Cytotechnology **29:** 135–148, 1999.

A microcarrier-based cell culture process for the production of a bovine respiratory syncytial virus vaccine

Enda Moran*
MFM Laboratories Ltd., 4 Warner Dr., Springwood Industrial Estate, Braintree, Essex, CM7 2YW, UK.

Key words: anchorage dependent, animal cell, bioreactor, Cultispher S, Cytodex 3, microcarrier, respiratory syncytial virus, vaccine

Abstract

Veterinary viral vaccines generally comprise either attenuated or chemically inactivated viruses which have been propagated on mammalian cell substrates or specific pathogen free (SPF) eggs. New generation vaccines include chemically inactivated virally-infected whole cell vaccines. The NM57 cell line is a bovine nasal turbinate persistently infected (non-lytic infection) with a strain of the respiratory syncytial virus (RSV). The potential of microcarrier technology for the cultivation in bioreactors of this anchorage dependent cell line for RSV vaccine production has been investigated. Both Cytodex 3 and Cultispher S microcarriers proved most suitable from a selection of microcarriers as growth substrates for this NM57 cell line. Maximum cell densities of 4.12×10^5 cells $ml^{-1}$and 5.52×10^5 cells ml^{-1} respectively were obtained using Cytodex 3 (3 g l^{-1}) and and Cultispher S (1 g l^{-1}) in 5 l bioreactor cultures. The fact that cell growth was less sensitive to agitation rate when cultured on Cultispher S microcarriers, and that cells were efficiently harvested from this microcarrier by an enzymatic method, suggested Cultispher S is suitable for further evaluation at larger bioreactor scales (>5 l) than that described here.

Abbreviations: BHK, baby hamster kidney; FMD, foot and mouth disease; MDBK, Madin Darby Bovine Kidney; RSV, respiratory syncytial virus; SPF, specific pathogen free; TCA, tricarboxylic acid.

Introduction

Bovine respiratory syncytial virus (RSV) is an economically important causative agent of respiratory disease in cattle world-wide (Fenner et al., 1987; Thomas et al., 1982). The virus (family *Paramyxoviridae*, genus *Pneumovirus*) contains a single strand of negative sense RNA within a lipid bi-layered envelope. A glycoprotein on the surface of the virion, the fusion or F protein, fulfils a particularly important role in the pathogenesis of this virus. The F protein mediates fusion of the viral envelope to the host cell membrane following attachment of the virion to a surface receptor. In addition, it promotes cell-cell fusion allowing intercellular spread of infectious virus without cell lysis. This glycoprotein stimulates protective immunity to both bovine and human RSV disease (Stott et al., 1984; Taylor et al., 1984).

Typical cytopatholgy of RSV *in vitro* involves syncytium formation in which cells fuse together to form large multi-nucleate masses. RSV may also cause persistent infections in certain cell lines in which viral antigen accumulates in the cytoplasm and at the surface of the cell with minimal or no cytopathology (Pringle et al., 1978). Persistently infected cells are routinely identified by immunofluorescent staining of whole or fixed cells using monoclonal antibodies or polyclonal sera directed at the viral antigen of interest. These cells have potential as viral vaccines following appropriate treatments to inactivate the cell-associated live virus (Onuma et al., 1984).

* Present address: GlaxoWellcome Research & Development, Biotechnology Development Laboratories, South Eden Park Rd., Beckenham, BR3 3BS, UK. Telephone:+44 (0) 181 639 6551; Fax: +44 (0) 181 639 6149; E-mail:ebm41807@glaxowellcome.co.uk

The use of microcarriers for the cultivation of anchorage dependent mammalian cells in suspension culture has now become firmly established (Van der Velden-de Groot, 1995) since the early publications detailing the production of viral vaccines from anchorage dependent cell lines grown on microcarriers, for example polio vaccine on primary monkey kidney cells (Van Wezel et al., 1980) and on Vero cells (Montagnon et al., 1983). While it has proved possible to cultivate the foot and mouth disease virus (FMD) in a baby hamster kidney (BHK) suspension-adapted cell line in sufficient quantities to satisfy the large world market for this animal vaccine (Barteling and Vreewijk, 1991), most veterinary virus vaccines remain produced in anchorage dependent cell culture systems. These cell cultivation systems still include now relatively old technology such as roller bottle culture, or multi-tray cell factories (Long 1994).

The benefits of using microcarrier culture for vaccine production including cost savings in labour, process consumables, laboratory space, and the possibility of greater yields through process control in bioreactors has prompted vaccine manufacturers to consider microcarrier technology as an important process improvement. Many types of microcarriers, for example solid, microporous and macroporous, are commercially available for use in different bioreactor configurations including batch, fluidized-bed and perfusion systems. This paper describes a rapid study designed to investigate the feasibility of using microcarrier technology in a mammalian cell culture process for the production of a bovine RSV vaccine; this vaccine has previously been manufactured in both roller bottle culture and cell factories.

Materials and methods

Cell line

The NM57 cell line used in this work was derived from organ cultures of bovine foetal nasal mucosa infected with strain 127 of RSV and was originally named SW129 NM7 (European Patent 0 043 272). This persistently infected diploid line bears the highly immunogenic viral fusion protein (F antigen) at the cell membrane. Glutaraldehyde-fixed SW129 NM7 cells were first demonstrated to confer protection to cattle from RSV challenge by Stott and others (1984) and subsequently by Howard et al. (1987). The currently licensed bovine vaccine, Torvac® , comprises inactivated NM57 cells in an adjuvanted formulation for the active immunisation of cattle against RSV disease.

Microcarriers

The Cytodex 3 (Pharmacia, Sweden) and Rapidcell C (ICN Pharmaceuticals Ltd., USA) microcarriers are collagen-coated dextran and polystyrene microcarriers respectively. Rapidcell P and Biosilon (Nunc, UK) are polystyrene microcarriers. Cultispher S is a macroporous, cross-linked gelatin microcarrier (Percell Biolytica AB, Sweden). The properties of all microcarriers used are shown in Table 1. All microcarriers were washed and autoclave sterilised in phosphate buffered saline (PBS) according to the manufacturers instructions.

Cell culture

All reagents were obtained from Life Technologies Ltd., UK unless otherwise stated. The basal medium contained glutamine-free Eagles minimal essential medium supplemented with 5% irradiated foetal calf serum, buffered with sodium bicarbonate and HEPES and containing polymixin (100 units ml^{-1}) and neomycin (100 μg ml^{-1}). Variations of this medium were supplemented with glucose, glutamine, bovine serum albumin (BSA) and lactalbumin hydrolysate, or certain combinations of these. All raw materials of bovine or porcine origin were obtained from certified sources in the US, New Zealand or Australia.

In order to compare the performance of the microcarriers in spinner flask cultures, different quantities of each microcarrier giving equivalent amounts of available surface area for cell growth were used or alternatively, cultures were seeded with similar numbers of cells per microcarrier. The choice of method was dependent on the manufacturer data available. Cell inocula for all cultures were harvested from 80–90% confluent roller bottle cultures between passage numbers 19 and 32. Spinner cultures (Techne, UK) containing microcarriers and cell inoculum (2.54×10^7 total cells) in medium at 50% of the final working volume were interval stirred (cycles of stirring at 30 rpm for 10 min followed by 50 min without agitation) for 18 h at 35 °C, after which time fresh, pre-warmed medium was added to 300 ml. Cultures were then stirred continuously at 30 rpm.

