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MEDIUM AND METHOD FOR VIRAL PROPAGATION AND MULTIPLICATION

The present invention relates to a medium and to a method for viral propagation and multiplication on cells in culture, in particular for producing vaccines.

The production of viral vaccines, whether they are simply attenuated, inactivated, subunits or recombinant, involves the large scale production of viruses.

This production can be carried out, depending on the nature of the virus, on various supports which allow the replication of the virus; thus, for example, for most of the currently commercially available anti-influenza vaccines, virus proliferation is carried out on embryonated hens' eggs, whereas for some vaccines against Japanese encephalitis, this step is carried out on mouse brains.

However, the use of such supports poses many problems: availability, reproducibility, but especially safety because of possible contaminations with viruses, mycoplasmas or any other undesirable element originating from the support. Increasingly, attempts are thus being made to dispense with such supports and, whenever possible, cell cultures are used which are infected with a small amount of the virus to be produced, and which are then maintained under conditions which promote virus replication inside the infected cells, as well as the propagation of the infection from one cell to another. The viruses produced are then harvested either from the culture medium in which the cells bathe, when it involves intracellular viruses which leave the cell after their budding (which in particular has the advantage of making it possible to carry out several virus harvests with the same culture cells), or by treating the cells themselves when it involves intracellular viruses.

These techniques entail having cell cultures of good quality; many developments of the prior art have

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thus related to improving cell cultures and in particular to the culture media used. In order to limit the risks of contamination of the cells with mycoplasmas, viruses, bovine spongiform encephalopathy agents or any other unconventional transmissible agent, the use of serum-free, and even protein-free, culture media has been proposed, as is described in Patent Application WO 96/15231. Alternatively, application FR 2,732,347 proposes the use of cell culture medium which is free of animal serum, but which contains plant extracts.

Thus, due to the use of such media for the cell culture step, the safety of the products obtained using the cells thus produced is increased.

However, when it is a matter of producing viruses forming part of the composition of vaccines, the steps which are consecutive to this cell culture phase should themselves also exhibit a maximum degree of safety.

Specifically, conventionally and as described in US Patent 4525349, when cells in culture are used for producing viruses, once the cell culture phase has been carried out, the medium which was used for the cell culture is removed and then replaced with a new medium in which the cells bathe for a moment in order to be washed; this new medium is then removed; this cell washing procedure can optionally be repeated a certain number of times.

Next, a new medium is introduced into the cell culture, which will allow the cells firstly to survive and to be infected with the viruses, and secondly to have at their disposal elements which are sufficient to be the support for the replication of these viruses. However, as is described in the article "Protein-free culture of Vero cells: A substrate for replication of human pathogenic viruses", *Cell Biology International, Vol. 17 No. 9, 1993 pp. 885-895*, even when the cell culture is carried out in protein-free medium, the culture medium used in the prior art in this viral

multiplication and propagation step generally contains foetal calf serum or, to limit the contamination problems, at least human albumin. The use of human albumin at this stage of a method for manufacturing viral vaccines is considered to be without risk for the quality of the vaccine. However, with the aim of completely eliminating all risk, even theoretical, it would be desirable to have a medium which is suited to the propagation and multiplication of viruses on a support consisting of cells in culture, which is free of human or animal protein, even of recombinant origin, but which nevertheless allows production yields which are compatible with industrial requirements so as not to increase the costs of producing vaccines comprising viruses produced on cells in culture.

The aim of the invention is in particular to provide such a medium.

Another aim of the invention is to provide a medium and a method for viral propagation and multiplication which then allows, when it is desired, a virus inactivation step to be carried out.

To achieve these various aims, a subject of the present invention is a medium for viral propagation and multiplication on cells in culture, characterized in that it is free of human or animal proteins, even of recombinant origin, and in that it comprises elements of plant origin.

