concentration was calculated using the following formula: Average cell number x dilution x 10^4 = cells/ml. Finally the cell suspension was diluted to the desired working concentration.

Example 3: Effect of the addition of a factor selected from growth factors and fibronectin to a serum free culture medium on the formation of a CEF-monolayer

In preliminary experiments the inventors have shown that CEF cells do not attach to the surface of cell culture vessels if medium 199 is used that does not comprise FCS. Moreover, no monolayers are formed. Normal monolayer formation is observed if medium 199 containing 7% FCS is used. It was analyzed whether attachment and growth of CEF cells in serum free medium 199 can be achieved if additives are added to the medium.

The tested additives comprise recombinant Epidermal Growth Factor (r-hEGF) and Fibronectin (FN).

For the experiments CEF cells were grown in medium 199 with the different additives alone or in combination. Cells grown in medium 199 without any additives served as negative control. Cells cultivated in medium 199 comprising 7% FCS served as positive control. All experiments were conducted in 6-well cell culture plates with 3 ml medium. The additives were treated according to the data sheets of the supplier before being used for the cell culture. Fibronectin was allowed to adsorb to the surface of the cell culture plates for 25 minutes before use. Fibronectin was used in a concentration of 3 $\mu g/cm^2$ and EGF was used in a concentration of 10 ng/ml. Before adding any cells the cell culture plates were brought into contact with the fibronectin-containing medium for 25 minutes.

Every culture medium plus the additives to be tested was cultured in duplicates. The 6-well cell culture plates were incubated for 4 days at 37° C. From day 1 to 4 the attachment and growth of the cells was evaluated using a microscope.

For the positive control a normal attachment and growth of the CEF-cells has been observed. For Medium 199 without additives nearly no attachment of CEF cells could be observed.
A crucial improvement in the forming of a monolayer was seen by the use of EGF added to Medium 199 compared to Medium 199 without additives. It was found that the cells attached and formed the typical fibroblast morphology. Furthermore, a continuous growth could be observed over the whole period of 4 days.
An improvement of cell attachment was also achieved by adding fibronectin to the culture medium. The addition of both, EGF and Fibronectin resulted in a slight improvement compared to the addition of EGF only and Fibronectin only.

In summary, it has to be concluded that monolayer formation of CEF cells in the serum-free Medium 199 can be supported by the use of the additives EGF and Fibronectin.

Moreover, in parallel sets of experiments 1 x 10^7 CEF cells were seeded in medium comprising 10% FCS, medium not comprising FCS and medium not comprising FCS but comprising EGF. The cell number was counted 2 days after seeding. The number of cells amounted to 42%, 6% and 44%, respectively, of the cell number used for seeding. Thus, the results for the cells seeded in serum free medium comprising EGS were as good as the results obtained with medium comprising FCS and significantly better than with medium neither containing serum nor EGF.

In addition the medium comprising EGF was compared to various standard serum free media, such as DMEM, Opti-Mem or 293-SFM. To this end 1 x 10^7 CEF cells were seeded in the various serum-free media and cultivated for 4 days. The number of cells cultivated in medium comprising EGF was 24, 5 and 12 times higher than the number of cells cutivated in serum free DMEM, Opti.Mem and 293-SFM, respectively.

Example 4: Infection of CEF cells with MVA

CEF cells were infected four days after seeding in roller bottles. At that time point cells have grown to an adequate monolayer. Cells were infected with a MOI of 1 or 0.1 MVA. For the infection the growth medium was removed from the flasks. The desired amount of virus per roller bottle was diluted in 20ml of the appropriate infection medium without serum. At this stage the serum free medium may or may not comprise a factor selected from growth factors and fibronectin. Cells were incubated with the virus for 1 hour at 37°C at 0.3-0.5rpm in a roller bottle incubator. After 1 hour the roller bottles were filled with the appropriate serum free growth medium to a total volume of 200ml per roller bottle. At this stage the serum free medium may or may not comprise a factor selected from growth factors and fibronectin. Virus replication was stopped after 48 or 72 hours by freezing the roller bottles to –20°C.

Example 5: Preparation of Viral Extracts from Infected CEF Cells and Titration of MVA

The frozen roller bottles were thawed at room temperature. During the thawing process the cells detach from the surface of the roller bottles and can mechanically be removed by shaking the flasks. Virus/cell suspension was harvested and aliquoted to smaller volumes. To release the virus from the infected cells, virus/cell suspensions were 3 times freeze/thawed. The freeze/thawed virus samples were used for titration.

Titrations were performed on 1st passage CEF cells in 96-well plates, using 10-fold dilutions of viral suspension and 8 replicates per dilution. After the infection, infected cells were visualized with an anti-vaccinia virus antibody and an appropriate staining solution.

