



## Production of high titre disabled infectious single cycle (DISC) HSV from a microcarrier culture \*

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### Abstract

Disabled Infectious Single Cycle (DISC) HSV-2 has been cultured in the complimentary cell line CR2 to provide high titre bulk material suitable for the purification of the virus as a live viral vaccine. CR2 cells are cultured on the microcarrier Cytodex-1 at  $5 \text{ g l}^{-1}$  in small scale (1 l) and larger scale (15 l) reactors. The cells are infected at an MOI of  $0.01 \text{ pfu cell}^{-1}$  and the culture harvested 60–72 h later. The infected cells are removed from the microcarriers by the addition of a hypotonic saline and the virus released by low-pressure disruption techniques. Virus titres achieved are compared to the standard roller bottle process. The resulting material is the starting point for the purification of the DISC-HSV virus.

### Introduction

The incidence of genital herpes is high and increasing world-wide (Corey, 1993). An important aim in combating the disease is achieving an effective vaccine which can act against both the primary and recurrent disease caused by herpes simplex virus. The company strategy has therefore been to adapt live viruses by genetic manipulation to introduce an acceptable margin of safety through the development of DISC (Disabled Infectious Single Cycle) virus vaccines. These are viruses which lack an essential gene, and are therefore unable to undergo multi-cycle replication in a vaccinated host. They can, however be prepared in a way that allows them to go through a single cycle of replication in cells of the vaccinee, leading to an effective immune response. It has been shown that herpes simplex viruses lacking the essential glycoprotein H (*gH*) gene can be used as effective vaccines (Farrell et al., 1994; McLean et al., 1994). The virus is therefore genetically inactivated so that it is unable to spread within the host.

Currently the DISC-HSV is propagated in a complementing cell line derived from a Vero (African Green monkey kidney) cell line approved by the World Health Organisation (WHO) for use in vaccine manufacture (WHO, 1989). The Vero cells were modified to contain the HSV-2 *gH* gene under the control of the HSV-1 glycoprotein D (*gD*) gene promoter (Boursnell et al., 1997). The complementing cell line was designated CR2. Because the *gD* promoter requires additional HSV proteins for its induction, *gH* is regulated so that it is only produced in the cell following an infection with virus. Virus produced from CR2 cells can infect normal cells, but can only perform one cycle of replication. The progeny virus from this replication cycle lack the *gH* protein and are therefore non-infectious (Figure 1).

Previously it has been shown that a DISC *gH*-deleted HSV-1 can protect against HSV-1 challenge in the mouse ear model (Farrell et al., 1994). The DISC virus, by virtue of its capacity for a single round of replication in normal cells, is more potent than a non-replicating, inactivated virus preparation. A *gH*-deleted HSV-2 virus has also been tested as a vaccine in a guinea pig model. Animals vaccinated with DISC HSV-2 showed complete protection against primary HSV-2 induced disease, even when challenged six

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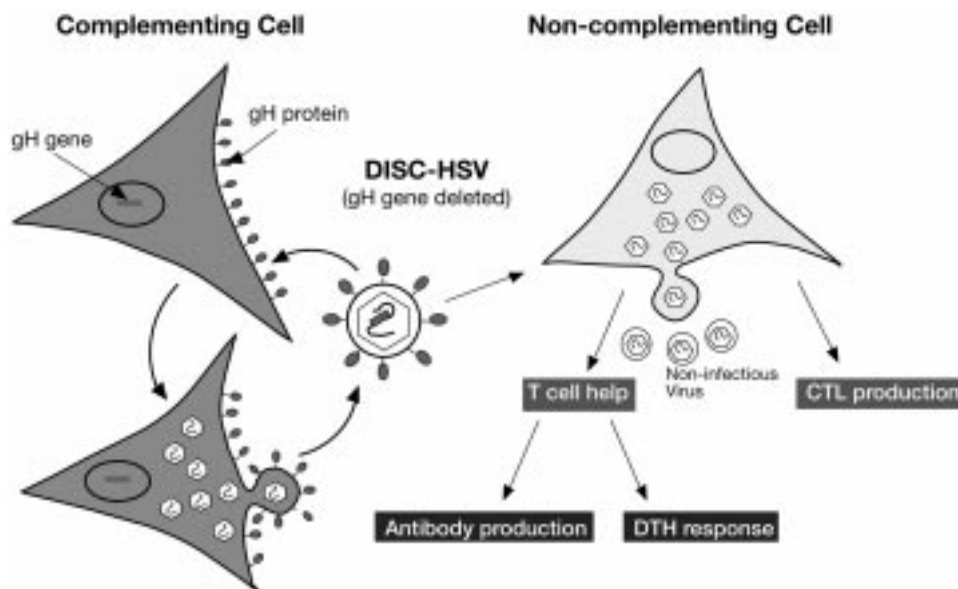