Cell fermentations were carried out in glass bioreactors of 5 l working volume and controlled using

Table 1. Properties of microcarriers used in this study

Microcarrier	Supplier	Size range (μm)	Material	Density (g cm^{-3})	Surface area (cm^2 g^{-1})
Cytodex 3	Pharmacia	(141–211)	Collagen coated, cross linked dextran	1.04	2700
Rapidcell P	ICN Pharmaceuticals	(150–210)	Polystyrene	1.02	–[1]
Rapidcell C	ICN Pharmaceuticals	(150–210)	Collagen coated polystyrene	1.03	–[1]
Biosilon	Nunc	(160–300)	Polystyrene	1.05	255
Cultispher S	Percell Biolytica	(130–380)	Cross linked gelatin, macroporous	–[1]	–[2]

[1] Data not available from the manufacturer.
[2] Not measurable due to the macroporous nature of the Cultispher S microcarrier.

BIOLAB CP hardware (B. Braun Biotech, Germany). Temperature and dissolved oxygen in all cultures were maintained at 35 °C and 30% of medium saturation with air respectively. Agitation and pH were maintained at 60 rpm and pH 7.2 unless otherwise indicated. The agitator comprised two sets of pitch-bladed impellers spaced three inches apart on the agitator shaft. The initiation phase of the culture was carried out in a reduced volume of 1.5 l to facilitate efficient attachment of the cells to the microcarriers. For fermentations using Cytodex 3, the procedure involved ten cycles of agitation for ten minutes at 75 rpm followed by a settling phase of fifty minutes, then continuous stirring thereafter. The cultures were next filled to 5 l with pre-warmed medium at 20 h post-inoculation. A number of different attachment phase programs in 1.5 l medium were used for Cultispher S cultures: these were AP1 – 4 cycles of 12 min at 75 rpm + 28 min settling, and AP2 – continuous stirring at 75 rpm. Following these attachment phases, the cultures were treated in a similar manner to the Cytodex 3 cultures. Medium changes were carried out by allowing the microcarriers to settle under gravity for ten minutes before pumping 4.5 l spent medium to waste and re-filling with a similar volume of fresh pre-warmed medium. When the performance of a certain growth medium was being investigated, cells were cultured for one passage in roller bottles in that particular medium before harvesting and seeding to the microcarrier culture. Data presented for both spinner and fermentation cultures are the averages of two replicates. All cultures tested free of mycoplasma and microbial contamination.

Cell recovery from bioreactor cultures

The gelatinous matrix of the Cultispher S microcarriers enabled their complete dissolution *in situ* by enzymatic digestion. On the day of harvest, agitation was stopped and the confluent microcarriers allowed to settle to the bottom of the fermenter. 4.5 l of spent medium was removed to waste through a headplate-mounted harvest tube. Following 2×3 l washes of the microcarriers with phosphate buffered saline (PBS), 1.2 l of 0.5% trypsin/EDTA solution was added and trypsinisation allowed to proceed for 30 min at 60 rpm and pH 7.6. Growth medium containing serum was next added to the concentrated cell suspension to neutralise the trypsin activity. Finally, cells were recovered from the bioreactor, centrifuged and re-suspended in Earles balanced salts solution (EBSS) to a concentration of 2×10^6 cells ml^{-1}.

Analytical methods

Cells removed from microcarriers by trypsinisation were enumerated using a neubauer haemacytometer. Cell viability was determined using a trypan blue staining procedure (0.4%, Sigma, UK). Visual monitoring of Cultispher S cultures was carried out using MTT (Sigma, UK) staining of the colonised microcarriers. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted by mitochondrial dehydrogenases in viable cells to an insoluble purple formazan product. Hence, the intensity and spread of purple staining of the microcarriers pro-

vided an indication of their degree of confluency when examined microscopically.

Glucose and lactate concentrations were quantified using a Microzym-L analyser (SGI, France). The analyser used the enzymatic reactions of glucose oxidase and lactate dehydrogenase in the presence of potassium ferricyanide for glucose and lactate quantification respectively. Ammonium ion was measured using a DirectION electrode (Merck, UK).

RSV was assayed using calf testis cells in a 96-well microtitre plate $TCID_{50}$ assay using the limiting dilution method. Four wells were inoculated per dilution of virus. After 72 h incubation at 37 °C, the presence of RSV was determined by UV microscopy after acetone fixing of the cell monolayers and indirect immunofluorescent staining using a mouse anti-F antigen monoclonal followed by a sheep anti-mouse IgG-FITC conjugate (Sigma, UK). Viral titres were determined by the Reed & Muench procedure.

The number of NM57 cells expressing the RSV F antigen at the cell membrane was determined by washing the cells in PBS, immunolabelling with the antibodies described above and counting the fluorescing cells using a UV microscope at X10 magnification. Three hundred cells were counted for each determination and the result expressed as a percentage of the total number of cells in the population. This characteristic is referred to in the text as the %IF of the cell population.

Results and discussion

Microcarrier selection. I

An initial assessment of the suitability of the microcarriers for the cultivation of NM57 cells showed that Cytodex 3 was an obvious candidate for further development work, Figure 1. Cultispher S microcarriers were evaluated later in the development cycle and will be described in a subsequent section of this paper. In a previous attempt at the culture of the NM57 cell line on Cytodex 3 microcarriers, a cell density of 2.5×10^5 NM57 cells ml^{-1} at 1.5 g l^{-1} Cytodex 3 was achieved in 500 ml spinner cultures (Hayle, 1986). However, no further increase in cell density was observed on increasing the concentration to 2 g l^{-1}. Using a growth medium and spinner culture conditions not too dissimilar to that used by Hayle, it has proved possible to culture NM57 cells to 100% confluency at concentrations of 3 and 4 g l^{-1} Cytodex 3 in our laboratory.

It was initially suspected that cell proliferation on Rapidcell C and Cytodex 3 would be comparable given that both present a collagen-layered substratum to the cells – however, this proved not to be the case. Maximum cell densities on these microcarriers were 4.82×10^5 NM57 cells ml^{-1} and 0.8×10^5 NM57 cells ml^{-1} on Cytodex 3 and Rapidcell C respectively. The fact that cell growth was equally poor on different size ranges of Rapidcell C (150–210 μm, Figure 1; 90–150 μm, data not shown) suggests that the degree of spherical surface geometry is not a crucial parameter affecting cell attachment and proliferation on this microcarrier. Appreciable cell-mediated aggregation of microcarriers similar to that reported by Varani et al. (1996) was observed for both the Rapidcell P and Rapidcell C cultures. In repeats of these cultures, a progressive increase in agitation rate from 30 rpm to 40 rpm (day 1), to 50 rpm (day 2) and finally to 60 rpm (day 3 onwards) failed to disrupt these clumps comprising on average 6–10 microcarriers. It was routinely observed that cells trypsinised from microcarriers for the purpose of counting showed viabilities greater than 95% even in the cultures showing poor growth, prompting the conclusion that only viable cells remain attached to the microcarriers during culture. The Biosilon microcarrier supported cell proliferation but showed extensive clumping on steam sterilisation. These clumps were disrupted by 30 min sonication in PBS prior to the initiation of the spinner cultures but this occurrence excluded this microcarrier from consideration for large scale applications.