According to one characteristic of the invention, the medium for viral propagation and multiplication comprises proteins or glycoproteins extracted from potatoes or from cucumber, having a mitogenic potential and having a molecular weight between 1000 and 200,000 daltons.

According to one particular embodiment, the medium according to the present invention comprises plant hydrolysates.

According to one particular characteristic of the invention, the proteins or glycoproteins are extracted from potatoes.

According to another characteristic of the invention, the proteins or glycoproteins are obtained in the following way:

- washing and grinding of plants,
- dilution in aqueous medium,
- heat-coagulation of the mixture obtained,
- purification by chromatography.

According to one characteristic of the invention, the plant hydrolysates are obtained by chemical and/or enzymatic hydrolysis of raw materials such as cotton, soybean, wheat or rice. Particularly good results have been obtained with the products of the HyPep™ range supplied by the company Quest International, in particular with the products comprising a high proportion of peptides with a molecular mass lower than 1000 daltons.

According to another characteristic, the medium for viral propagation and multiplication according to the invention is particularly suited to viral multiplication on Vero cells.

According to another characteristic, the medium for viral propagation and multiplication according to the invention is particularly suited to the multiplication of Japanese encephalitis viruses, poliomyelitis viruses and rabies viruses.

A subject of the present invention is also a method for manufacturing viral vaccines which comprises a phase of viral production on the cells in culture, characterized in that the phase of virus propagation and multiplication is carried out in the presence of a medium which is free of human or animal protein, even of recombinant origin, and which comprises plant extracts.

The present invention will be better understood upon reading the description which will follow.

The medium and the method according to the invention, are suited to the production of all viruses which are able to replicate on cell cultures. They can in particular be viruses belonging to the following

families: orthomyxovirus, paramyxovirus, reovirus, picornavirus, flavivirus, arenavirus, herpesvirus, poxvirus and adenoviruses. They can also be recombinant viruses. In particular, the invention is particularly suited to the production of viruses which can form part of the composition of vaccines, and in particular of human vaccines for which the safety requirements are maximal. They can in particular be the poliomyelitis, rabies, Japanese encephalitis, yellow fever, rubella, mumps, Dengue fever or measles viruses, the viruses of the various forms of hepatitis, the AIDS or chickenpox viruses, herpesvirus, viruses of diseases caused by the respiratory syncytial virus, cytomegalovirus, EBV, rotaviruses or the influenza.

The present invention is particularly advantageous for producing the viruses which are responsible for poliomyelitis, rabies, Japanese encephalitis, rubella, chickenpox, hepatitis A, influenza, Dengue fever, measles or mumps.

The cells which can be used for virus production according to the invention are all cells which can multiply in culture and which are permissive to the virus whose production is desired. They can in particular be Vero cells, CV-1 cells, LLC-MK2 cells, MDCK cells, MDBK cells, WI-38 cells, MRC5 cells (human fibroblasts) or BHK21 cells. Particularly good results have been obtained with Vero cell cultures or MRC5 cell cultures.

The cell culture can be carried out according to the various techniques usually used; in a cell-cultivator, in roller bottles, in Roux flasks, in Multitrays™, in Cell-Cubes™. For industrial reasons, it is preferred to carry out the cell culture in cell-cultivators using microsupports consisting of, for example, Cytodex™ beads.

This cell culture can be carried out under various conditions, in particular with regard to the medium used; it is in fact possible to implement the

present invention even if the culture medium used for the cell growth phase contained serum or at least human or animal proteins; in this case, of course, it will be necessary to carry out thorough washing of the cells before their infection, so as not to lose the advantage provided by implementing the invention with regard to the absence of risk of contamination. However, the use of cell cultures obtained using a cell culture medium which is also free of proteins of human or animal origin is preferred.

Depending on the nature of the cells used and the viruses to be produced, the most appropriate moment for carrying out the virus infection with an inoculum of the virus may vary. Generally, this procedure is carried out rather in the middle or at the end of the exponential growth phase; it has in fact been noticed that, under these conditions, the results obtained are particularly satisfactory.