Figure 1. Disabled Infectious Single Cycle (DISC) virus.

months after vaccination (Boursnell et al., 1997). The animals were also almost completely protected against recurrent disease.

For the vaccine to become a viable option as a commercial product, it must be manufactured on a large scale with appropriate processing to meet the demands of the regulatory requirements of safety and efficacy. Currently the DISC-HSV is propagated in the CR2 cell line. The CR2 cell line is an adherent cell and therefore routine culture was performed in roller bottle cultures. Whilst this method of cell growth and virus production is suitable for development work it is not a desirable system for the manufacture of the virus on a larger scale. The use of microcarriers as a support for anchorage-dependent cells has been reported previously (Hu et al., 1985; Van Wezel, 1973). Anchorage-dependant cell lines have been cultured to produce a variety of viruses on a large scale (Talbot et al., 1989; Lesko et al., 1993; Bajot et al., 1987; Meignier, 1978). Some cell lines such as Vero and MRC5 have been propagated on microcarriers to produce human viral vaccines (Fabry et al., 1989; Griffiths et al., 1980; Montagnon et al., 1981). Griffiths et al. (1982) have demonstrated HSV-2 production from cells cultured on low ( $2 \text{ g l}^{-1}$ ) concentrations of Cytodex microcarriers. The yields obtained were ten-fold lower when compared to those achieved from a roller bottle system. It was hoped that our current microcarrier system would produce equivalent productivity when compared to the standard roller bottle process.

The aim of this work was to evaluate the production of DISC-HSV in a microcarrier based culture system in comparison to a conventional roller bottle culture process. The work set out to determine the conditions for the optimum growth of cells and ultimately, production of high titre virus. Production of the virus was initially determined in small-scale (1 l) cultures. Scale-up of the production system was then demonstrated at the 15 l scale.

This paper details the way in which we have approached this issue of manufacturing DISC-HSV only as far as the initial upstream bulk harvest product and does not discuss the subsequent virus purification.

## Materials

**Viruses:** The DISC-HSV virus, grown in gH-expressing CR2 cells was constructed at Cantab Pharmaceuticals, Cambridge, U.K.

**Cells:** Two cell lines are used in our study. One has been designated CR1 and the other CR2. The CR1 cell line is a line used for the assay of the virus only. It is a Vero derived cell line that has been modified to express the glycoprotein H (gH) gene derived from HSV-1 this gene is under the control of the HSV-1 glycoprotein D (gD) promoter. CR2 cells are a modified Vero cell from the WHO Vero accredited bank (No. 88020401; European Collection of Animal Cell

Cultures ECACC, Porton Down, U.K.) that has been modified to express the glycoprotein H (gH) gene derived from HSV-2. This gene is under the control of the HSV-1 glycoprotein D (gD) promoter. The CR1 cells are solely used to assay the virus in the TCID<sub>50</sub> assay whereas the CR2 cells are the production cells for manufacture of the DISC-HSV. Cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM with high glucose, Life Technologies, U.K.) supplemented with 5% Foetal Bovine Serum (PABCO, New Zealand origin).

*Roller bottles:* The bottles used had a total surface area of 850 cm<sup>2</sup> and were obtained from Corning.