Culture of NM57 cells under different conditions

It is well established that the successful culture to confluency of anchorage dependent cells is in part conditional on supplying a minimum number of cells per microcarrier at culture initiation and that this ratio typically varies with each cell line/microcarrier combination (Clark and Hirtenstein, 1981). This ratio for the culture of NM57 cells on Cytodex 3 was determined to be seven cells per microcarrier, Figure 2. Under these conditions, microscopic examination showed that all microcarriers were colonised 24 h after inoculation and carried on average approximately 9 cells per microcarrier whereas the equivalent culture inoculated at 4 cells per microcarrier showed a significant proportion ($\sim$20%) of uncolonised microcarriers 24 h post-inoculation. The cultures initiated at a density of seven or nine cells per microcarrier showed typical diploid fibroblastic morphology close to confluency

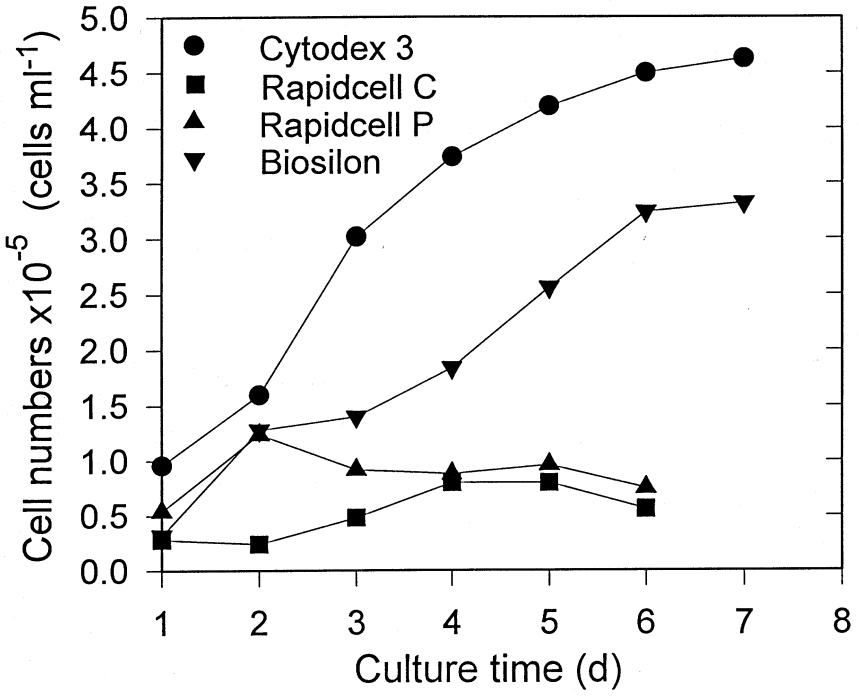

Figure 1. Growth of NM57 cells on different microcarriers in 300 ml spinner cultures using basal medium supplemented with BSA and lactalbumin hydrolysate. The cultures contained Cytodex 3 (3 g l^{-1}), Rapidcell C (3 g l^{-1}), Rapidcell P (8 g l^{-1}) and Biosilon (32.3 g l^{-1}). The Cytodex 3, Rapidcell C and Rapidcell P cultures were initiated with inocula of 10 cells/microcarrier in medium at 50% of the final working volume. The total of 9.7 g Biosilon in these 300 ml cultures provided equivalent surface area for cell growth to those cultures using Cytodex 3 (≈2430 cm^2) – the cell inoculum of 2.54×10^7 cells therefore amounted to equivalent inoculation densities of 1.04×10^4 cells cm^{-2} for these cultures. All cultures were interval stirred for 18 h at 35 °C (cycles of stirring at 30 rpm for 10 min followed by 50 min without agitation) after which time fresh, pre-warmed medium was added to 300 ml and continuous stirring set at 30 rpm.

at day four of the fermentation – cells were aligned in 'contact-inhibited' parallel arrays and showed no signs of multi-layering which is often evident during prolonged culture of more established cell lines such as Madin Darby Bovine Kidney (MDBK) or HEp-2 on microcarriers.

It is clear that the growth of NM57 cells on Cytodex 3 in fermentation culture is quite sensitive to relatively small variations in microcarrier concentration, pH and agitation rate, Figure 3. The optimal concentration of Cytodex 3 proved to be 4 g l^{-1}. The productivities of the 3 g l^{-1} and 4 g l^{-1} Cytodex 3 cultures at pH 7.2 & 60 rpm were almost identical – 3.83×10^4 (day 5) and 3.82×10^4 (day 4) NM57 cells cm^{-2} respectively. Microcarriers at day 4 in the 3 g l^{-1} culture were approximately 70–80% confluent. Full confluency was not reached until day 5, 24 h after the 4 g l^{-1} culture reached confluency. Culture cell numbers at day 1 were as follows: 3 g l^{-1} Cytodex 3/4.9 cells $microcarrier^{-1}$, 4 g l^{-1} Cytodex 3/9.6 cells $microcarrier^{-1}$. This lower degree of microcarrier coverage at day 1 resulted in the longer time required to achieve full confluency in the case of the 3 g l^{-1} culture.

The 5 g l^{-1} culture controlled at pH 7.2 & 60 rpm yielded an average of 3.05×10^4 NM57 cells cm^{-2}. A significant proportion of microcarriers (approximately 15%–20%) in duplicate 5 g l^{-1} Cytodex 3 cultures showed a degree of confluency of 50% or less on day 4 (visual observation using light microscopy). Hence, the heterogeneity of microcarrier coverage resulted in the lower average cell yield per unit surface area. This phenomenon has been observed previously. Chun et al. (1996) could only achieve a maximum average of 26 cells per Cytodex 1 microcarrier when culturing human embryonic lung cells at a concentration of 15 g l^{-1}, compared to a yield of approximately 36 cells per microcarrier at a concentration of 5 g l^{-1} Cytodex 1. One reason for this may be the greater chance of collisions occurring in cultures with high concentrations of microcarrier, thus increasing the probability of dislodgement of weakly attached mitotic cells.

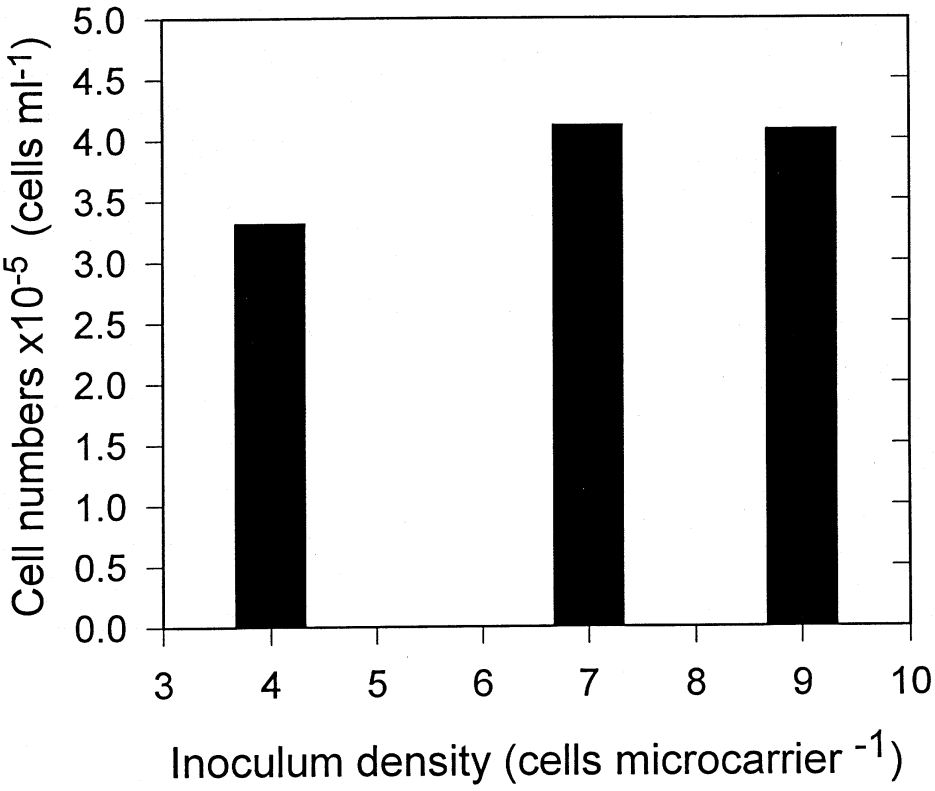

Figure 2. Effect of inoculum level on maximum NM57 cell densities on Cytodex 3 at a concentration of 4 g l^{-1} in 5 l bioreactor cultures. All cultures were carried out using basal medium supplemented with BSA and lactalbumin hydrolysate and controlled at pH 7.2, 30% DO (dissolved oxygen), 35 °C and 60 rpm.