According to the invention, the culture medium used for the step of viral propagation and multiplication is a medium which is free of human, animal or recombinant protein, but which comprises proteins or glycoproteins extracted from potatoes or from cucumber, having a mitogenic potential and having a molecular weight between 1000 and 200,000 daltons and/or hydrolysates of plant extracts. This medium is a medium which can comprise all or part of the elements conventionally used for viral multiplication, such as HAM F 12 medium, but which is supplemented with plant extracts obtained for example in the following way:

- reduction of the plant pulp,
- treatment of the pulp obtained with a solvent,
- evaporation of the solvent,
- purification of the extract obtained by chromatography.

The mitogenic potential of the proteins or glycoproteins which are suitable for the purposes of the invention can be assessed in particular using

assays for analysing cell growth and mitogenic activity such as M.T.T. coloration assays.

The plant extracts which are suitable for the purposes of the invention are in particular those described in Patent Application FR 2,732,347. The products sold by the company Biomedia under the name GCR1003, TCR1005 or BCR1008 can also be used, alone or in combination, or the product Prolifix which is also sold by this company and which comprises all the nutrient elements of conventional HAM F 12 medium supplemented with suitable plant extracts.

Alternatively, it is possible to use a conventional HAM F 12 medium supplemented with plant hydrolysates such as the HyPep™ products in a proportion of 5 g/l.

It is also possible to use a medium comprising both the proteins or glycoproteins obtained by heat-coagulation of the plant pulp and plant hydrolysates such as those mentioned above.

In addition, it is possible to add, to these media, any other element which is likely to improve or facilitate one of the steps leading to the production of a vaccine, just as it is possible to slightly modify this medium without changing the properties thereof.

The examples which follow illustrate embodiments of the invention, in a non-limiting way.

EXAMPLE 1: PRODUCTION OF VACCINES AGAINST JAPANESE ENCEPHALITIS

Vero cells are used to produce the virus which is responsible for Japanese encephalitis. The cells used are cells derived from the strain distributed by ATCC (American Type Culture Collection) under No. ATCC-CCL 81-VERO F 1415, which is at the 124th passage and which is taken to the 137th passage in the conventional way. The virus whose production is desired is the virus of P3 strain, at the 88th passage, supplied by NVSI.

The reservoir of a 15-litre cell-cultivator is filled with Iscove medium supplemented with 4% calf serum containing *Cytodex 1*™ microcarriers at a concentration of 3.5 g/l. The medium is seeded with an inoculum of Vero cells in a proportion of 200,000 cells/ml.

The culture is amplified and subcultured twice a week every 4 to 5 days. The cell concentrations which are obtained are between 2 and 3×10^6 per ml. The cell suspension obtained is then rinsed with the medium for viral propagation, with the aim of removing the maximum amount of residual calf serum.

In order to test a medium according to the invention comparatively with a medium of the prior art, the same procedure is carried out in parallel, in several identical cell-cultivators, with 2 different media:

- a medium according to the invention consisting of HAM F 12 medium (supplied by Life Technologies Gibco) supplemented with Prolifix II medium supplied by the company Biomedia, at a concentration which is 10 times that indicated in their 1996 catalogue, in a proportion of 1 volume of 10-fold concentrated Prolifix II per 10 volumes of HAM F 12,
- a medium according to the prior art consisting of Iscove medium (supplied by Life Technologies Gibco) supplemented with human albumin to obtain a final albumin concentration in the medium of 0.3%.

After rinsing, a virus inoculum is introduced into each of the cell-cultivators in an amount corresponding to an MOI (Multiplicity Of Infection) of 1/6000.

The contents of the cell-cultivators are then kept stirring at a temperature of 37°C for 3 days, and then the 1st harvest is carried out by recovering the medium for viral propagation, which has become a viral suspension, which is replaced in each of the cell-cultivators with an equal amount of fresh medium of the same nature; a harvest of the medium for propagation is

then carried out every 24 hours, until 5 harvests have been recovered. Each of the harvests is filtered through a filter with a cut-off threshold of 0.22 μm, and stored at +5°C while waiting to be mixed with the 4 other harvests from the same cell-cultivator.

