Retroviral Vector Production for Gene Therapy Applications

by

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A thesis submitted to
The University of Birmingham,
for the degree of
Doctor of Philosophy

School of Chemical Engineering
The University of Birmingham
December 2000

Abstract

The production of retroviral vectors for gene therapy applications faces a number of challenges. Of primary concern is the low titre of vector stocks produced by packaging cells in culture and the inherent instability of retroviral vector activity.

A systematic investigation of culture parameters that can effect vector titre was conducted. Physical and chemical factors including temperature, pH, medium composition, dissolved oxygen and serum concentration were all assessed. In addition, a number of studies were undertaken to assess the effects of packaging cell growth rate on vector production.

The use of a packed bed, as a novel system for large-scale vector production, was also investigated. Prolonged production of retroviral vector stocks was demonstrated in the packed bed system with immobilised packaging cells. Determination of the critical culture parameters allowed optimisation of culture conditions, which can be continuously controlled in the packed bed system, thereby ensuring optimal vector production throughout the production period. Furthermore, vector decay rates can be reduced by the immediate collection of vectors into a recovery vessel.

The studies within this thesis will aid the development of appropriate procedures for the large-scale production and handling of retroviral vector stocks for human gene therapy applications.

Dedicated to Duncan

Acknowledgements

I would like to thank Dr. Mohamed Al-Rubeai for his guidance throughout this research. My thanks also go to all the members of the Animal Cell Technology Group who helped to create an enjoyable working atmosphere in the laboratory with their continual good humour.

Finally, I am eternally grateful to my husband, Duncan, without whom this Ph.D. would not have been possible.

This Ph.D. was supported by Ashby Scientific Ltd, Leicestershire, U.K.

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Chapter 1: Introduction

1.1 Gene Therapy

Gene therapy was originally conceived as a treatment for genetic disorders. In such cases, the principle of gene therapy is the introduction of a normal functional copy of a defective or missing gene into the genome, in order to restore normal cell function and thereby eliminate the disease symptoms. A number of diseases, of known molecular pathology, have been targeted for gene therapy research. These include adenosine deaminase deficiency (Blaese et al., 1990), cystic fibrosis (Knowles et al., 1995), haemophilia A and B (Fallaux and Hoeben, 1996), insulin-dependent diabetes mellitus (Bailey et al., 1999), Duchenne muscular dystrophy (Parrish et al., 1999), emphysema and Gaucher's disease (Morgan, 1995). Today, the delivery of genes is also under investigation for the treatment of infectious diseases such as Acquired Immunodeficiency Syndrome (Todd et al., 2000), Herpes and Hepatitis (Weatherall, 1995). A number of neurological degenerative diseases are also being targeted (Parasrampuria, 1998). Other complications such as atherosclerotic plaque formation (Fan et al., 1999) and organ transplant rejection (Moore, 1999) may also benefit from gene therapy.

Gene therapy has also been recognised as a potential cure or treatment for cancer (Fujiwara et al., 1994). While cancer may be considered a genetic disorder due to the numerous mutations in evolving clones of tumours, any attempt to correct these mutations by gene therapy would be impossible in most cases, due to the huge range and complexity of mutations. Instead, gene therapy has been identified as a potential method for the selective killing of cancer cells (Avalosse et al., 1995; Davis et al., 1996).

A number of approaches to kill cancer cells using gene therapy have been identified. For example, genes encoding a novel 'pro-drug activating enzyme' can be transferred to tumour cells, resulting in the expression of this novel enzyme by these cells (Davis et al., 1996; Green et al., 1998; Palù et al, 1999). This enzyme has the ability to convert a non-toxic 'pro-drug' administered to the patient into a cytotoxic agent, thereby killing any cells producing that enzyme (and neighbouring cells). Obviously such an approach would be limited to localised tumours and gene transfer would ideally need to be specific to the tumour cells only. Such approaches have a great advantage over conventional chemotherapy, in that cytotoxicity is more targeted to the tumour cells.

Alternatively, the transfer of genes encoding immuno-stimulatory proteins (such as various cytokines or the co-stimulatory protein B7/CD80) into tumour cells can result in a systemic immune response against these tumour cells (Fujiwara et al., 1994; Davis et al., 1996; Gilligan et al., 1998). The resultant tumour cell-specific cytotoxic T-cells can identify and kill the tumour cells in the body and induce systemic immunity.