An agitation rate of 60 rpm created a homogenous suspension of microcarriers under low shear conditions in a 4 g l^{-1} Cytodex 3 fermentation. In contrast, cells did not grow to confluency when the culture was agitated at a rate of 80 rpm. Much work has described the effects that hydrodynamic forces due to fluid turbulence, sparging and microcarrier collisions, have on cell growth, removal and death in microcarrier cultures (Aunins et al., 1986; Croughan et al., 1988; Croughan and Wang, 1989). In general, the deleterious effects of the above phenomena on cell cultures e.g. cell death and removal from the microcarriers, have been shown to become more pronounced in direct proportion to the agitation rate.

Studies during the course of this work revealed no significant difference between growth rates of NM57 cells in static flasks (150 cm^2) or roller bottles (900 cm^2) in the range of pH 7.0 – 7.4. These cultures were carried out by using media of different starting pH and carrying out medium changes every 48 h in order to keep the pH reasonably constant over the course of the cultures. However, this robustness was not observed in fermenter cultures. The fermentation carried out at pH 7.0 failed to thrive, yielding a maximum cell concentration of 2.17×10^5 cells ml^{-1} at day four. This maximum cell density at pH 7.0 represented an approximately three-fold increase in cell numbers over the course of the culture, a significantly poor performance, compared to the parallel culture controlled at pH 7.2. From a process control point of view, this pH sensitivity did not pose a problem – the pH controller comfortably maintained pH in these 5 l fermentations at pH 7.2±0.02 by acid or alkali addition (both at 0.2 mol l^{-1} concentration).

Nutrient and oxygen requirements

NM57 cells readily use glucose as a carbon and energy source, Figure 4. At a starting concentration of 4.48 mmol l^{-1} glucose, cells grew to confluency within 96 h. During the exponential growth phase, lactate was produced and accumulated in the medium.

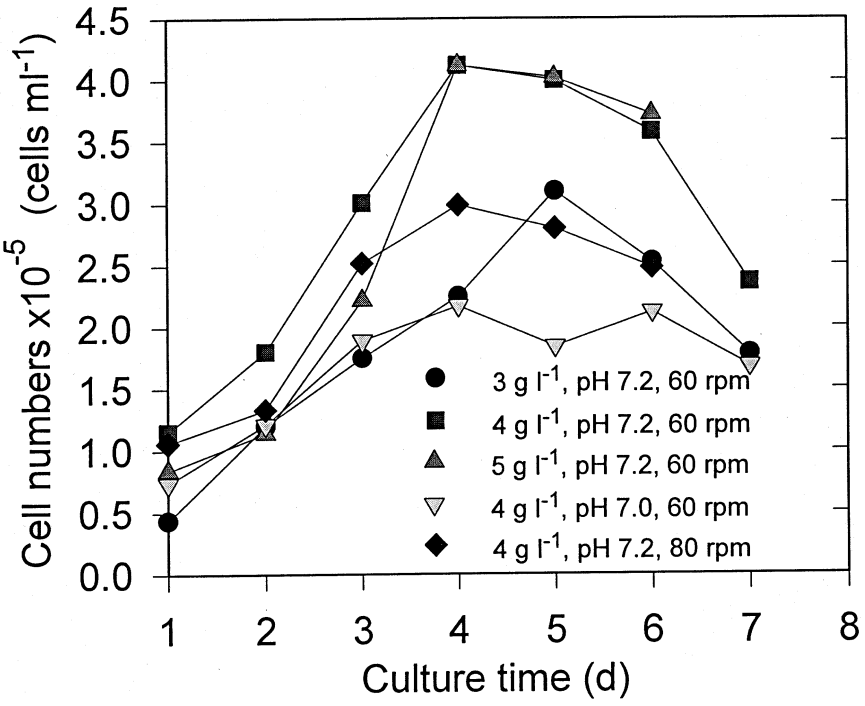

Figure 3. Growth of NM57 cells on Cytodex 3 in 5 l bioreactor cultures controlled at 30% DO, 35 °C and using the various conditions indicated. All cultures used basal medium supplemented with BSA and lactalbumin hydrolysate and were inoculated at a density of seven cells per microcarrier.

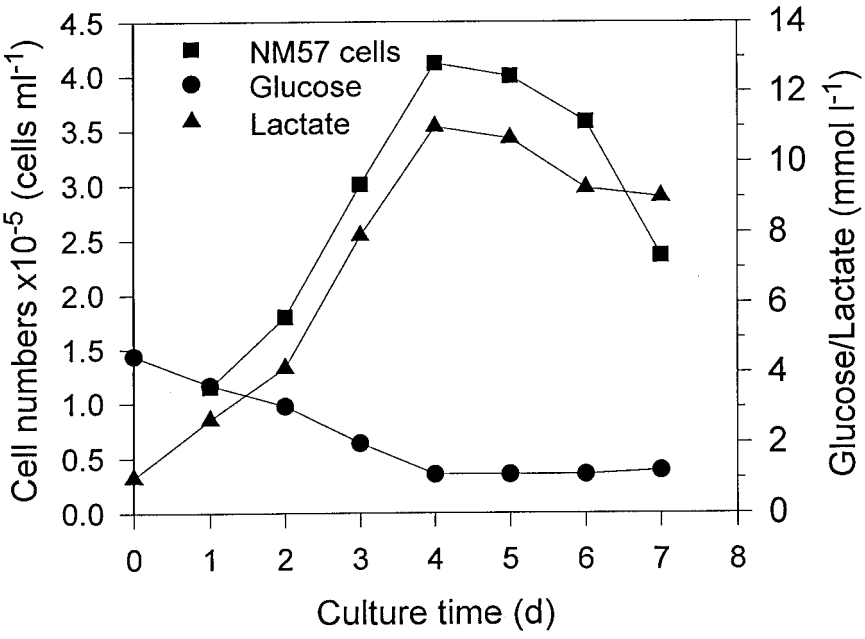

Figure 4. Glucose and lactate concentrations during culture of NM57 cells on Cytodex 3 at a concentration of 4 g l^{-1} in 5 l bioreactor cultures. This culture was inoculated at a density of seven cells per microcarrier. Other conditions were as described in the caption to Figure 2.

The main concern with the accumulation of lactate is the inhibitory effect that this by-product of cellular metabolism may have on the cells producing it. The proliferation of cells to confluency suggests that the concentrations of lactate at 72 h and 96 h (7.92 mmol l^{-1} and 11.0 mmol l^{-1} respectively) were not inhibitory to cell growth, although inhibition studies have not been carried out to show that newly passaged cells would survive in medium containing lactate at these concentrations.

The oxygen demand of this culture was estimated at 100% confluency (day 4) using the dynamic measurement method (Fleischaker and Sinskey, 1981). The following assumptions were made in the calculation; a) negligible oxygen transfer both into and out of the medium from the reactor headspace compared to that due to cellular consumption at maximum cell density and b) the dissolved oxygen concentration of saturated growth medium was 197 μmol l^{-1} (Miller et al., 1988). The calculated utilisation rate of 0.031 μmoles 10^{-6}cells h^{-1} is quite low compared to typical values for transformed cell lines, for example 0.18 μmoles 10^{-6}cells h^{-1} for a hybridoma (Miller et al., 1989), but not too dissimilar to the value determined for other diploid fibroblasts such as FS-4 cells (0.05 μmoles 10^{-6} cells h^{-1}) as determined in Fleischaker and Sinskey's study (1981).

