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Method for the Passaging of Microcarrier Cultures to a Production Scale for Producing High Titre Disabled Infectious Single Cycle-Herpes Simplex Virus Type-2

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Abstract A complementary cell line CR2 is currently used to propagate the Disabled Infectious Single Cycle Herpes Simplex Virus Type 2 (DISC HSV-2) on a small laboratory scale upto 15 L. These cultures are initiated by passaging the cells from roller bottle cultures. Whilst this is suitable for the laboratory scale it is totally impractical for use in seeding an industrial manufacturing scaled version of the culture. It is paramount to have a robust system for passaging cells from a small microcarrier culture system to a larger one by a serial subculturing regime. Here we report on the successes we have had in our laboratory in scaling up our production system for the DISC HSV-2 from small 1-L cultures to a 50-L vessel with the maintenance of the viral productivity. Ease of use, reproducibility and the need to minimise overall production times were factors which were taken into consideration whilst developing our procedures We were aware of the need to keep a production train simple and as short as possible as this was the small scale study for an envisaged manufacturing process.

Keywords. DISC HSV-2, microcarrier culture, passaging regime, industrial manufacturing, CR2 cells, viral productivity

Introduction

It has been considered that an effective vaccine against both the primary and recurrent disease caused by herpes simplex virus is an essential requirement. Genital herpes has a high incidence in populations, which is increasing worldwide [1] Cantab's vaccine has been developed with this consideration in mind. The strategy has been to alter live natural viruses by the use of genetic manipulation to introduce an acceptable margin of safety through the development of Disabled Infectious Single Cycle (DISC) virus vaccines. The DISC virus is a virus that lacks an essential gene. This loss of such a gene renders the virus disabled and stops its ability to have a normal multicycle replication phase in a vaccinated host. The viruses can be processed in a way that allows them to only go through one single cycle of replication in the vaccinee. This single cycle grants the provision of an effective immune response. It has been reported that a herpes simplex virus lacking the essential glycoprotein H (gH) gene could be used as effective vaccines [2]. The virus is cultured using a complementary cell line (CR2) expressing the essential gH protein. Virus produced from the complementary cell line can infect normal cells. However, the virus can only perform a single cycle of replication (Fig. 1) Progeny viruses from this round of replication lack the gH and are therefore non-infectious. A DISC, gH-deleted HSV-1 has been used to protect against HSV-1 challenge in the mouse ear model [2] demonstrating the potential of the virus as an effective vaccine in the treatment of herpes. A gH-deleted HSV-2 virus has also been tested as a vaccine in a guinea pig model [3] Animals vaccinated with DISC HSV-2 showed complete protection against primary HSV-2 induced disease, even when challenged six months after

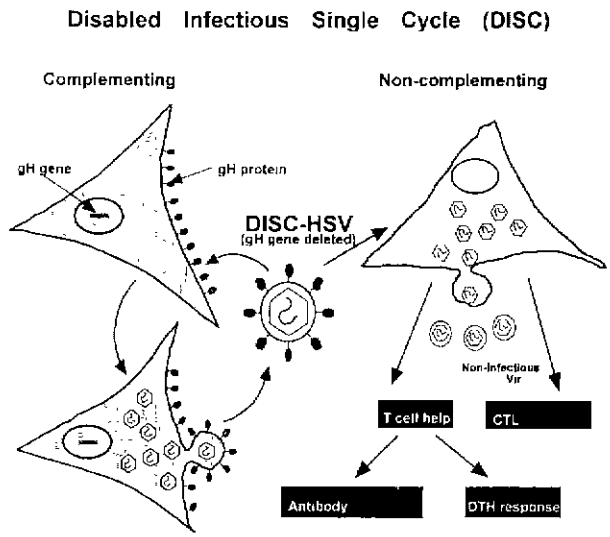

Fig. 1. Disabled Infectious Single Cycle (DISC) virus concept. The DISC–HSV-2 virus can only replicate to generate infectious virus when cultured in the complementary cell line

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vaccination The animals were also almost completely protected against recurrent disease The complementary cell line (CR2) used to propagate the DISC HSV-2 virus was derived from a Vero (monkey kidney) cell line approved by the World Health Organisation (WHO) for use in vaccine manufacture. The CR2 cells contain one integrated copy of the HSV-2 gH gene under the control of the HSV-1 glycoprotein D (gD) gene promoter. Because the gD promoter requires other HSV proteins for its induction gH is, therefore, only produced in the cell following infection with the virus. This is another element of the safety considerations undertaken in the vaccine concept.