The huge potential for gene therapy to cure a wide range of diseases has lead to high expectations and a great increase in research efforts in this area. The first human gene therapy protocol was conducted in 1990 by W. French Anderson and showed promising results (Blaese et al., 1995). Over the following four years, more than 100 gene therapy protocols were approved for clinical trials (Gordon and Anderson, 1994) and by 1999, 184 gene therapy clinical trials were ongoing, illustrating the rapid growth of this field. As of September 2000, the FDA (Food and Drug Administration) have allowed 386 gene therapy INDs (Investigational New Drugs) to proceed to clinical trial (personal communication, Julie

Zawisza, Centre for Biologics Evaluation and Research, FDA). Furthermore, with the advent of the first draft of the human genome sequence (see Coghlan and Boyce, 2000) and the development of advanced technologies for the identification of genes and their function (Kost, 1999), the number of candidate diseases for gene therapy is likely to dramatically increase. However, to date, evidence of clinical improvement has been proven in only a handful of patients undergoing gene therapy (Palù et al., 1999). This apparently poor outcome can be attributed to the number of challenges faced by gene therapy, such as the ability to reproducibly transduce a sufficient number of target cells with the therapeutic gene and to obtain and sustain sufficient levels of the gene product to alleviate the disease symptoms. The efficient transfer of a therapeutic gene into human cells will depend upon the technology used for gene delivery. A number of delivery systems have been identified, which either involve physical delivery of naked DNA (non-vector delivery), or the use of vectors (viral and non-viral). The advantages and disadvantages of each are outlined below.

1.2.1 Gene Delivery

1.2.1 Non-Vector Delivery

Naked DNA can be physically transferred into cells by a number of methods, including microinjection (Capecchi, 1980), electroporation (Chu et al., 1987), calcium phosphate precipitation (Loyter et al, 1982) and microparticle bombardment (Burkholder et al., 1993). Such methods rarely lead to integration of the DNA into the host genome, and hence expression is usually only transient (non-integrated DNA is readily degraded).

Microinjection is not suitable for most gene therapy applications due to the limited number of cell nuclei one can directly inject with naked DNA. Conversely, under optimal

conditions, electroporation can allow up to 90% of a cell sample to be transfected with foreign DNA. Electroporation involves the exposure of cells to a high voltage, thereby allowing the passage of DNA inside the cells. However, transfection of these cells is usually only transient. While transfection efficiencies may not be as high, an alternative method involves the co-precipitation of DNA with calcium phosphate, thereby enabling some of the DNA to be taken up into cells by non-specific endocytosis. However, a number of cell types appear to resist such transfection. Again, DNA transferred by this method does not usually integrate into the host chromosomes, but transiently resides in the nucleus as an episome. Finally, cells can be bombarded with microparticles onto which DNA has been precipitated. These particles enter the cells and the DNA is gradually released from the particles to allow its transient expression (Burkholder et al., 1993).

1.2.2 Non-Viral Vectors

Despite the great number of advantages of viral vectors (discussed below), the development of non-viral vectors is still ongoing. The use of non-viral vectors avoids the potential risks of viral infections and immune responses, which could result from the use of viral vectors for gene delivery. However, whilst non-viral vectors are essentially non-pathogenic, they are less effective than viral vectors for gene delivery *in vivo* (Robbins, 1998).

Most non-viral vectors involve DNA-conjugates (a complex of positively charged molecules with the negatively charged nucleic acids). These methods of gene delivery will require significant modifications before they are able to reach the current efficiency achievable with viral vectors.

1.2.3 Viral Vectors

Currently preferred methods for gene delivery involve the use of viral vectors (Mitani and Caskey, 1993; Gordon and Anderson, 1994; Robbins et al., 1998; Palù et al., 1999). This is because genes are delivered more efficiently by viral vectors, compared to DNA transfection (Hwuang and Gilbo, 1984; Kim et al., 1998). Viruses have evolved to efficiently transfer their own genome into a host cell, thereby using the cell's machinery for their own reproduction. This unique process has been exploited in the development of viral vectors for gene delivery (Morgenstern and Land, 1990; Morgan, 1995; Duisit et al., 1999; Palù et al., 1999; Peng and Russell, 1999). Vectors derived from retroviruses are used in the majority of gene therapy clinical trials to date (Gordon and Anderson, 1994; Vos, 1995; Andreadis et al., 1999). However, vectors derived from adenovirus, adeno-associated virus and herpesvirus are receiving increasingly more attention in the field of gene therapy (Mitani and Caskey 1993; Vos, 1995; Parasrampuria, 1998). The properties of each of these vectors are described in brief, with emphasis on retroviral vectors, for the purposes of this thesis.

1.2.3.1 Retrovirus Vectors

Retroviral particles consist of 2 identical copies of single stranded, positive sense RNA (typically 7-10 kilo base pairs in length), plus integrase and reverse transcriptase enzymes, contained within a protein shell (capsid) which is surrounded by a lipid membrane. This outer membrane is studded with glycoprotein projections (envelope proteins). Some classes of retrovirus also possess an intermediate layer between the protein shell and viral membrane (Nermut and Hockley, 1996). The diameter of a typical retrovirus particle has been reported to range from 90 to 140nm.