Medium changes are often carried out to replenish spent nutrients and remove toxic metabolites in microcarrier cultures. A 4.5 l medium change (90% of working volume) after 96 h replenished glucose and reduced lactate levels close to pre-culture concentrations, but the lactate levels rose rapidly in the 72 h following the medium change to 10.15 mmol l^{-1}, Figure 5. The difference in starting cell densities of 0.3×10^5 cells ml^{-1} at day 1 (Figures 4 and 5) is most likely the reason that cell numbers are not equivalent in both cultures at day 4, the time of the medium change. The reason for this difference is not entirely clear but may be due to the fact that the NM57 cells used for the culture in Figure 5 (medium change) were at one passage from the end of their useful lifetime in culture, while the NM57 cells used for the culture depicted in Figure 4 were four passages from revival. The lower cell density (day 1, Figure 5) following culture initiation may thus be a 'cell-age' related effect. However, the growth rates are approximately equivalent in these cultures up to the point of the medium change on day 4 (μ=0.43 d^{-1} / Figure 4; μ=0.41 d^{-1} / Figure 5) and cell densities were similar at the maximum of both cultures (4.12×10^5 cells ml^{-1} / Figure 4; 4.07×10^5 cells ml^{-1} / Figure 5). Therefore, the inference from these data is that the batch fermentation without any manipulation following inoculation is neither limited by nutrient supply or waste product accumulation.

NM57 cells cultured in medium supplemented with glucose to a concentration of 2 g l^{-1} did not reach comparable densities to that previously achieved in medium containing 1 g l^{-1} glucose, Figure 6. In addition, although the specific growth rates of the cells in these cultures were similar, the cells in the supplemented medium showed an increased specific glucose utilisation rate, Table 2. It is not unusual for mammalian cells in culture to increase their glucose utilisation rate in response to elevated glucose concentrations (Duvar et al., 1996; Miller et al., 1989). In this instance however, a concomitant increase in NM57 cell numbers and progression to 100% confluency was not observed. It would appear that a greater proportion of glycolytic energy is used to provide maintenance energy than for biosynthesis and cell growth as the concentration of glucose is increased.

NM57 cells did not grow on Cytodex 3 in basal medium, but grew to varying degrees of confluency when cultured in basal medium supplemented with protein hydrolysates or glutamine, Figure 7. The cells cultured in medium supplemented with 2 mmol l^{-1} glutamine failed to grow to confluency. This is unusual as glutamine is a preferred energy and carbon source for mammalian cells (Reitzer et al., 1979). However, glucose and glutamine may be metabolised in a competitive manner if both are present in the medium and glutamine inhibits the oxidation of the glucose–6 carbon through the TCA (tricarboxylic acid) cycle in diploid fibroblast cultures (Zielke et al., 1978). Thus, it may be the case that the cell yield on glucose (present at 1 g l^{-1} as part of the EMEM component of the basal medium) is inhibited to some extent by glutamine. Daily supplementation to a total concentration of 0.4 mmol l^{-1} glutamine in an attempt to limit possible accumulation of inhibitory ammonium ion produced from both cell metabolism and spontaneous decomposition of glutamine failed to yield confluent cultures. Ammonium ion concentrations in these cultures rose to a maximum of 1.5 mmol l^{-1}. These data indicate that glutamine is not efficiently metabolised as a primary nutrient source by NM57 cells. Since the development of this cell line in 1973, these cells have been maintained and cultured in protein and lipid-rich media. It is therefore most likely that certain unidentified peptides, lipoproteins, lipids or other essential factors in the proteinaceous supplements are crucial

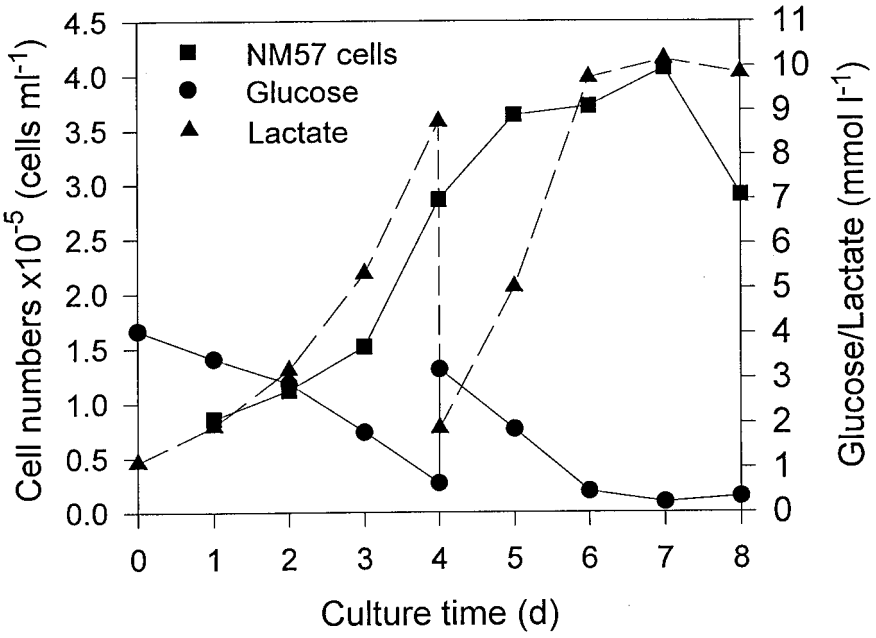

Figure 5. Glucose and lactate concentrations during culture of NM57 cells on Cytodex 3 at a concentration of 4 g l^{-1} in 5 l bioreactor cultures. This culture was inoculated at a density of seven cells per microcarrier and received a 4.5 l medium change at day four. Other culture conditions were as described in the caption to Figure 2.

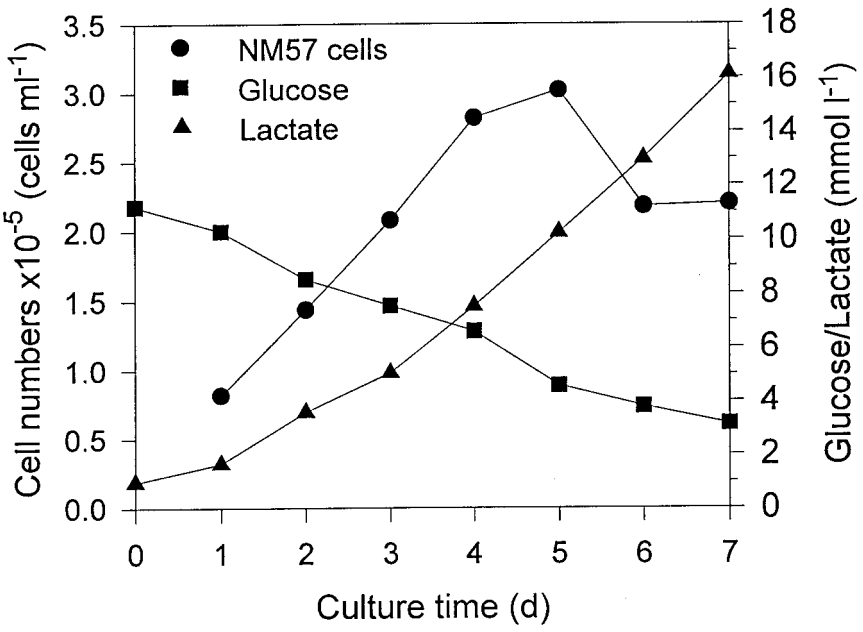

Figure 6. Cell growth and metabolite concentrations during culture of NM57 cells on Cytodex 3 at a concentration of 4 g l^{-1} in 5 l bioreactor cultures. This culture was inoculated at a density of seven cells per microcarrier and carried out in basal medium supplemented with BSA, lactalbumin hydrolysate and containing glucose at a total concentration of 2 g l^{-1}.