*Cytodex 1 microcarriers:* (Pharmacia Biotech, U.K.): The microcarriers were used at a density of 5 g l<sup>-1</sup> and were prepared according to the manufacturers instructions. Before use they were pre-conditioned in DMEM (5% FBS).

*Hypotonic saline:* The solution comprised of Na<sub>2</sub>HPO<sub>4</sub> 2.29 g l<sup>-1</sup>, NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O 0.599 g l<sup>-1</sup> NaCl 0.58 g l<sup>-1</sup>. The reagents were sourced from BDH.

## Methods

### *Roller bottle culture production systems*

#### *Cultivation of cells in roller bottles*

Roller bottle cultures were seeded with a total of  $2 \times 10^7$  CR2 cells per roller bottle. The cultures contained 100 ml of DMEM (5% FBS) per bottle culture. Cultures had a five day cell growth period at 37 °C prior to infection with DISC-HSV.

#### *Roller bottle infection*

At infection the cell monolayers were washed with Dulbecco's PBS to decrease the level of any contaminating BSA from the FBS. Serum-free DMEM was added to the cells as a maintenance medium throughout the virus production phase. Cultures were infected with a working seed preparation of DISC-HSV at a multiplicity of infection (MOI) of 0.01 pfu cell<sup>-1</sup>. The temperature of the cultures during the virus production phase was decreased and maintained at 34 °C.

#### *Roller bottle harvest*

Approximately 64–68 h post infection cell monolayers have between 90–100% CPE. This observed level

of CPE is ideal for harvesting the DISC-HSV. The harvest method involved pouring off the medium and adding 10 ml of hypotonic saline. The cultures were then incubated at 34 °C for 5–10 min. The cells and virus were collected by scraping the surface with a plastic scraper.

### *Production of DISC-HSV in microcarrier culture 1 l and 15 l scale*

#### *Microcarrier preparation*

The microcarriers were prepared as according to instructions from Pharmacia. They were prepared in a siliconised Schott bottle of suitable size. All microcarriers were conditioned by washing with DMEM (5% FBS) prior to addition to the vessel. The microcarriers for the 1 l cultures were washed twice with 250 ml of complete medium whilst the microcarriers for the 15 l cultures were washed twice with 1500 ml of medium.

#### *Reactor vessels*

All vessels were obtained from FT Applikon Ltd. For the 1 l cultures a 2 l total volume jacketed glass vessel was used. A BioBench 20 l total volume, jacketed stainless steel vessel was used to culture the 15 l cultures. Both vessels had spin filters (76 µm mesh size) and used reverse marine impellers. The 2 l vessels had an H:D ratio of 1.5 and the 20 l vessel had an H:D ratio of 2.2.

#### *Cell growth*

The growth of our CR2 cells was examined on several commercially available microcarriers. The range used included the following: – Cytodex 1 (Pharmacia), Cytodex 2 (Pharmacia), Cytodex 3 (Pharmacia), Cultisphere (Cellon Sarl), Cytopore 1 (Pharmacia), Cytopore 2 (Pharmacia), Cytoline 2 (Pharmacia) and FACT (Solohill).

The microcarrier Cytodex 1 was chosen as the production carrier because the cell density obtained using this carrier was the greatest from the least amount of microcarriers used. Cytodex 3 microcarriers gave a similar cell number as expected but this microcarrier has a pig skin collagen layer on the bead. Cytodex 1 was selected in preference to the other microcarriers because of good cell growth and to remove the issue of animal products in the manufacturing process. We currently use the Cytodex 1 at a level of 5 g l<sup>-1</sup>. The growth medium used was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% Foetal