The envelope proteins play a pivotal role in determining the specificity of the retrovirus and are responsible for the attachment to and fusion with target cell membranes (allowing internalisation of a retroviral particle into the target cell). Alternatively, some retroviruses gain entry, after binding to the target cell, by endocytosis. Retroviral RNA is reverse transcribed into DNA, through action of the reverse transcriptase enzyme (characteristic of retroviruses). The double stranded viral DNA (a precursor to the integrated proviral form) passes into the nucleus of the infected cell and is integrated into the host cell genome (via integrase action). The retroviral DNA permanently resides within the chromosomes of the infected cell, allowing the expression of viral genes and proviral DNA replication along with the cell's own DNA. Hence retroviral particles use the host cell's machinery for their own replication. Proviral DNA is also passed onto the cell progeny. The production of new particles requires the transcription of integrated viral DNA into RNA and the processing of retroviral proteins. The core proteins and envelope proteins assemble at the plasma membrane of the infected cell, where retroviral RNA is packaged and the particles bud off from the cell. Alternatively, some types of retrovirus particles assemble in the host cell cytoplasm before moving towards the plasma membrane where they become enveloped and bud off from the cell (Nermut and Hockley, 1996). Wild type retrovirus particles are constitutively released from an infected cell, usually without any deleterious effects to the cell itself. The life cycle of a typical wild-type retrovirus is illustrated in Figure 1.2.3.1.