Table 2. Specific growth and glucose utilisation rates of NM57 cells on Cytodex 3 microcarriers (4 g l^{-1}) in basal medium supplemented with BSA, lactalbumin hydrolysate and containing either 1 g l^{-1} or 2 g l^{-1} glucose

Growth/Metabolic Parameter		Glucose Concentration	
		1 g l^{-1} [1]	2 g l^{-1} [2]
Specific growth rate, μ	(d^{-1})	0.43	0.41
Specific glucose utilisation rate, $q_{glucose}$	(μmoles 10^{-6} cells d^{-1})	1.1	2.3

[1] Calculated using data from days 1 to 4, Figure 4.
[2] Calculated using data from days 1 to 4, Figure 6.

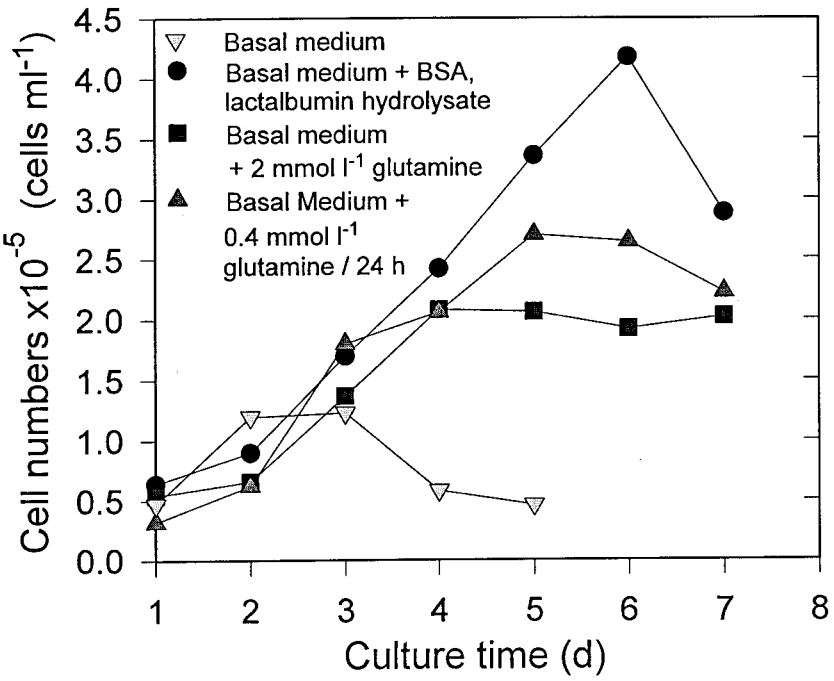

Figure 7. Growth of NM57 cells in 300 ml spinner cultures using basal medium containing various supplements. All cultures used 3 g l^{-1} Cytodex 3 and were inoculated at a density of seven cells per microcarrier. The 'Basal medium + 0.4 mmol l^{-1} glutamine / 24 h' culture was fed daily from a sterile 200 mmol l^{-1} stock solution to a total concentration of 0.4 mmol l^{-1}.

to the successful culture of this cell line. The limited number of useful passages from revival for production purposes, ruled out any attempts to adapt the cell line to a protein-free medium.

Microcarrier selection. II

The formulation of this RSV vaccine is based on supplying a minimum number of viral F antigen-bearing cells per dose. The successful recovery of intact NM57 cells from the microcarriers following fermentation was thus an important requirement. In this regard, the continued use of Cytodex 3 proved problematic – attempts to separate the cells and microcarriers following trypsinisation using stainless steel sieves of differing mesh sizes resulted in clogging and poor cell recoveries. This prompted the consideration of the Cultispher S microcarrier as an alternative to Cytodex 3. An inoculum level of 105 cells per microcarrier proved sufficient to yield a confluent culture of NM57 cells using Cultispher S at a concentration of 1 g l^{-1}, Figure 8. This is significantly larger than the corresponding figure of 7 NM57 cells per Cytodex 3 microcarrier. However, the lesser number of Cultispher S microcarriers per dry gram of material, and the use of continuous agitation at the initiation phase

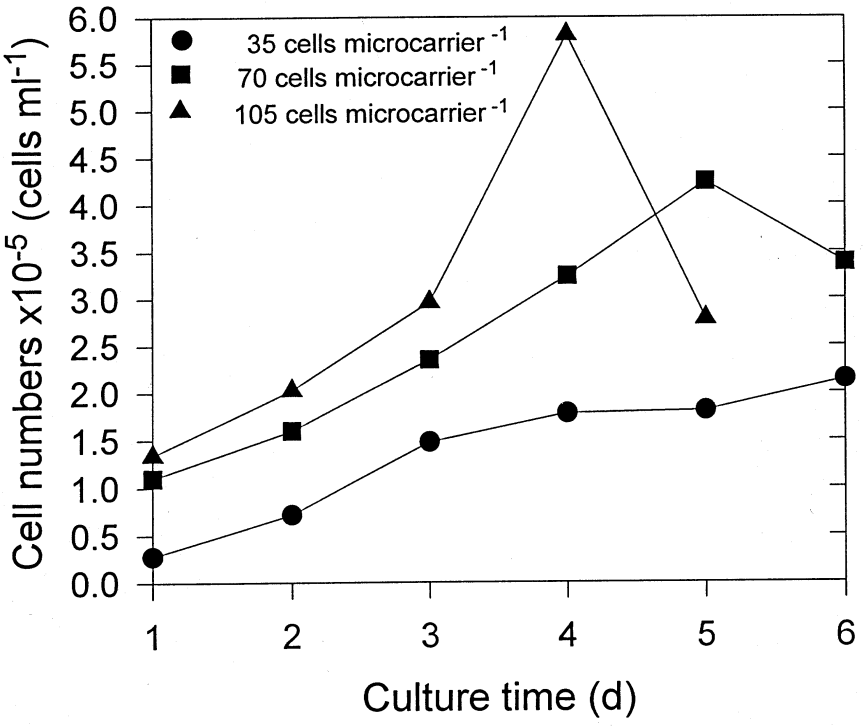

Figure 8. Effect of inoculum level on the growth of NM57 cells on Cultispher S at a concentration of 1.5 g l^{-1} in 300 ml spinner cultures. All cultures were carried out using basal medium supplemented with BSA and lactalbumin hydrolysate. These cultures were initiated by inoculating the cells to 150 ml medium containing microcarriers and using a modified attachment phase of continuous stirring at 30 rpm for 18 h at 35 °C, after which time fresh, pre-warmed medium was added to 300 ml.

of these cultures, necessitated the increase in inoculum size. NM57 cells propagated to greater densities on Cultispher S (5.52×10^5 cells ml^{-1}) in 5 l bioreactor cultures compared to those using the Cytodex 3 microcarrier, Figure 9. When using attachment phase AP1 (intermittent stirring over 4 h in reduced volume), considerable sticking and clumping of colonised microcarriers to each other and to the vessel sides was observed, even though the glass vessel was siliconized beforehand. The introduction of a continuously stirred attachment phase (AP2) eliminated this problem. MTT staining of samples from these Cultispher S fermentations indicated weak purple staining of individual cells on the microcarrier surface at day 1 whereas all beads stained an intense purple at day 5 indicating full colonisation of the microcarriers with viable cells. The data in Table 3 indicate the acceptable performance of the Cultispher S microcarriers as a substratum for NM57 cell growth in direct comparison with Cytodex 3.