In detail, at day zero of the assay primary CEF cells (see section "preparation of Chicken Embryo Fibroblast (CEF) cells") were trypsinized and counted as described in the section "counting cell density". The cells were diluted to $1x10^5$ cells/ml in RPMI medium with 7%FCS. Following this dilution, 100 µl were seeded in each well of the 96-well plates using a multichannel pipette. Cells were incubated over night at 37°C and 5% CO_2. The virus samples to be titrated (see section "preparation of viral extracts from infected CEF cells) were serially diluted in 10-fold steps from $10^{-1} - 10^{-12}$ using RPMI without serum. This serial dilution is carried out by adding 900 µl RPMI to all the wells of a 96-deep-well plate. 100µl of virus sample was added to all the wells of the first row and mixed. Thereafter, 100µl of each sample were transferred to the next row of wells using a multi-channel pipette. The 96-deep-well plates were kept on ice when performing the dilutions. Plates were incubated for 5 days at 37°C and 5%CO_2 to allow the infection to proceed. After 5 days, cells were immunohistochemically stained with a vaccinia virus specific antibody. For the staining, the culture medium was removed by turning the 96-well plate upside down over a receptacle. Cells were fixed with 100 µl/well methanol/acetone (1:1) mixture for 10 minutes at room temperature. The fixing solution was

removed and plates were air-dried. After drying, cells were washed once with PBS and incubated for 1 hour at room temperature with the anti-vaccinia virus antibody (Anti-Vaccinia virus antibody, rabbit polyclonal, IgG fraction (Quartett, Berlin, Germany #9503-2057) diluted to 1:1000 in PBS with 3% FCS. After removing the antibody, cells were washed twice with PBS and incubated for 1 hour at room temperature with HRP-coupled (Horse Radish Peroxidase couple) anti-rabbit antibody (Anti-rabbit IgG antibody, HRP coupled goat polyclonal (Promega, Mannheim, Germany # W4011) diluted to 1:1000 in PBS with 3% FCS. Again, cells were washed with PBS and stained either with o-Dianisidine or TMB. For using the o-Dianisidine staining method, cells were incubated with 100µl/well staining solution consisting of 5mg o-Dianisidine and 180 µl 30% H_2O_2 per 60ml of 50mM phosphate-citrate buffer. Cells were incubated at room temperature until they stained brown. Infected cells were clearly visible after 1-3 hours. Using the TMB staining method, cells were incubated with 30µl/well 1.2mM TMB (Seramun Diagnostica GmbH). After 15 minutes incubation time, the TMB solution was removed and cells were washed once with PBS. Infected cells appear dark blue. The plates were scored for infected cells. The viral titre was calculated using the formula of Spearman and Kaerber. For the calculation of the $TCID_{50}$ every well showing brown or blue cells was marked positive. Because assay parameters are kept constant, the following simplified formula was used:

$$\text{Virus titre } [TCID_{50}/ml] = 10^{[a+1.5+xa/8+xb/8+xc/8]}$$

a = dilution factor of last column, in which all eight wells are positive
x_a = number of positive wells in column a+1
x_b = number of positive wells in column a+2
x_c = number of positive wells in column a+3

Example 6: Optimal seeding density for CEF cells in serum free medium and optimal amount of MVA for infection of CEF cells

An optimal seeding cell density of $7.5x10^7$ cells/$850cm^2$ (surface of one roller flask) was determined for serum-free CEF growth. Cells were able to build a good monolayer without forming big clumps at day four after seeding and could be infected at this time point.

Experiments were carried out to determine the best level of viral inoculation and length of the infection for the maximum production of MVA from CEF cells cultured in a serum-free process. CEF cells were seeded at a density of $7.5x10^7$ cells/$850cm^2$ in medium according to the present invention. At day 4 after seeding, cells were infected with different amounts of MVA in the range of 0.05 to 1.0 $TCID_{50}$/cell of MVA. Best results were obtained with 0.1 $TCID_{50}$/cell of MVA.

Example 7: Optimal pH of serum free medium for culturing and infection with MVA

MVA and other poxvirus infections are sensitive pH below 7.0. Poxviruses are not stable at acid pH and it is recommended that purified poxviruses are stored in a buffered solution above pH 7.0 to ensure stability and viral integrity upon storage as a liquid viral preparation. Experiments were carried out to determine the effect on virus yield when carrying out infection at different starting pH. Roller-bottles were seeded with CEF cells in the usually way in serum free medium comprising 10ng/ml EGF plus 4mM L-Glutamine and cultured for 4 days. Cells were infected with MVA at 0.1 $TCID_{50}$/cell in serum free medium comprising 10 ng/ml EGF plus L-Glutamine and 1mM Asparagine at different pH's ranging from 6.5 to 9.0. At 72 hours post infection, the pH of the medium was measured and viral yields were determined by titrating cell extracts in the usual manner. The results are presented in the following table, which shows the effect of initial pH of the medium at the start of the infection on virus yield.