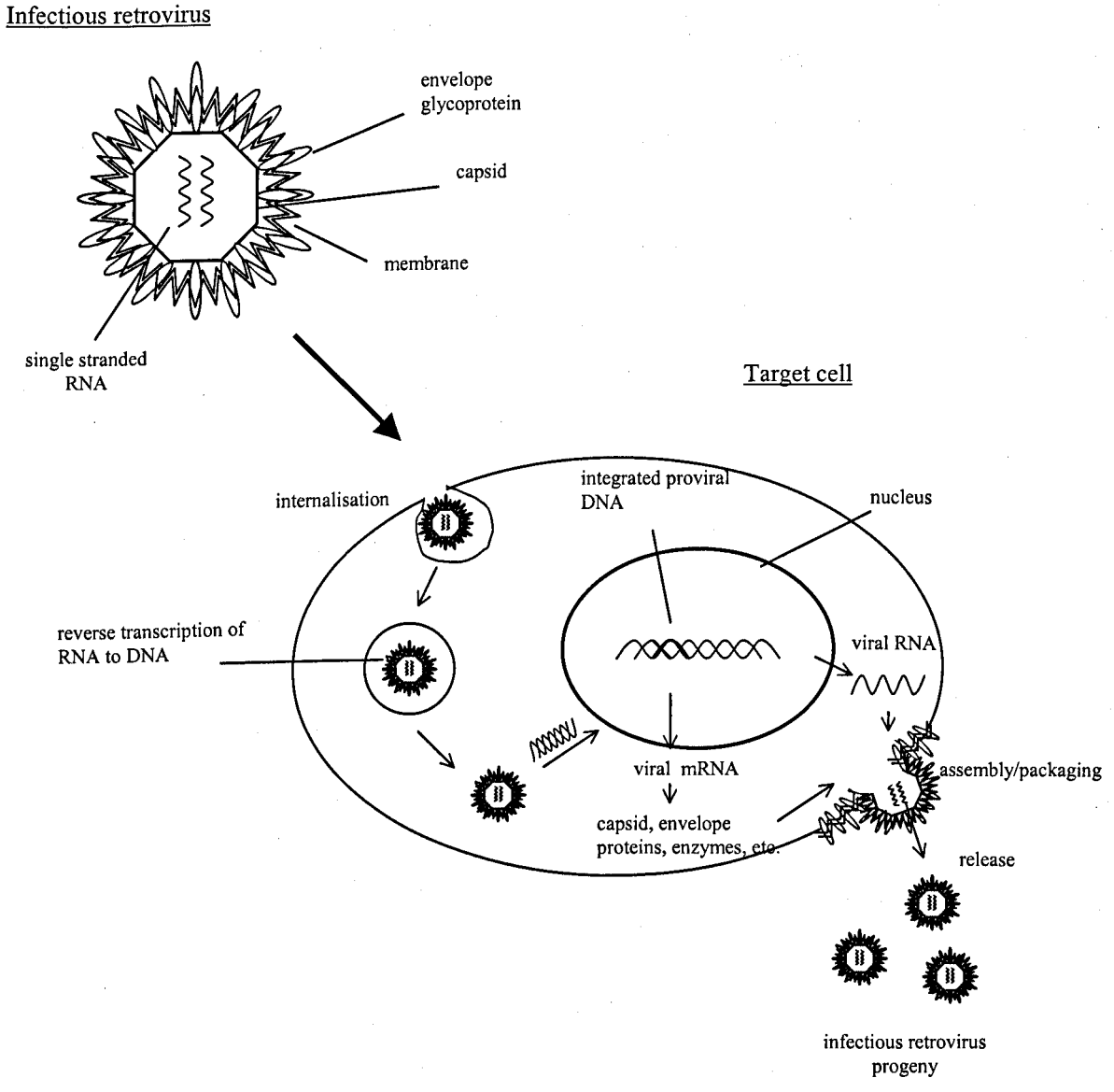


Figure 1.2.3.1

Life cycle of a typical wild-type retrovirus (approximately 100nm diameter):

Retroviral particles attach to target cell receptors via envelope proteins and become internalised (usually via endocytosis). Viral RNA is reverse transcribed into DNA, which enters the nucleus and integrates with the target cell genome (to become proviral DNA). The target cell's machinery is used for the transcription of proviral DNA and the translation of viral proteins, allowing the packaging of viral RNA and the assembly of new retrovirus particles which 'bud off' from the target cell membrane.

The retroviral genome contains three regions, *gag*, *pol* and *env*, which encode for the capsid proteins, the viral enzymes (reverse transcriptase, integrase and protease) and the envelope proteins, respectively. The lipids and carbohydrates of the virus particle are derived from the host cell plasma membrane (during budding). An important feature of the retroviral genome is the long terminal repeats (LTRs) repeated at each end of the genome. These sequences are essential for the initiation of viral DNA synthesis, integration of proviral DNA and the regulation viral gene transcription (Fritz 1996). In addition, the *psi* packaging sequence (ψ) is essential for the packaging of retroviral RNA during the synthesis of retroviral progeny.

Three sub-groups of retrovirus exist: oncoretroviruses (e.g. Moloney Murine Leukaemia Virus, MoMLV), lentiviruses (e.g. Human Immunodeficiency Virus, HIV) and spumaviruses (also known as foamy viruses). Retroviruses have been extensively studied for a number of years. Prior to the incidence of widespread HIV-induced disease in humans, MoMLV was the most commonly studied retrovirus. However, the last two decades has seen a dramatic increase in HIV research. Until recently, spumaviruses have received little attention due to their lack of significant association with disease (Russell and Miller, 1996). The pre-existing wealth of knowledge on the structure and function of retroviruses has resulted in the majority of vectors developed for gene therapy being constructed from these viruses (namely MoMLVs). In fact, over 60% of gene therapy clinical trials in 1999 used retroviral vectors for gene delivery (Andreadis et al., 1999).

The Moloney Murine Leukaemia Virus (MoMLV) is considered to have one of the simplest genomes of the retroviruses. In contrast, HIV is considered to have one of the most complex genomes of most retroviruses. However, while many other genes encoding for non-

structural proteins exist, the LTRs and *gag, pol* and *env* genes still predominate the HIV genome. The important features of the MoMLV *gag, pol* and *env* genes and their encoded proteins are described below.

The majority of structural viral proteins are encoded by the *gag* gene (group antigen gene). A '*gag* polyprotein' is expressed and post-transcriptional modification yields four proteins: p10 (nucleocapsid), p12, p15 (matrix protein) and p30 (capsid protein). Adjacent to the *gag* gene is the polymerase (*pol*) gene which encodes the integrase, reverse transcriptase and protease enzymes. Finally the envelope (*env*) gene, adjacent to the *pol* gene encodes a polyprotein which is cleaved by protease enzymes to yield gp70 (surface glycoprotein) and p15E (transmembrane protein). The fact that these protein-coding domains are separate from the regulatory elements of the viral genome, makes these viruses amenable to modification for gene transfer vectors.

In order to use retroviruses as gene delivery vehicles, the life cycle of the retrovirus must be altered such that these particles will not replicate within the target cell (to avoid spread of the virus *in vivo*). By deleting the viral genes *(gag, pol* and *env*) from the retroviral genome and replacing them with a therapeutic foreign gene, the modified virus can still enter a target cell and insert its genome. However, further particles cannot be produced due to the lack of the essential gene functions. Hence the virus is rendered replication incompetent. In addition, a selectable marker gene is usually inserted to enable detection of successfully infected target cells. The LTRs and *psi* packaging sequence (ψ, essential for encapsidation), are retained in such retroviral vectors. The *production* of such vectors for gene delivery is made possible

through the use of so-called packaging/producer cells which contain the viral structural and functional genes as separate trans-complementing expression cassettes. (see Section 1.3).