It was possible to agitate these Cultispher S fermentations at higher rates than the Cytodex 3 fermentations without compromising final cell yields. It is likely that the growth of cells within the macroporous matrix of the Cultispher S microcarriers affords protection from harmful shear effects that impact on cells grown on the solid, non-porous microcarriers. Indeed, similar observations have been recorded by Ng et al. (1996) when culturing Vero cells on Cytodex 1 and Cultispher G microcarriers. The final density of the 1 g l^{-1} Cultispher S / AP2 fermentation corresponds to a cell yield of 733 NM57 cells per microcarrier. While this yield is quite substantial, Ohlson and co-workers (1994) succeeded in cultivating CHO-K1 cells to approximate densities of 4500 cells per Cultispher S microcarrier. This fact seems to confirm the suspicion of a degree of contact inhibition prohibiting NM57 cell proliferation to the high cell densities commonly observed with continuous cell lines. *In situ* enzymatic digestion of the cell-laden Cultispher S microcarriers at the 5 l scale greatly facilitated cell recovery – under the conditions described in a previous section, a single attempt at this exercise proved that it was possible to recover 86% of whole, intact cells from the fermenter

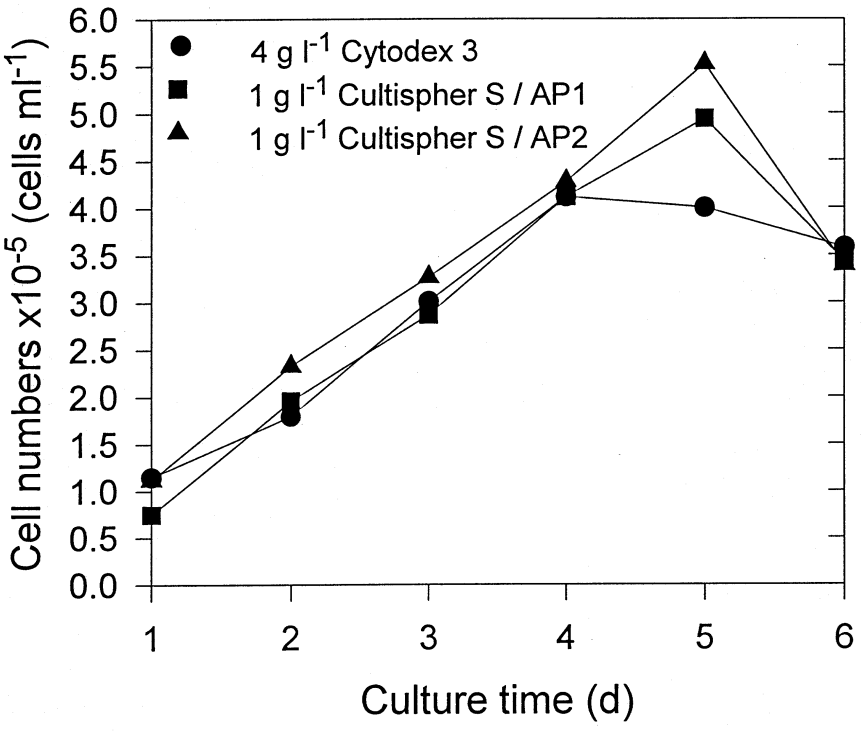

Figure 9. Growth of NM57 cells on Cultispher S at a concentration of 1 g l^{-1} in 5 l bioreactor cultures. All cultures were carried out using basal medium supplemented with BSA and lactalbumin hydrolysate. The Cultispher S cultures were initiated by seeding with 105 NM57 cells per microcarrier and used different attachment phases in 1.5 l medium. These were; AP1 – 4 cycles of 12 min at 75 rpm + 28 min settling, and AP2 – continuous stirring at 75 rpm. The cultures were then filled to 5 l with pre-warmed medium at 20 h post-inoculation. The Cultispher S fermentations were controlled at pH 7.2, 30% DO, 35 °C and 75 rpm whereas the Cytodex 3 culture, shown here for comparison, used the above conditions with the exception of an agitation rate of 60 rpm.

Table 3. Growth parameters of NM57 cells on Cytodex 3 (4 g l^{-1}) and Cultispher S (1 g l^{-1}) microcarriers in 5 l fermentation cultures under similar conditions of pH (7.2), temperature (35 °C) and dissolved oxygen (30%). The Cytodex 3 cultures were inoculated with 7 cells per microcarrier and agitated at 60 rpm while the corresponding conditions for the Cultispher S cultures were 105 cells per microcarrier and 75 rpm respectively

Growth Parameter		Cytodex 3[1]	Cultispher S[2]
Specific growth rate, μ	(d^{-1})	0.43	0.38
Doubling time, t_d	(d)	1.61	1.82
No. of generations		2.48	2.74

[1] Calculated using data from days 1 to 4, Figure 9.
[2] Calculated using data from days 1 to 5, Figure 9 (1 g l^{-1} Cultispher S / AP2).

suitable for chemical inactivation. The productivities of various culture systems used for the cultivation of this cell line are summarised in Table 4. A lower culture productivity per unit surface area for certain cell types cultivated on microcarriers compared to growth on non-spherical surfaces is not unusual. However, the greater percentage immunofluorescence (%IF) of cell populations grown on Cytodex 3 microcarriers (80%) compared to roller bottle and cell factory populations (50%) was unexpected. This phenomenon might be explained by the following: a common characteristic of persistently infected cell lines in culture is the re-

Table 4. Comparison of NM57 cell productivities and the relative number of vaccine doses obtained from a selection of different culture systems

Culture Type	Culture Productivity (NM57 cells cm^{-2})	%IF	Vaccine Doses [1]	Relative cost /dose[2]
Roller bottle (900 cm^2)	5.7×10^4	50	1	– [4]
Cell Factory (24000 cm^2)	5.05×10^4	50	23	1
Fermentation (5 l) (4 g l^{-1} Cytodex 3)	3.83×10^4	80	64	1.14
Fermentation (5 l) (1 g l^{-1} Cultispher S)	– [3]	55	62	0.33

[1] All figures are relative to the number of doses obtainable from a single roller bottle culture that is arbitrarily designated as one unit.
[2] Cost figures are relative to the cost of producing the number of doses obtainable from a single cell factory culture which is arbitrarily designated as one unit.
[3] Not measurable due to the macroporous nature of the Cultispher S microcarrier. The number of doses in this instance was calculated from the average maximum cell density of 5.52×10^5 cells ml^{-1} in 5 l bioreactor cultures.
[4] Not determined.