A suitable production process has been developed at the laboratory scale. The process involves culturing the CR2 cells on Cytodex 1 microcarriers [4,5]. At the laboratory scale the cultures work effectively at producing high titre DISC HSV-2 at the 1, 5, 10, and 15 L scale. However if the vaccine is to be successful during the clinical trials there will be a need to increase the scale of production by at least an order of magnitude. This causes a problem in itself as the laboratory scale can be initiated by passaging of cells from roller bottle cultures, at the manufacturing scale envisaged, currently 500 L, this will not be possible. If it were then almost 3,000 roller bottle cultures would be required to seed the culture This is a number that is highly impractical and carries an inherent risk at the trypsinisation and cell collection stages unless expensive sophisticated robotic equipment is used. A suitable scaled passaging regime from small-scale microcarrier cultures to industrial sized microcarrier cultures must be the only viable method to use. This passaging regime must be robust and practical [6,7] It must be capable of delivering conditions for suitable high cell growth with a final virus production phase that has a level equivalent to or better than the best laboratory scale culture. The virus production must be maintained on a virus produced per cell basis. We have reported [4,5] on the initial attempts in our laboratory at the passaging regime we have developed. Here we intend to discuss the further development of such a manufacturing passage regime and not other conditions that enable production of high titre virus harvests

MATERIALS AND METHODS

Viruses

The DISC HSV-2 virus, grown in gH-expressing CR2 cell line was constructed at Cantab Pharmaceuticals (Cambridge, UK). Routine working research stocks were prepared and held at –80°C at Cantab ready for use in all development work.

Cells

The CR2 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with high (4.5 g/L) glucose (Gibco, Life Technologies, Paisley, UK) supplemented with 5% foetal bovine serum (FBS) of New Zealand origin (Biogen Autoclear, Salisbury, UK) Cells were expanded from a cryopreserved ampoule into a 25-cm^2 flask. When a confluent monolayer was observed the culture was expanded through several flasks to generate initial roller bottle cultures. Roller bottles with a surface area of 850 cm^2 were supplied by Corning. Roller bottle cell cultures were maintained by routinely passaging them on a weekly cycle. The passage regime used trypsin/EDTA (Gibco, Life Technologies, Paisley, UK) to dislodge the cells from the roller bottle surface. The cells were harvested from the roller bottle cultures by removing the culture medium, washing the monolayer with three 50 mL portions of calcium and magnesium free Dulbecco's phosphate buffered saline (DPBS) (Gibco, Life Technologies, Paisley, UK) to remove any proteins which would inhibit the action of trypsin. A 10 ml portion of trypsin/EDTA was added to each roller bottle culture, which was then re-incubated at 37°C. They were incubated for 10 min. After this incubation period the cell monolayers were observed to ensure that the cells had become dislodged. The action of the trypsin/EDTA was stopped by the addition of 25 mL of complete growth medium. Cells harvested from all the roller bottles were pooled ready for passaging into fresh roller bottles or as an inoculation into microcarrier cultures. Roller bottles were seeded at 2×10^7 CR2 cells per roller bottle. Cells that were to be used for inoculation of initial microcarrier cultures were harvested 5 days post seeding into roller bottle cultures. All roller bottle cultures were purged with air/10% CO_2 for 30 sec prior to closure of the lid. The cultures were incubated in a roller incubator at 37°C and 0.2 rpm. Cytodex 1 microcarriers (Amersham Pharmacia Biotech, Welwyn, UK) were used at a density of 5 g/L and were prepared by hydrating the carriers in 1 L DPBS for a minimum of 4 hours. The volume of DPBS was changed and the swollen microcarriers autoclaved for 20 min at 121°C. Before use they aseptically pre-conditioned with two 1 L volumes of DMEM (5% FBS).

The sterile pre-conditioned microcarriers were delivered into the reactors by use of a seeding bottle able to be connected.