Modifications to this basic retroviral vector design have been made to improve the production in packaging cells. For example, it has been observed that the complete removal of the *gag, pol* and *env* sequences and insertion of the foreign genes directly adjacent to the ψ sequence can result in a significantly reduced vector titre released by packaging cells, compared to wild-type virus (Armentano et al., 1987). The inclusion of extra sequences from the *gag* gene adjacent to the ψ sequence (so-called ψ+ region) was subsequently demonstrated to improve vector titre, such that levels were similar to those of wild-type virus (Armentano et al., 1987; Morgenstern and Land, 1990), so long as the ψ and *gag* sequences were kept together. Retroviral vectors such as this are continually being modified and improved to increase both productivity and safety (see Section 1.3 for safety improvements in retroviral vectors).

Retroviral vectors possess a number of advantages for human gene therapy applications. Firstly the gene of interest can be permanently integrated into the host genome, allowing the *potential* for permanent gene expression and hence a 'cure' for genetic disorders. However, in reality this has become difficult to achieve, due to the frequent loss of gene expression observed *in vivo*. A number of mechanisms for the reduced transgene expression *in vivo* have been proposed, including the methylation of viral promoters, hence cellular promoters have been investigated in an attempt to circumvent this problem (Kurian et al., 2000). Kitamura (1999) has demonstrated that Murine LeukaemiaVirus (MLV) Long Terminal Repeats can be silenced by TNF-α and TGF-β1 released by activated macrophages. Hence

if cells transduced by MLVs are in the vicinity of activated macrophages, or cytokines released by other immune cells, the transgene expression could be silenced.

The gene transfer by retroviral vectors is however highly efficient and up to 8 kilobases of DNA can be inserted. They also have a wide host range, which is an advantage with respect to the number of gene therapy applications, but a disadvantage where gene insertion is required to be restricted to one cell type. Further disadvantages include the risk of insertional mutagenesis or activation of oncogenes as a result of random integration of the viral genome into the host cell chromosomes. Random integration can also lead to varying levels of therapeutic gene expression, as this is dependent on the site of integration (Gordon and Anderson 1994). Furthermore, integrated transgene expression is highly susceptible to transcriptional silencing by adjacent heterochromatin. Ongoing research into the use of various promoters and enhancers aims to allow better control over the levels of expression of retroviral vector delivered genes. For example, certain promoters, such as the cytomegalovirus (CMV) promoter are more powerful than others at driving gene transcription (Soneoka et al., 1995). The use of Locus Control Regions (LCRs) is also under investigation to overcome this problem. LCRs are *cis*-acting elements that have the ability to confer position independent, cell-type specific expression of linked sequences (Cunliffe et al., 1995). This is brought about by inducing an 'open' chromatin domain, which is transcriptionally active. Hence LCRs have great potential in controlling the expression of genes randomly inserted by retroviral vectors in specific cell types (provided vector size is not limiting). LCRs are tissue specific and may therefore be suitable for inducing the expression of transgenes in certain cell types. Unlike LCRs, so-called UCOEs (Ubiquitous

Chromatin Opening Elements) are not tissue specific and hence are more suitable where prolonged expression of transgenes is required in *all* cell types (Antoniou, 2000).

Difficulties in obtaining high titre vector supernatants from packaging cell cultures also hinders the use of retroviral vectors for clinical applications (see Section 1.3). Furthermore, retroviral vectors derived from the oncovirus subgroup can only infect actively dividing cells (limiting the number of cell types that can be targeted by these vectors). However, vectors derived from the lentivirus subgroup of retroviruses can infect slowly dividing and quiescent cells (Lewis and Emerman, 1994). This is supposedly afforded by their possession of nuclear localisation signals which allow their entry into the nucleus without requiring nuclear fragmentation (Ferderico, 1999). Vectors derived from this subgroup of viruses (e.g. Human Immunodeficiency Virus, HIV; Simian Immunodeficiency Virus, SIV, and Equine Infectious Anaemia Virus, EIAV) are therefore currently receiving more attention in a number of research groups (Dutton, 1999). Due to the safety concerns over using HIV-derived vectors, many groups are turning to non-primate lentiviruses (Naldini, 1998; Federico, 1999).