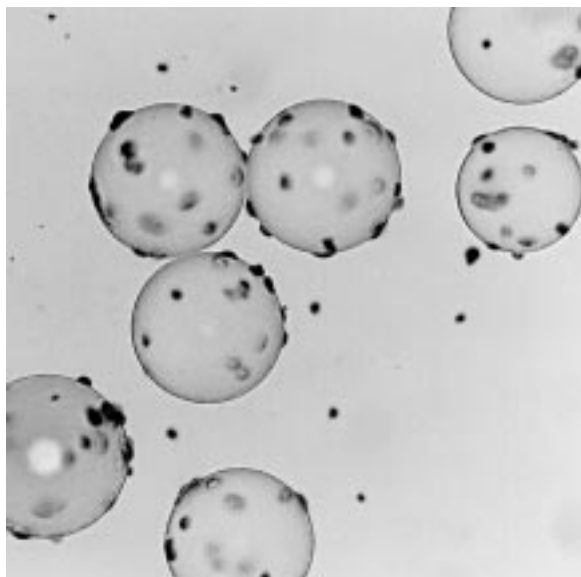


Figure 2. Cell attachment to cytodex microcarriers, 1 h post inoculation.

Bovine Serum. The growth medium used for 15 l cultures was DMEM with 25 mm HEPES (5% FBS).

Approximately 75% of the final volume of cultivation medium and  $5 \text{ g l}^{-1}$  of microcarriers were pre-incubated in the culture vessel overnight. The culture parameter set points were controlled by using the Applikon 1030 controlling system for both vessels. The culture pH was maintained by controlling at a set point of  $7.2 \pm 0.2$  by sparging air/ $\text{CO}_2$  (90:10%) into the spin filter. Dissolved oxygen concentrations were kept above a minimum set point of 30% saturation by sparging when necessary into a spin filter with air for the 1 l cultures and with pure oxygen in the 15 l cultures. The culture temperature was maintained at  $37^\circ\text{C}$  throughout the cell growth period by use of a recirculating thermocirculator attached to the jacket of the 2 l vessels and in the case of the 20 l vessels the Applikon controlling system used a similar integral recirculating thermocirculator.

The cell inoculum for cultivation on microcarriers was prepared from late exponential CR2 cultures in  $850 \text{ cm}^2$  roller bottles. Cultures were inoculated at a density of approximately ten CR2 cells per microcarrier and  $5 \text{ g}$  of Cytodex  $1 \text{ l}^{-1}$  (Figure 2). Cell growth was monitored by estimating nuclei released from a sample of microcarrier culture taken from the reactor after incubation in  $0.1 \text{ M}$  citric acid containing  $0.1\%$  (w/v) crystal violet (Sanford et al., 1951). Glucose and lactate concentrations were determined using an

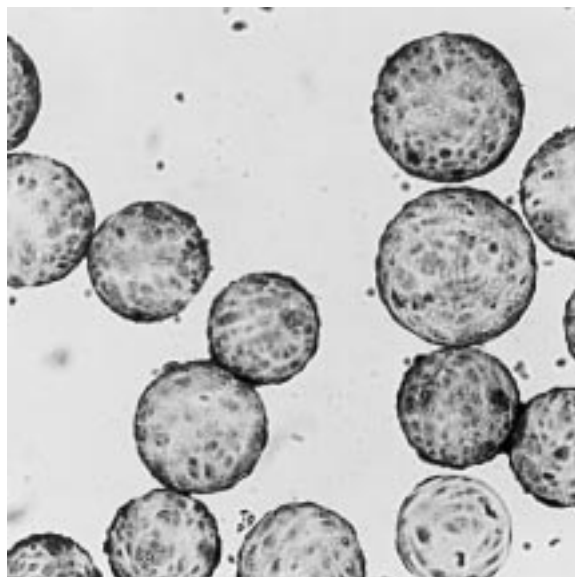


Figure 3. A confluent monolayer of CR2 cells growing on Cytodex 1. At this stage the cells are infected with the DISC-HSV virus.