Culture Conditions

The 50-L production vessel was a New Brunswick BioFlo 6000 skid mounted vessel It was fitted with a 1/3 diameter uplifting impeller. The temperature of the culture was maintained at 37°C ± 0.5°C throughout the growth phase. There was a pH setpoint of pH 7.1 maintained on the culture for cell growth. This was controlled by the use of 1 0 M NaOH and sparging of the culture with CO_2 (0.01 vvm) The dissolved oxygen set point was held at 50% saturation with respect to air The set point was controlled by sparging in oxygen, on demand, at a rate of 0 02 vvm. The agitation rate for the growth cycle was 57 rpm

Cultivation of Cells on Microcarriers

Approximately 75% of the final volume of cultivation medium and 5 g/L of microcarriers were pre-incubated in the culture vessel overnight. The pH was controlled to setpoint of pH 7.2 ± 0.2 with either air/10% CO_2 or the addition of 1 M NaOH. Dissolved oxygen concen-

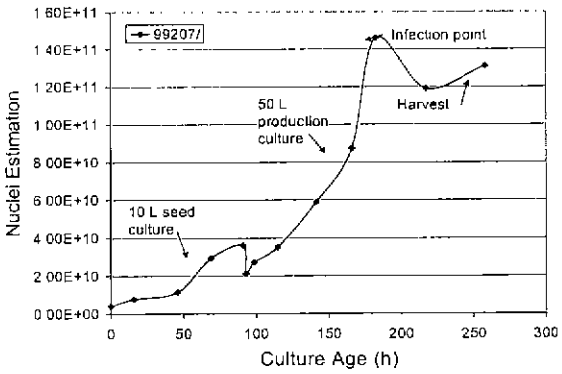

Fig. 2. Typical nuclei estimation profile for a production system using a 1:5 passage regime from a 10-L culture to a 50-L culture

trations were set at a minimum of 30% with respect to air. The cell inoculum for cultivation on the initial microcarrier culture was prepared in late exponential cultures in 850-cm^2 roller bottles. Microcarrier cultures were inoculated at approximately sixteen CR2 cells per microcarrier Cell growth was monitored by estimation of cell nuclei that were released from samples incubated in 0.1 M citric acid containing 0.1% (w/v) crystal violet [8]. During the growth phase of the cells a 50% complete medium change was made on days 2 and 3 post cell inoculation This medium change was used to ensure that the level of lactate in the culture did not become too high and inhibitory and also to ensure adequate levels of glucose to the cells. The medium was removed using a large seed bottle and fresh medium was added using a separate seed bottle. The use of individual seed bottles enabled aseptic removal and addition of the medium. Glucose and lactate concentrations were determined using the YSI 2700 (YSI Limited, UK) and were monitored pre- and post- a 50% media change on days 2 and 3 of the growth period.

Microcarrier Passaging Regime

The procedure outlined in Fig 2 is the method currently used on a passage regime for scaling up the microcarrier process from 10-L cultures to a 50-L production vessel. The trypsin/EDTA solution to be used was prewarmed at 37°C prior to use. The culture medium was removed from the vessel through the internal 76-μm sieve to a waste container by pumping out or over-pressuring the vessel. When all the media had drained out as fully as possible from the vessel the DPBS is pumped into the reactor Approximately 6 L volume of DPBS should be added to the reactor. The DPBS inlet line was clamped off and microcarriers which may have become trapped around the 76-μm sieve were dispersed by gently agitating the reactor vessel or by blowing air back through a 0.22-μm filter connected to the 76-μm internal sieve. The vessel was then drained of DPBS as before. The washing procedure was repeated with three further 6 L aliquots of the DPBS.

After the fourth DPBS wash the trypsin/EDTA bag is removed from the incubator and aseptically connected to the vessel The contents of the bag were pumped into the reactor. This point indicates the start of the trypsinisation incubation period. The impeller speed was increased to 180 rpm during the trypsinisation incubation. The culture was incubated for 8 min. After this time a sample is taken to estimate the detachment of the cells from the microcarriers. If the majority of the cells are not detached the incubation must be continued for a further 2 min, then sampled and observed again. The culture was sampled at 2 min intervals until the vast majority of cells are detached.

When approximately 90% cells have detached, the action of trypsin/EDTA is stopped by the addition of the complete production medium. At this point the agitation setpoint is decreased to the standard growth and infection speed (96 rpm). This trypsinisation time is a critical parameter of the passaging regime. If the culture incubation time is too short not all the cells become detached from the microcarriers. This would result in a poor passage, as there would not be enough free cells to come into contact with the fresh microcarriers, an uneven culture would result. If the trypsinisation incubation time is too long then the cells may be completely digested and a low number of cells are transferred to the new culture causing a long lag phase. The long incubation time may also cause clumping of the cells again resulting in uneven plating of the fresh microcarriers.