lease of amounts of infectious virus to the medium (Holland et al., 1976; Rima and Martin, 1976). RSV has been routinely isolated from NM57 cultures – for example, average yields of virus from NM57 cells grown in our different culture systems were determined and shown to be similar, for example $10^{3.61}$ $TCID_{50}$ ml^{-1} in roller bottles and $10^{3.56}$ $TCID_{50}$ ml^{-1} in microcarrier cultures. This virus has been shown to readily infect various cell types including MDBK, HEp-2 and calf testis cells in our laboratory. Maintenance of persistence infection is mediated by distribution of virus to daughter cells during mitosis and cell fusion, but also to a certain degree by infection of uninfected cells by free virus during culture – it may be the case that the controlled environment of the bioreactor facilitates the maintenance of a greater number of RSV-infected NM57 cells compared to the environment of the roller bottle and cell factory systems. The fact that virus production in cell systems is typically pH dependent supports this theory – while pH is controlled at pH 7.2 in microcarrier fermentations, pH often drops to pH 6.8 and lower in the later stages of roller and cell factory cultures. However, the initiation and maintenance of persistent viral infections in cells systems is a complex process dependent on many variables – no published information currently exists regarding the maintenance of the virus-carrier state of cell lines in large scale production systems. In contrast to the Cytodex 3 cultures, the %IF of cell populations from Cultispher S cultures are significantly lower (55%). The production of lytic viruses on cells grown in macroporous microcarriers is generally poor compared to that on cells grown on solid microcarriers. This may be due to the diffusional resistance within the confines of the pores of the microcarrier. Although the strain of RSV used in this study is a non-cytopathic virus, the mechanism of cellular infection is similar to lytic virus i.e. passive transport of the virus to the cell membrane followed by adsorption to the specific cell surface receptor. Thus, widespread distribution of the RSV F-antigen in the NM57 cell population may be obstructed by diffusional resistance to the virus within the microcarrier. Regardless of the underlying mechanisms at work here, fermentations using Cytodex 3 and Cultispher S showed similar productivity from the point of view of the number of vaccine doses obtained from each culture system. In addition, the use of a microcarrier cell culture process using Cultispher S showed a significant cost saving (0.33 cost units) in comparison to the currently used technique of static culture using cell factories (1 cost unit) for the production of this RSV vaccine, Table 4. This simple costing was based on the cost of materials such as the disposable multi-tray cell factories, microcarriers and culture growth medium – other pertinent cost considerations including labour, laboratory space etc. would in fact make the microcarrier cell culture process even more favourable from a purely economic viewpoint.

Finally, it is worth noting that the conditions of cell recovery, specifically proteolytic digestion of the

Cultispher S microcarriers and a proprietary virus chemical inactivation procedure (validated to inactivate both cell-associated and free form RSV – data not shown), did not affect either the number of cells expressing RSV F-antigen or the immunogenicity of this antigen. These facts have been confirmed by immunolabelling of inactivated cells *in vitro*, but more importantly, from bioequivalence studies conducted in the field comparing vaccine produced from the different culture systems listed in Table 4. This work has thus demonstrated the potential of producing a vaccine, previously produced in traditional culture systems, in bioreactors using microcarriers. The obvious benefits of transferring this process to bioreactor technology, such as greater containment, economic savings in labour, materials and laboratory space are significant. Work is currently underway involving the development of this process to full production scale.

Acknowledgements

The author wishes to thank Grampian Pharmaceuticals Ltd. and MFM Laboratories Ltd. for allowing the publication of this article.

References

Aunins JG, Croughan MS, Wang DIC and Goldstein JM (1986) Effects of paddle impeller geometry on power input and mass transfer in small scale animal cell culture vessels. Biotechnol Bioeng 34: 1127–1132.

Barteling SJ and Vreewijk J (1991) Development of foot and mouth disease vaccines. Vaccine 9: 75–88.

Chun B-H, Park S-Y and Moon H-M (1996) Production of live attenuated varicella-zoster virus in human embryonic lung cells using microcarrier. Biotechnol Tech 10: 151–156.

Clark JM and Hirtenstein MD (1981) Optimizing culture conditions for the production of animal cells in microcarrier culture. Ann NY Acad Sci 369: 33–46.

Croughan MS, Hamel J-FP and Wang DIC (1988) Effects of microcarrier concentration in animal cell culture. Biotechnol. Bioeng 32: 957–982.

Croughan MS and Wang DIC (1989) Growth and death in overagitated microcarrier cell cultures. Biotechnol Bioeng 33: 731-744.

Duvar S, Muthing J, Mohr H and Lehmann J (1991) Scale-up cultivation of primary human umbilical vein endothelial cells on microcarriers from spinner vessels to bioreactor fermentation. Cytotechnology 21: 61-72.

Fenner F, Bachmann PA, Gibbs EPJ, Murphy FA, Studdert MJ and White DO (1987) Veterinary Virology. Academic Press, Inc., London.

Fleischaker RJ and Sinskey AJ (1981) Oxygen demand and supply in cell culture. Eur J Appl Microbiol Biotechnol 12: 193-197.

Hayle AJ (1986) Culture of respiratory syncytial virus infected diploid bovine nasal mucosa cells on Cytodex 3 microcarriers. Arch Virol 89: 81-88.

Holland JJ, Villarreal LP, Welsh RM, Oldstone MBA, Kohne D, Lazzarini R and Scolnick E (1976) Long-term persistent vesicular stomatitis virus and rabies infection of cells *in vitro*. J Gen Virol 33: 193-211.

Howard CJ, Stott EJ, Thomas LH, Taylor G and Gourlay RN (1987) Protection against respiratory disease in calves induced by vaccines containing respiratory syncytial virus, parainfluenza type 3 virus, *Mycoplasma bovis* and *M dispar*. Vet. Record 121: 372-376.

Long B (1994) The Production and Regulation of Veterinary Virus Vaccines. In: Animal Cell Biotechnology. Vol. 6. (pp 281-313) Academic Press Ltd., London.

Miller WM, Wilke CR and Blanch HW (1988) Transient responses of hybridoma metabolism to changes in the oxygen supply rate in continuous culture. Bioprocess Eng 3: 101–111.

Miller WM, Wilke CR and Blanch HW (1989) Transient responses of hybridoma cells to nutrient additions in continuous culture: I. Glucose pulse and step changes. Biotechnol Bioeng 33: 477–486.

Montagnon BJ, Vincent Falguet JC and Janget B (1983) Thousand litre scale microcarrier culture of Vero cells for killed polio virus vaccine. Promising results. Dev Biol Stand 55: 37–42.

Ng Y-C, Berry JM and Butler M (1996) Optimization of physical parameters for cell attachment and growth on macroporous microcarriers. Biotechnol Bioeng 50: 627–635.

Ohlson S, Branscomb J and Nilsson K (1994) Bead-to-bead transfer of Chinese hamster ovary cells using macroporous microcarriers. Cytotechnology 14: 67–80.

Onuma M, Hodatsu T, Yamamoto S, Higashihara M, Masu S, Mikami T and Izawa H (1984) Protection by vaccination against bovine leukemia virus infection in sheep. Am J Vet Res 45: 1212–1215.

Pringle CR, Shirodaria PV, Cash P, Chiswell DJ and Malloy P (1978) Initiation and maintenance of a persistent infection by respiratory syncytial virus. J Virol 28: 199–211.

Reitzer LJ, Wice BM and Kennel D (1979) Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J Biol Chem 254: 2669–2676.

Rima BK and Martin SJ (1976) Persistent infection of tissue culture cells by RNA viruses. Med Microbiol Immunol 162: 89–118.

Stott EJ, Thomas LH, Taylor G, Collins AP, Jebbett J and Crouch S (1984) A comparison of three vaccines against respiratory syncytial virus in calves. J Hyg 93: 251–261.

Taylor G, Stott EJ, Bew M, Fernie BF, Cote PJ, Collins AP, Hughes M and Jebbett J (1984) Monoclonal antibodies protect against respiratory syncytial virus infection in mice. Immunology 52: 137–142.

Thomas LH, Gourlay RN, Stott EJ, Howard CJ and Bridger JC (1982) A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves. Res Vet Sci 33: 170–182.

Van der Velden-de Groot CAM (1995) Microcarrier technology, present status and perspective. Cytotechnology 18: 51–56.

Van Wezel AL, Van der Velden-de Groot CAM and Van Herwaarden JAM (1980) The production of inactivated polio vaccine on serially cultivated kidney cells from captive bred monkeys. Dev Biol Stand 46: 151–158.

Varani J, Josephs S and Hillegas WJ (1996) Human diploid fibroblast growth on polystyrene microcarriers in aggregates. Cytotechnology 22: 111–117.

Zielke HR, Ozand PT, Tildon JT, Sevdalian DA and Cornblath M (1978) Reciprocal regulation of glucose and glutamine utilisation by cultured diploid fibroblasts. J Cell Physiol 95: 41–48.