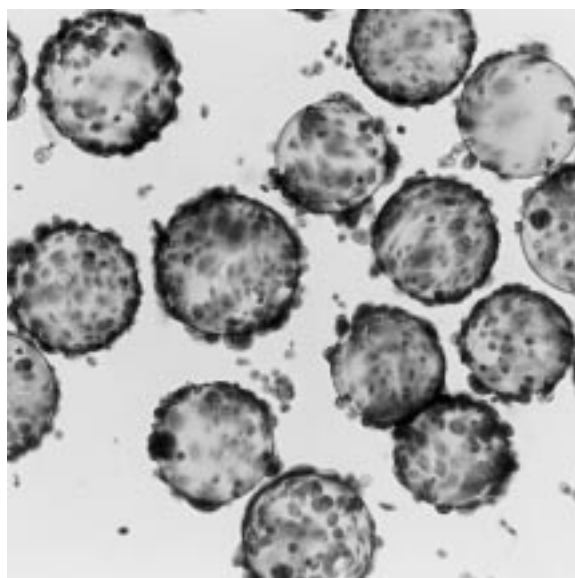


Figure 4. Culture showing 100% cytopathic effect prior to the addition of hypotonic saline. All cultures stained with crystal violet/acetic acid ( $0.1\% \text{ w/v} : 0.1 \text{ M}$ ).

off-line YSI 2700 glucose/lactate analyser (YSI, U.K. Ltd). Glucose and lactate concentrations were controlled by partial media changes on days 2 and 3 for the 1 l culture and days 2, 3, 4 and 5 for the 15 l culture. The 15 l culture was maintained for an additional 48 h period compared to the small scale 1 l cultures in

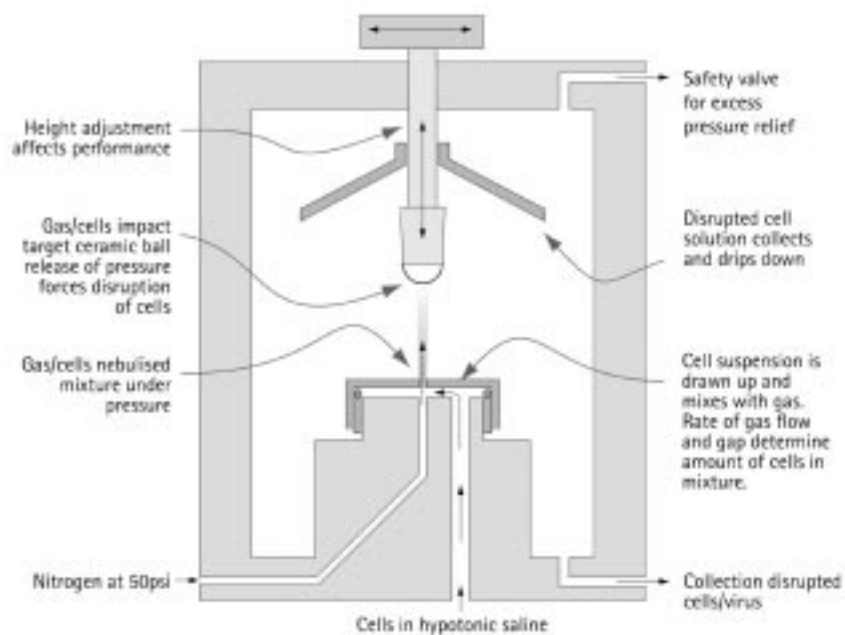


Figure 5. The bionebuliser is a low pressure shear disruption method which uses nitrogen as a carrier gas. The disrupter is depicted here schematically indicating how the process disrupts cells. Using a carrier gas pressure of 50 psi cells are drawn up and nebulised. This cell mixture is directed to a ceramic ball bearing where they impact. The cells are disrupted to release the virus and the whole disrupted cell/virus solution is collected.

order to provide a maximal cell density at the point of infection.

#### *Virus infection of microcarrier cultures*

Confluent microcarriers (Figure 3), approximately 100 h post inoculation in the 1 l cultures and approximately 140–160 h in the 15 l cultures were infected with DISC-HSV at an MOI of 0.01 pfu cell<sup>-1</sup>. The agitation was stopped and the microcarriers were allowed to settle out. The medium was then removed to waste. The 1 l cultures were washed by addition of 1 l of Dulbecco's PBS with agitation. The PBS wash was removed and this step repeated a further two times. The 15 l cultures were washed by addition of 10 l of Dulbecco's PBS with agitation. The PBS wash was removed to waste and this step was repeated a further two times. After washing, the original culture volume was replenished with serum free DMEM. The temperature during the infection period was decreased and maintained at 34 °C.