When the 5 harvests have been carried out, they are mixed together and this mixture is then concentrated by filtration up to a factor of 100. The mixture consisting of the 5 harvests from the same cell-cultivator constitutes a batch.

In order to determine the amount of viruses produced, an infectious activity assay is carried out for each of the batches produced. The assay is carried out in 2 different ways: either by infectious activity measured on a cell layer, or by infectious activity measured in mice by intracerebral inoculation into the mice, observation of the animals for 14 days and calculation of the LD50.

The results are expressed as Log 10 of infectious particles per litre of cell-cultivator. When the titration on cells is considered, the mean of the results obtained for the various batches produced using a medium according to the invention is 8.84, whereas the mean obtained for the batches produced using a medium according to the prior art is 7.85. If the titration in mice is considered, the mean for the batches produced according to the invention is this time 9.22, whereas the mean for the batches produced with a medium according to the prior art is 9.30. It is thus observed, unexpectedly, that the results obtained with the medium according to the invention are equivalent to those obtained with a medium of the prior art, whereas the consequence of the deficit of albumin, which is a protein considered to play an important role in transporting nutrient elements inside the cell, was expected to be a considerable decrease in the amount of viruses produced.

The batches obtained were then inactivated by adding a formaldehyde solution in an amount giving a

final concentration in the mixture of 1/4000 and kept at room temperature with continuous stirring for 14 days. The verification of the inactivation by means of a control according to the protocol recommended by the WHO (Technical Bulletin 771 from 1988) showed that the batches produced complied with the WHO recommendations.

These inactivated batches were then purified by ion exchange chromatography and by gel filtration in order to remove therefrom all the viral proteins and residual impurities which are not desired in the vaccines. The mixture obtained was then formulated so as to allow the production of vaccine doses. The good quality of the vaccines produced was verified using the assay of immunogenicity in mice which is recommended by the WHO for the vaccines against Japanese encephalitis, and which consists in determining the amount of neutralizing antibodies produced by immunized mice. Since the activity of the vaccines produced according to the method of the invention is not lower than that of the vaccines of the prior art, this confirms that it is possible, using the medium and the method according to the invention, to produce viral vaccines under conditions of increased safety by dispensing with the use of albumin.

EXAMPLE 2: COMPARISON OF VARIOUS MEDIA ACCORDING TO THE INVENTION

To comparatively test various media, 150-cm^3 flasks are used, which are filled with 50 ml of Iscove medium supplemented with 4% calf serum. This medium is seeded with a Vero cell inoculum in a proportion of 250,000 cells/cm^2. The flasks are left for 4 to 5 days at 37°C and are then rinsed by means of the medium for viral multiplication and propagation.

The media used are as follows:

- a medium A, which is a medium according to the prior art, consisting of Iscove medium (supplied by Life Technologies Gibco) supplemented with human albumin

to obtain a final albumin concentration in the medium of 0.3%,

- a medium B consisting of HAM F 12 medium supplemented with the following mitogenic molecules:
  * BCR 1008 in a proportion of 10^{-3} g/l
  * TCR 1005 in a proportion of 10^{-4} g/l
  * GCR 1003 in a proportion of 10^{-4} g/l
- a medium C consisting of the abovementioned medium (HAM F 12 supplemented with BCR 1008, TCR 1005 and GCR 1003), but also comprising 1 g/l of the HyPep 4602 product which consists of gluten hydrolysate supplied by the company Questel International,
- a medium C' which is identical to the medium C, but in which the HyPep 4602 concentration is 5 g/l
- a medium D consisting of HAM F 12 medium supplemented with HyPep 4602 in a proportion of 1 g/l, but lacking the mitogenic molecules BCR 1008, TCR 1005 or GCR 1003
- a medium D' which is identical to the medium D, but in which the HyPep 4602 concentration is 5 g/l.