Approved clinical protocols using retroviral vectors for gene delivery have included a wide range of diseases e.g. haemophilia, ADA-SCID, HIV infection and Gaucher's disease (Morgan, 1995). Other diseases also targeted for retroviral vector gene delivery include β-thalassaemia (Cone et al., 1987), muscular dystrophy and Parkinson's disease (Dutton, 1999). The ability of vectors derived from the oncovirus group to selectively transduce cancer cells (which are usually more proliferative than normal cells) is an advantage of this type of retroviral vector for use in cancer treatment. Hence these vectors have been investigated for the treatment of a range of cancers including ovarian cancer, breast cancer,

brain tumours and lung cancer (Morgan, 1995; Dutton, 1999). Phase I clinical trials, using retroviral vectors for gene delivery, have demonstrated no toxicity (Nemunaitis et al., 1999), which is a major advantage of this type of vector over adenoviral vectors.

1.2.3.2 Adenovirus Vectors

Adenoviruses are non-enveloped and possess a linear double stranded DNA genome, approximately 30-35k base pairs in length. This is encapsidated in a 70nm diameter icosahedral shell. The replication cycle of the adenovirus can be divided into 'early' and 'late' phases. During the early phase the virus undergoes absorption onto the target cell, penetration into the cells (through receptor mediated endocytosis), transcription of the viral DNA, translation of the 'early genes' (E1, E2, E3 and E4) and finally the induction of macromolecule synthesis by the host cell. The onset of DNA replication occurs during the late phase, as does initiation of the expression of 'late genes', which allow the assembly of the virus progeny. Unlike retroviruses, the replication and release of adenovirus particles from an infected cell, requires cell lysis.

Adenoviruses can also be rendered replication incompetent for gene therapy use, by removing early region genes. The E1 gene, which is important for the induction of E2, E3 and E4 promoters, can be removed, rendering the virus replication incompetent (Robbins et al., 1998). Deletion of the E3 region provides space for the insertion of the therapeutic gene (Trapnell and Gorziglia, 1994). The production of such adenoviral vectors is performed in helper cells such as the human embryonic kidney cell line, HEK293, which constitutively expresses the E1 proteins (Benihoud et al., 1999). However, there is a danger of homologous recombination between the adenoviral vector and the trans-complementing

cassette of the HEK293 helper cells, which can result in the production of replication competent adenovirus particles (Zhu et al., 1999). A reduction in the risk of such recombination has been achieved by Fallaux et al. (1998) through the engineering of embryonic retinoblasts (PER.C6) which possess an E1 locus that does not overlap with the adenoviral vector sequences.

In contrast, some research groups are also looking at the use of replication *competent* adenoviral vectors, where expression of the E1 gene is controlled by a tumour-specific promoter. In theory, the E1 gene in these vectors should only be expressed in tumour cells, where replication of adenoviral particles will occur and kill the tumour cells either directly (via cell lysis), or through the delivery of a 'suicide gene' (Robbins et al., 1998).

Adenoviruses have a reported immunogenic and cytotoxic potential (Benihoud et al., 1999). Furthermore, most adenoviral vectors are E1/E3 deleted and the E3 region is involved, in part, in evasion of the immune response. Hence $E3^-$ vectors are actually more immunogenic than $E3^+$ vectors.

Current targets for adenoviral vector delivery of genes include the cells of the airway epithelium in cystic fibrosis patients (Knowles, 1995). Delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to these cells is not feasible *ex vivo,* and *in vivo* delivery requires high concentrations of the gene delivery vehicle. The ease of producing high titre adenoviral vector stocks is therefore a great advantage of these vectors (Tapnell and Gorziglia, 1994). Other advantages of adenoviral vectors include their increased stability compared to retroviral vectors (due to the lack of envelope and to the

possession of a DNA genome, as opposed to RNA) hence these vectors are more amenable to purification and concentration processes. They also possess the ability to infect both dividing and quiescent cells and the occurrence of integration is rare, minimising the risks of insertional mutagenesis or activation of oncogenes. This lack of gene integration means that gene expression in the target cell is only transient. In some applications transient expression may be more desirable (such as the delivery of suicide genes to kill off selected cells). However, where transient expression does not elicit sufficient gene expression to alleviate disease symptoms, repeat administration of the vectors may be required. A major disadvantage of the use of adenoviral vectors is their immunogenic and immuno-reactive potential. Where an immune response to the vector is observed, repeated administrations are unlikely to be feasible. Adenoviruses have been shown to cause both acute viral toxicity and a vigorous immune response, resulting in inflammation. In fact the first and only death to date, resulting from viral vector delivery of a therapeutic gene, involved the use of adenoviral vectors (Boyce, 1999). During a clinical trial led by James Wilson last year, the patient receiving the highest dose of adenoviral vectors suffered severe toxicity effects, leading to his death. This apparent dose-dependent toxic response to an adenoviral vector is not the first to be reported (Tapnell and Gorziglia, 1994; Knowles, 1995), but is the first known fatal response. More recently however, the reported success in the reduction of head and neck tumours, using adenoviral vectors, has revived the faith in these vectors for human gene therapy applications (Meek, 2000).