#### *Harvest of microcarrier cultures*

Infected cells (Figure 4) were harvested from the microcarriers 60–72 h post infection when the cells on the majority of the microcarriers showed 100% CPE. Harvesting was accomplished by removal of the media

from the culture, addition of hypotonic saline at a volume equal to 10 ml hypotonic saline per roller bottle equivalent of surface area of microcarriers used in the culture. The detached cells were separated from Cytodex 1 by filtration through a sterilisable 70 µm mesh in the bottom of the vessel. The resulting cell/virus supernatant was subjected to low pressure disruption (Degouys et al., 1997), (Figure 5), to release the DISC-HSV virus from the cells and cellular debris. Virus titres were calculated using an in-house TCID<sub>50</sub> assay with subsequent conversion to pfu ml<sup>-1</sup>.

#### *Cell disruption and virus release by bionebulisation*

The cell and virus harvests from either pooled roller bottle cultures or microcarrier cultures were passed through a bionebuliser (Glas-Col) at a pressure of 50 psi using sterile filtered nitrogen as the carrier gas. The carrier gas causes a vacuum over the hole leading to the cell suspension. This vacuum draws the cell suspension up the tubing and allows the cell suspension to become mixed with the carrier gas. The mixture then flows through the orifice to impact on the target a ceramic ball to disrupt the cells in the suspension. The flow rate of the gas determines the density of cells in the mixture and the target ceramic ball can be adjusted to maintain a good bionebulisation of the cells.

**Table 1.** Comparison of the virus titres achievable in the two microcarrier cultures used versus the current roller bottle process. Based on available surface area for cell attachment, the 1 l culture is equivalent to approximately  $25 \times 850 \text{ cm}^2$  roller bottles and the 15 l culture approximately  $388 \times 850 \text{ cm}^2$  roller bottle equivalents in terms of potential virus production

	Total pfu	
	1 l	15 l
Microcarriers	$1.5 \times 10^{10}$	$7.08 \times 10^{11}$
Expected roller bottle equivalent	$2.5 \times 10^{10}$	$3.88 \times 10^{11}$

The process is depicted in Figure 5. This low-pressure shear system has been used previously to disrupt cells (Degouys et al., 1997).

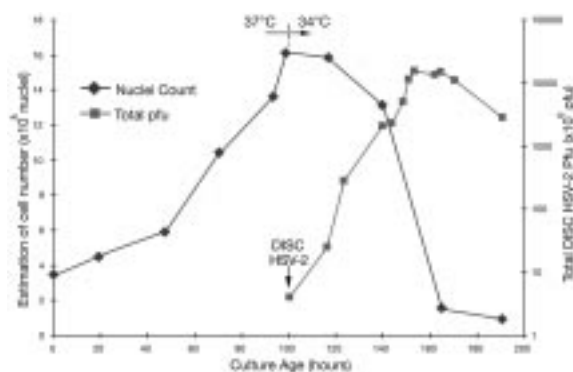
#### Virus titration

The DISC virus infectious titre ( $\text{TCID}_{50} \text{ ml}^{-1}$ ) was estimated in CR1 cells on 96-well plates infected with serial tenfold dilutions of virus material. The CPE was read 3–4 days post infection using a  $\text{TCID}_{50} \text{ ml}^{-1}$  method (Reed and Muench, 1938). The  $\text{TCID}_{50} \text{ ml}^{-1}$  assay is used with a subsequent conversion to pfu  $\text{ml}^{-1}$  as described by Dougherty (1964). The  $\text{TCID}_{50}$  dose can be measured by a number of means and the method of Reed and Muench (1938) was employed in this case. This  $\text{TCID}_{50}$  is the usual end point for many virus assays. The conversion to pfu  $\text{ml}^{-1}$  takes into account the distribution of virus in the suspension according to Poisson's Law. According to Poisson distribution the  $\text{TCID}_{50}$  would contain 0.69 infectious particles per unit volume. Therefore this correction factor can only be applied when chance distribution of virus in suspension occurs (Dougherty, 1964).