	serum free medium comprising 10 ng/ml EGF	
Starting pH	pH at 72h p.i.	Titre [$TCID_{50}$/ml]
6.5	7.05	0.56×10^7
7.0	7.34	10.0×10^7
7.5	7.53	5.60×10^7
8.0	7.68	8.60×10^7
8.5	7.75	7.80×10^7
9.0	8.03	0.65×10^7

For the infections carried out in serum free medium comprising 10 ng/ml EGF supplemented with L-Glutamine and Asparagine, the viral production was relatively constant with a starting pH from 7.0 to 8.5 but viral productions were low at starting pH of 6.5 and 9.0. Best yield was obtained at starting pH 7.0. Commercially available standard serum free media usually have a pH of 7.4. Therefore adjusting the pH of the serum free medium to 7.0 can help to improve virus yield.

Example 8: Effect of added Asparagine to the serum free medium

Preliminary experiments have revealed that the amount of asparagine may be limiting during the cultivation of CEF cells and the infection of CEF cells with MVA. To overcome the depletion of Asparagine in the serum free media during the culturing and infection process, extra Asparagine was added to the medium as a supplement before infecting CEF cells. To determine the optimal amount of Asparagine to supplement the medium with, roller-bottles were seeded with CEF cells (7.5×10^7 cels/850cm^2) in serum free medium comprising 10 ng/ml EGF plus 4mM L-Glutamine. Four days after seeding cells

were infected with MVA at 0.1 $TCID_{50}$/cell in serum free medium comprising 10 ng/ml EGF plus 4 mM L-Glutamine supplemented with different Asparagine concentrations (0.5, 1.0 and 1.5 mM). Viral replication was stopped at 72 hours post infection and viral titres were determined. The results are shown in the following table that shows the production of MVA from CEF cells supplemented with different levels of Asparagine for the infection stage. The titres represent the averages of 3 roller-bottles per Asparagine supplementation.

Supplement Asparagine	Viral titres after 72 hours infection [$TCID_{50}$/ml]
0.0 mM	1.8×10^8
0.5 mM	1.3×10^8
1.0 mM	6.8×10^8
1.5 mM	1.0×10^8

The results demonstrate that supplementing the serum free medium comprising 10 ng/ml EGF medium with Asparagine could improve viral production and that supplementation to 1mM for the infection process was optimal.

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Claims

1. Method for the amplification of a poxvirus comprising the following steps:
   - cultivation of primary avian cells in a serum free medium comprising a factor selected from the group consisting of growth factors and attachment factors
   - infection of the primary avian cells with the poxvirus
   - cultivation of the infected cells in serum free medium until progeny poxvirus is produced,

wherein the primary avian cells are cells allowing the productive replication of the poxvirus.

2. Method according to claim 1, wherein the primary avian cells are chicken embryo fibroblasts (CEF).

3. Method according to anyone of claims 1 to 2, wherein the growth factor is an epidermal growth factor (EGF), in particular recombinant-human EGF

4. Method according to claim 3, wherein the concentration of EGF is in a range of 5 to 20 ng/ml medium.

5. Method according to anyone of claims 1 to 4, wherein the attachment factor is fibronectin.

6. Method according to claim 5, wherein the concentration of fibronectin is in the range of 1 to 10 µg/cm^2 surface of the cell culture vessel

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7. Method according to anyone of claims 1 to 2, wherein the medium comprises two or more factors selected from growth factors and attachment factors.

8. Method according to claim 7, wherein the medium comprises EGF and fibronectin in concentration ranges as defined in claims 4 and 6.

9. Method according to anyone of claims 1 to 8, wherein the medium further comprises one or more additives selected from microbial extract, plant extract and extract from a non mammalian animal.

10. Method according to claim 9, wherein the microbial extract is yeast extract or yeastolate ultrafiltrate.

11. Method according to claim 9, wherein the plant extract is rice extract or soja extract.

12. Method according to claim 9, wherein the extract from non-mammalian animal is fish extract.

13. Method according to anyone of claims 1 to 12, wherein the poxvirus is an orthopoxvirus.

14. Method according to claim 13, wherein the orthopoxvirus is a Vaccinia virus.

15. Method according to claim 14, wherein the Vaccinia virus is Modified Vaccinia virus Ankara.

16. Method according to anyone of claims 13 to 15, wherein the poxvirus is an attenuated virus or a recombinant virus.

17. Method according to anyone of claims 1 to 16, wherein the serum free medium in one or both of the steps of

- infection of the primary avian cells with the poxvirus and
- cultivation of the infected cells in serum free medium until progeny poxvirus is produced

lacks the factors selected from growth factors and attachment factors.

18. Method according to anyone of claims 1 to 17, wherein subsequent to the step of cultivation of the infected cells in serum free medium until progeny poxvirus is produced one or more purification steps are made.