1.2.3.3 Adeno-Associated Virus Vectors

Adeno-associated viruses cannot replicate alone and require the presence of either adeno-viruses or herpes-viruses to complete their life cycle. For this reason they are often referred

to as 'satellite' viruses (Fritz, 1996). These viruses are a defective sub-group of the parvovirus family. Their genome consists of single stranded DNA (either sense or anti-sense), which is contained within an icosahedral particle of only 18-26nm diameter. The so-called *rep* and *cap* genes of the adeno-associated viral genome encode for polypeptides, which are important in virus replication and encapsidation, respectively.

Replication of the adeno-associated virus involves host cell lysis, however in the absence of helper viruses the adeno-associated virus remains latent and cell lysis does not occur. Latent infection can last for years but, if during latent infection a helper virus is added, the life cycle of the adeno-associated virus progresses and cell lysis ensues.

Vectors derived from adeno-associated viruses (where the *rep* and *cap* genes are deleted) are becoming increasingly popular in the development of new vectors for gene delivery. Current targets include haemophilia (Dutton, 1999) and cystic fibrosis (Samulski, 1995). While these vectors may not be suitable for all cell types, high transduction efficiencies have been observed in muscle, brain and liver cells. Adeno-associated viral vectors offer a number of advantages as a gene delivery vehicles, namely the ability to induce gene expression for years (or perhaps permanently), the targeting of non-dividing cells, and the lack of apparent immunogenicity. Furthermore, natural infection of humans with adeno-associated virus appears to be asymptomatic (Clark et al., 1995). The expression of genes inserted into an adeno-associated viral vector can be easily regulated due to their ability to carry regulatory elements without interference from the viral genome. Adeno-associated viruses also contain unique inverted terminal repeats (ITRs) at each end of the their genome, which allow site specific integration of their DNA into the host genome (at a specific site on chromosome 19). Hence the potential to produce integrating viral vectors whilst avoiding the problems of

random integration is a great attraction of these viruses. However, to date, vectors derived form these viruses have lost this ability for site-specific integration, probably as a result of the deletion of important genes such as the *rep* gene (Robbins et al., 1998). Furthermore, removal of the *rep* and *cap* genes has sometimes been observed to reduce the rate of second strand synthesis and integration in some cell types is reduced, compared to the wild-type virus. Current research is focusing on ways to maintain site-specific integration sequences in recombinant vectors. Strategies to produce large amounts of adeno-associated viral vectors also require further development and simplification (Clark et al., 1995).

Disadvantages of adeno-associated viral vectors include the small genome, which allows the insertion of only 5kb or less foreign DNA. Preparations of these vectors must also be stringently screened for contaminating adeno- or herpes-viruses, which could render the adeno-associated viral vectors replication competent.

1.2.3.4 Herpesvirus Vectors

Herpesviruses are also undergoing increasing interest in the development of viral vectors for gene delivery. These viruses have a large genome (120-250kb), consisting of double stranded DNA. This is contained within 150-200nm diameter particles. Like adeno-associated viruses, herpesviruses can infect a wide range of cell types and their life cycle causes host cell death through lysis. However, non-replicating viruses can remain latent in the host cell for extremely long periods of time.

Herpes Simplex Virus, HSV, is a well-studied herpes-virus that replicates in the skin or mucous membranes of infected individuals, before infecting cells of the nervous system,

where latent infection ensues. Its specificity for latency within neuronal cells has led to significant research into the development of HSV vectors for the treatment of neurodegenerative disorders. However, the wide tissue tropism of these vectors makes it difficult to target specific neuronal cell types and avoid widespread infection of the nervous system (Vos, 1995).

Herpes viruses can be rendered replication defective by removal of half their genome (including specific 'immediate early' genes). These vectors allow the incorporation of large DNA inserts (up to 20-50kb), which is a major advantage in their use for the delivery of large human genes, such as the dystrophin gene, which cannot be accommodated in a number of the above viral vectors. Furthermore, multiple genes can be inserted into these vectors.