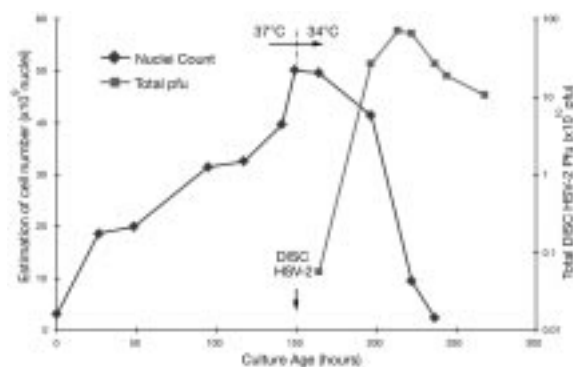
## Results

#### Roller bottle process

CR2 cells were cultured in roller bottles for 5 days until a cell density of  $1 \times 10^8$  cells was typically achieved. At this density 100% cell confluency was observed. Roller bottle cultures infected with DISC-HSV at an MOI of 0.01, exhibited 100% CPE after approximately 65 h. Harvesting the roller bottles with hypotonic saline it was possible to achieve  $10^9$  pfu per bottle.



**Figure 6.** Typical growth of CR2 cells and DISC-HSV production at 1 l scale. The total DISC-HSV virus titre produced in a 1 l vessel is approximately  $1.5 \times 10^{10}$  pfu.



**Figure 7.** Typical growth of CR2 cells and DISC-HSV production at 15 l scale. The amount of DISC-HSV virus titre able to be produced from a 15 l culture is  $7 \times 10^{11}$  pfu. This demonstrates a considerable scale up potential for a microcarrier production system.

#### 1 litre microcarrier cultures

Based on available surface area for cell attachment, a 1 l microcarrier culture is equivalent to approximately  $25 \times 850 \text{ cm}^2$  roller bottles in terms of potential virus production capability (Table 1). A typical growth curve for CR2 cells on Cytodex 1 microcarriers is shown in Figure 6. Cells multiplied exponentially during the first 4 days before reaching stationary phase. A cell density of  $1.5\text{--}2.0 \times 10^9$  total cells was typically obtained after approximately 100 h growth. At this density a confluent monolayer of CR2 cells covered the microcarriers (Figure 3). At 100% confluency the cells appeared elongated, resembling the surface of a golf ball. Cells ready for harvest exhibited complete CPE (Figure 4) 60–72 h after infection. The cultures were harvested before they became detached from the microcarriers. The morphological appearance of the cells at both critical stages was identical to the appearance observed in roller bottle cultures. Infectious

DISC-HSV released from the CR2 cells was quantified using a TCID<sub>50</sub> assay and compared to the amount obtained from the roller bottle culture harvests. The results are shown in Table 1. Typically  $1.5\text{--}2.0 \times 10^{10}$  total pfu from a 1 l culture was regularly achieved, with approximately a 20 hour period during which the culture could be harvested without significant decrease of the titre. The total surface area available in the culture is equivalent to 25 roller bottles. This enables a comparison in terms of productivity to be made. We routinely achieve  $2.0\text{--}2.5 \times 10^{10}$  total pfu from 25 roller bottle cultures. Therefore, the two production systems yield similar total amounts of DISC-HSV.

#### *15 litre microcarrier cultures*

Based on available surface area for cell attachment, a 15 l culture is equivalent to approximately  $388 \times 850 \text{ cm}^2$  roller bottles in terms of potential virus production (Table 1). A typical growth curve for CR2 cells on Cytodex 1 microcarriers grown at the 15 l scale is shown in Figure 7. Cells multiplied exponentially during the first 6 days before reaching stationary phase. Typically a cell density of  $4.0\text{--}5.0 \times 10^{10}$  total cells was obtained after approximately 150 h. After this growth period a confluent monolayer of CR2 cells (Figure 3) covered the microcarriers. When the cells exhibited complete CPE (Figure 4) 60–72 h after infection, the cultures were harvested. Infectious DISC-HSV released from the CR2 cells was quantified using a TCID<sub>50</sub> assay and was again compared to the titre achieved from the equivalent roller bottle culture harvests. The results are shown in Table 1. Typically  $4\text{--}7.0 \times 10^{11}$  total pfu from a 15 l culture was achieved, with a period of approximately 9 h during which this high titre was sustained. From an equivalent number of 388 roller bottles we would expect to achieve approximately  $3.88 \times 10^{11}$  total pfu. Our results, therefore, compare very favourably with the expectations from roller bottle cultures and the results achieved from the 1 l cultures.