The resulting suspension of detached cells and all the culture microcarriers (*i.e.* for a 10-L culture this would be 50 g microcarriers) was then transferred directly to 50-L working volume production vessel. The 50-L culture had been prepared with 200 g of microcarriers so that once passaged the total amount of microcarriers in the production vessel was 250 g or 5 g/L. An open down pipe was used to ensure a rapid and complete transfer of all the microcarrier and detached cells to the new vessel. Transfer of cells and carriers is completed by over-pressurisation of the seed vessel or by use of a peristaltic pump on the transfer line.

The cells are cultured in the same way as for the seed culture. On day 4 post inoculation the culture is ready for infection with the virus. The cell density required for infection is greater than 2 × 10^6 cells/mL. If this density is achieved the culture can be infected.

Virus Infection of the Microcarrier Culture

Prior to infection with DISC HSV-2, the cell cultures were washed with complete Dulbecco's PBS (Gibco, Life Technologies, Paisley UK) to reduce contaminating FBS levels. The virus infection and virus growth phase occurs in a serum free medium The cell growth medium is therefore removed. This removal is achieved by washing the confluent microcarriers three times with 33 L of prewarmed (37°C overnight) DMEM. The cultures are infected using a Multiplicity of Infection of 0.001 (one infectious virus particle to every 1,000 cells).

The infection regime outlined here is for our 50 L working volume culture. The control mechanisms for pH temperature and dissolved oxygen are temporarily stopped. The temperature setpoint was decreased to 34°C. The culture medium is removed from the vessel

through an internal 76-μm sieve to waste. A 33 L volume of prewarmed DMEM was pumped into the reactor The culture was agitated and any microcarriers which may have become trapped around the internal 76-μm sieve were dispersed by gently agitating the reactor vessel or by blowing air back through a 0 22-μm sterilising filter attached to the internal 76-μm sieve line This ensures that the microcarriers are well suspended in the culture facilitating the serum removal prior to infection. The washing procedure was repeated twice more. The final DMEM wash was drained from the vessel as previous and a complete working volume (50 L) of serum free DMEM was added to the vessel. The vessel control mechanisms for temperature, pH and dissolved oxygen are then reinitiated. The culture was allowed to equilibrate at the new set points. Once equilibrated the appropriate number of working seed virus ampoules to give an MOI of 0.001 were thawed and added to 500 mL of DMEM. The virus seed suspension was aseptically added to the culture. The virus growth phase is deemed to have started and lasts approximately 72 h.

Virus Harvest

The virus harvest procedure uses Dulbecco's DPBS buffer containing > 100 μg/mL dextran sulphate This is used in addition to the high shear forces generated by the greatly increased impeller speeds at harvest The released virus is then separated from the cells *via* simple depth filtration This harvest method is very gentle and does not cause as much cell-lysis as our previous methods we employed. This has the benefit of decreasing levels of contaminating cellular DNA and protein.

The volume of harvest buffer used was the working volume × 0.55. The final concentration of dextran sulphate is 100 μg/mL post addition. Any residual volume of medium that cannot be removed during the medium removal step is taken into account for the final buffer preparation. The buffer must be prewarmed to 37°C prior to addition to the vessel. The harvest buffer is left in the culture with continuous agitation at 141 rpm for two hours.

After two hours the virus is predominantly cell free the agitation and dissolved oxygen control are switched off The microcarriers are allowed to settle. The sparge inlet line is closed to prevent disturbance of the settled microcarriers The vessel is pressurised by pulsing air into the top of the vessel. The harvest buffer is then passed through the internal 76-μm sieve that retains the microcarriers. The microcarrier-free crude virus/cell suspension is then ready for clarification.

Clarification of the Cell/Virus Suspension from Microcarrier Cultures

The resulting virus cell suspension is clarified of cells and cellular debris by pumping the suspension through a 1 0-μm Sartorius PP capsule filter. We have successfully used small disposable filter capsules (0.05 m^2) and 10-inch cartridges. The filters are pre-wetted by flushing with DPBS containing 100 μg/mL dextran sulphate prior to the clarification of the virus/cell suspension.

Table 1. Comparison of DISC-HSV-2 production systems

Culture Size L	Source of Cell Inoculum	Final Total Cell Density × 10^{10}	Total Virus Produced (total pfu) × 10^{11}	Virus Titre/Roller Bottle Equivalent × 10^9 pfu/RB
Roller Bottle	Roller Bottle	0.08	0.01	1.0
1	Roller Bottle	0 15	0 1-0 2	0 8
15	Roller Bottle	3 7	3 9	0 99
15	3 L Microcarrier Culture	4 0	4 0	1 0
35	7 L Microcarrier Culture	8 5	10 0	1.1
50	10 L Microcarrier Culture	14.6	13 0	1 0

Post-clarification the filter again flushed with DPBS containing 100 μg/mL dextran sulphate to improve the total virus recoveries by expelling virus containing buffer from the filter housing, filter and associated tubing.