After rinsing, an inoculum of Japanese encephalitis virus which is identical to that of the previous example is introduced into each of the flasks in an amount corresponding to an MOI of 1/6000.

The contents of the flasks are then maintained for 3 days at 37°C, before the 1st harvest is carried out. The subsequent harvests are carried out in the same way as that described in the previous example.

The assays for determining the infectious titre are carried out on BHK21 cells and give the following results, which are expressed in $TCID_{50}$/ml (Log 10):

Medium A: 4.6
Medium B: 5.1
Medium C: 5.0
Medium C': 5.6
Medium D: 5.4
Medium D': 6.3.

These results confirm that the media according to the invention make it possible to obtain yields

which are as good as, if not better than, the media according to the prior art which contain albumin.

EXAMPLE 3: PRODUCTION OF VACCINE AGAINST RABIES

A Vero cell culture is prepared in a 2-1 cell-cultivator in conventional medium and, after washing, the cells are then infected with rabies virus with an MOI of 1/1000 and are maintained in a medium which is identical to that described in Example 1 (i.e. HAM F12 medium supplemented with Profilix™ II); the same experiment is carried out in an entirely identical manner, in another cell-cultivator of the same volume, the only difference being the use of a medium for viral propagation and multiplication of the prior art comprising human albumin. Harvests are successively carried out 4, 5, 6 and 7 days after infection. The counts carried out on the harvests obtained using the medium according to the invention and on the harvests obtained using the medium according to the prior art made it possible to obtain comparable results with both media; similarly, the infectious titres of the viruses obtained were equivalent.

These results thus demonstrate the possibility of producing virus against rabies on cells.

EXAMPLE 4: PRODUCTION OF VACCINE AGAINST POLIOMYELITIS.

Tests for production of type 1 poliomyelitis virus on Vero cells in culture were carried out. The infectious titres of the viruses obtained using a medium for viral propagation and multiplication according to the invention having the composition described in Example 1 were equivalent to the mean of the infectious titres obtained with the harvests carried out with the medium used for manufacturing the currently commercially available vaccines.

CLAIMS

1. Use of a culture medium which is free of human or animal protein, even of recombinant origin, and which comprises plant extracts for viral propagation and multiplication on cells in culture.

2. Use according to Claim 1, according to which the medium comprises mitogenic proteins or glycoproteins extracted from potatoes or from cucumber.

3. Use according to either of the preceding claims, according to which the plant extracts are obtained in the following way:

- reduction of potato or cucumber pulp,
- treatment of the pulp with a solvent,
- treatment by heat-coagulation,
- purification by chromatography.

4. Use according to one of the preceding claims, according to which the culture medium comprises plant extract hydrolysates.

5. Use according to one of the preceding claims, according to which the cells used for the support of the viral propagation and multiplication are Vero cells.

6. Use according to one of the preceding claims, according to which the viruses produced are Japanese encephalitis, rabies, poliomyelitis, hepatitis A, influenza, Dengue fever, measles, mumps, chickenpox and rubella viruses.

7. Use according to one of the preceding claims, according to which the viruses produced are Japanese encephalitis viruses intended to be used in human vaccines.

8. Method for manufacturing viral vaccines which comprises a phase of viral production on cells in culture, characterized in that the phase of virus propagation and multiplication is carried out in the presence of a medium which is free of human or animal protein, even of recombinant origin, but which comprises plant extracts.

9. Method according to the preceding claim, characterized in that the medium for viral propagation and multiplication comprises proteins or glycoproteins extracted from potatoes or from cucumber, having a mitogenic potential and having a molecular weight between 1000 and 200,000 daltons.

10. Method according to either of Claims 8 and 9, characterized in that the medium for viral propagation and multiplication comprises plant hydrolysates.