### **Discussion**

Cytodex 1 microcarriers can be used as a Vero cell culture support for the production of DISC-HSV virus. It could be envisaged that the microcarrier washing and conditioning procedure at a large manufacturing scale could be extremely time consuming. It is expected that the microcarriers will be sterilised *in-situ* inside the production vessels. Preliminary work at 35 l scale

employs the use of an internal sieve to aid the washing and conditioning process. At present at the 35 l scale this step takes approximately 2 h to complete. This washing and conditioning step may be an extensive time constraint at a manufacturing scale. Tackling the engineering problem in terms of pipe work and increasing flow rates could reduce this time. This is an issue that is under review and will require suitable development time to reduce it satisfactorily. Yields achieved from the microcarrier cultures were comparable to those obtained in the standard roller bottle culture systems (Table 1).

It has been demonstrated that a 15 l microcarrier culture can produce an equivalent amount of virus as that achieved from  $700 \times 850 \text{ cm}^2$  roller bottles despite having the surface area of only 400 roller bottles. This maintenance of the cell productivity has not always been observed when virus production systems have been scaled up. Griffiths et al. (1982) had a ten-fold decrease in cell productivity of HSV per cell when switching from roller bottle cultures to a microcarrier system using Vero cells. This may have been an artefact of poor virus release from the cells. In comparison, when culturing the HSV-2 on MRC-5 cells, a significant but smaller decrease in production levels was observed when changing from roller bottle cultures to microcarrier cultures. This observation reinforces the issues made in some early virus production work reported by Giard et al., 1977. They proposed that there are specific virus/cell line requirements coupled with a specific optimisation of the conditions not only for the growth of the cells but also for the virus production phase. The production of hepatitis A in microcarrier culture was reported to be 8-fold lower than when cultured in conventional flask cultures (Widell et al., 1984). The maintenance of productivity seen in this study is very encouraging in moving the culture system forward to a larger scale which may be suitable for the production of Phase III material and for the supply of commercial requirements. Due to the productivity's shown here, production at a 15 l scale would be large enough to supply material for Phase I and Phase II studies.

Additional benefits of this system arise in the need for decreased culture medium. The very large culture surface area to volume ratio offered by the microcarrier system provides high cell yields in a minimal volume. When compared with the roller bottle system approximately half the volume of medium is required to produce an equivalent cell density (Van Wezel, 1972). This leads to savings on the cost for

culture medium and in particular for costly serum additions.

The number of direct manipulations is also reduced when switching to a microcarrier based production system. This decreases the labour intensity of the process, minimising costs of materials and the overall process time. The virus is produced from a single batch culture with one set of aseptic manipulations throughout the process rather than multiple manipulations that are required when using a roller bottle system.

If the system can be operated at a larger scale (e.g. >50 l) and still maintain DISC-HSV productivity then it would become a viable production method. Passaging Vero cells to a large production vessel (500 l) has been suggested (Baijot et al., 1987), but it likely that the trypsinisation method will be all important in such a process.

The high productivity cultures using perfusion techniques, such as the production system used for the poliovirus production at 20 g l<sup>-1</sup> of microcarriers (Fabry et al., 1989), may also be employed for high titre virus production. However, further development work would be needed to adopt a similar production system for our DISC-HSV vaccine.

In our laboratory we are considering serial passaging and perfusion techniques to improve our production capabilities further. From the results reported in this present study we are confident that we have a productive scaleable microcarrier system for our DISC-HSV vaccine that will allow us to produce adequate material for both Phase III studies and commercial product.

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