Once clarified the virus is deemed a crude bulk and can be further processed by the Downstream Processing Group.

Virus Titration

The DISC HSV-2 virus infectious titre (in terms of pfu/mL) was estimated using a $TCID_{50}$ method based on the number of infected wells of CR1 cells in a 96-well plates infected with serial tenfold dilutions of virus material. The cytopathic effect (CPE) of the diluted virus was read 3-4 days post infection of the assay plates [9]

RESULTS AND DISCUSSION

This study shows the use of Cytodex 1 microcarriers as a suitable substrate for scaling up the culture of adherent cells for the purpose of our DISC HSV-2 vaccine. The results in Table 1 detail the expected virus titre and the virus production for each production system based on a surface area equivalence of the total number of roller bottles. This allows an easy comparison to confirm that the virus productivity per unit surface area is maintained as the size of culture is scaled up The Table 1 also shows how the cell density achieved in each culture slightly improves with the scale. This is an important trend for estimating the amount of working seed virus required for a manufacturing process. Total virus yields have been achieved that were comparable to those obtained in roller bottle culture systems [10]. This gives a good indication that the scale up method can be directly compared on the basis of roller bottle equivalents. The microcarrier process has previously been shown to be scaled up from a 1 L to 3 L to 15 L culture and also from 7 L to 35 L [10]. Here we demonstrate that the initial development of a passaging re-

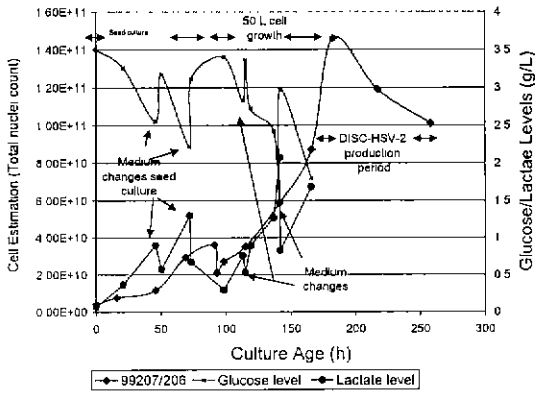

Fig 3. Cell growth curve with glucose lactate levels and indicated medium changes.

gime used previously [10] can be used effectively and improved to provide a means of scaling up to a 50-L working volume production vessel This knowledge that a suitable passage regime can be used here provides a system than can be the basis for a manufacturing scale up system. A 50-L working volume culture is a size of culture that we would deem similar to a manufacturing scale for production of Phase II and Phase III clinical trial material.

Our results in Fig. 2 show that the maximum cell density achieved in the passage of a 10-L to 50-L culture are high and conserved from the development systems. The time frame for the cells to attain a density suitable for virus infection is identical to the small scale processes We would like to refine our procedures to allow a greater split ratio than a 1:5 to be achieved. There is a loss on cell transfer to the larger culture as can be seen in Fig. 2 This loss is observed with every passage, we have minimised the loss substantially over our initial attempts [10]. We have improved the transfer recoveries over the laboratory scale passages by the use of specific agitation rates for the cell inoculation and growth phases of the culture. This has lead to a more consistent time frame for cell growth in the production vessel and for the phase for virus propagation. The medium changes in the seed culture and the production culture are indicated in Fig 3 They do demonstrate that the glucose/lactate levels are controlled effectively to ensure that the cell growth can be maintained and allow the cultures to achieve a density suitable for a passage and infection within reasonable time frames.

In conclusion we have demonstrated the ease with which microcarrier cultures can be used to seed larger microcarrier cultures firstly with our 1 L to 3 L to 15 L spilt ratios followed by the larger scale of a 7 L to 35 L process. We have now moved on to ensure that the process can be used at a small manufacturing scale of 10 L –50 L cultures. The final cell densities and the virus titres obtained in each of the systems are equivalent to those expected when a culture of this size is inoculated with roller bottles. We are now moving our efforts to improve our passaging regime to scale up the cultures to a 500-L working scale. The economics of the process and the labour intensive activities will be looked at to ptry and streamline the number of manipulations and allow such a process to fit well in the manufacturing GMP arena.

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