1.2.3.5 Poxvirus Vectors

Poxviruses have also been used for the development of gene delivery vectors (Cox et al., 1995; Palú et al., 1999). These viruses are large and oval in shape (200-400nm length), with a double stranded DNA genome. Members of this virus family replicate in the cytoplasm rather than the nucleus of the target cells. The Vaccinia virus, a member of the Poxvirus family, is the agent used to vaccinate humans against smallpox and has been widely studied in the laboratory. Viral vectors derived from these viruses are primarily used for the delivery of exogenous genes, usually those of pathogenic organisms, in order to elicit immunity against the pathogenic species (through the transient expression of antigens in host cells). This is quite a different approach in the use of gene therapy and is outside the realms of this review. However, Vaccinia virus vectors have more recently been developed for the

delivery of tumour associated antigens in an attempt to stimulate an immune response in cancer patients against tumour cells (Cox et al., 1995).

1.2.3.6 Alphavirus Vectors

Worth mentioning, but far less common are vectors derived from alphaviruses, namely Semliki Forest and Sindbis viruses. Alphaviruses are positive strand RNA enveloped viruses. The infection cycle of these viruses also takes place in the cytoplasm, thereby avoiding potential problems of RNA splicing or random DNA integration. Alphavirus vectors are currently being developed as prophylactic and therapeutic vaccines to treat infectious diseases and cancer (Schlesinger and Dubensky, 1999). Semliki Forest viruses have been reported to have a broad host range (Schlaeger and Lundstrom, 1998) and Sindbis virus replicons have been successfully targeted against neurons (Schlesinger and Dubensky, 1999). A major drawback of these vectors for use in human gene therapy applications is their pathogenic effects, hence there is a need to find variants that are non-cytopathic.

It is unlikely that one particular type of viral vector will be suitable for all gene therapy applications. Rather, the development of a range of vectors using a range of virus types will be necessary to fulfil the requirements of each treatment. For example, where 'suicide genes' need only be expressed for a short period of time, transient expression vectors such as adenoviral vectors may be more suitable. In contrast, the correction of a genetic disorder may require permanent expression of a therapeutic gene, hence retroviral vector gene delivery may be more suitable (to allow potentially permanent gene expression). The advantages and disadvantages of the viral vectors widely used for gene therapy applications are summarised in Table I. As a means of incorporating the benefits of a range of viral

vectors, chimeric vectors are also under development. These vectors contain the desirable features of two or more virus types (Robbins et al., 1998; Caplen et al., 1999; Duisit et al., 1999).

As mentioned earlier, retroviral vectors are currently the most common types of vector used in gene therapy clinical trials. As of September 2000, 165 of the 386 INDs allowed to proceed to clinical trial used retroviral vectors for gene delivery (personal communication, Julie Zawisza, CBER, FDA). The optimised production of these vectors requires immediate attention to enable their commercial scale manufacture (in the advent of product approval). The methods for production of retroviral vectors shall therefore be discussed, outlining the challenges in such production processes. The limited fundamental research, which has been conducted to date, on the factors affecting retroviral vector production by packaging cells is also reviewed.

1.3 Packaging Cells for Retroviral Vector Production

To enable the production of large numbers of retroviral vectors, replication competency is provided *in vitro* through the use of so-called packaging/producer cells. Packaging cells contain the viral helper genes (*gag*, *pol* and *env*), removed from the retroviral vector genome and inserted as trans-complementing expression cassettes. Thereby providing the viral proteins, both structural and functional, required for the synthesis of new particles. The retroviral vector is inserted into the packaging cells on a separate plasmid and particles containing the gene of interest and/or selectable marker genes are packaged and released from these cells in a similar fashion to that seen in wild-type infections. Particles released from the packaging cells maintain the ability to infect target cells and insert their genome,

but cannot go on to replicate within these cells due to the lack of the viral helper sequences (provided the target cells do not contain endogenous viral sequences). This production of retroviral vectors by packaging cells for gene delivery to target cells is illustrated in Figure 1.3.

The constitutive release of recombinant retroviral vector particles by such packaging cell lines allows the potential production of large volumes of well-characterised vector stock (Rigg et al., 1996). This is an advantage of using integrated retroviral vectors over transient transfection, where preparation of such stocks is not as easy (due to short production times and batch to batch variation). Furthermore, packaging cell cultures do not have to be sacrificed in order to harvest retroviral vector particles as is the case for many other vector types such as adenovirus vectors. However, the retroviral vector titre of stocks produced by packaging cells is relatively low, compared to other viral vector preparations and improvement of these titres through the modification of packaging cell lines and vector constructs is under investigation by a number of groups. In order to improve vector production by a packaging cell, many researchers have attempted to increase the expression of each viral helper gene. However, the over-expression of certain elements (such as gag proteins) has been shown to *reduce* the infectious titre of packaging cells, when not in balance with the expression of other vital genes (Duisit et al., 1999).

Virus type	Advantages	Disadvantages
	Oncovirus	
Retrovirus	• Integration of therapeutic gene • Highly efficient gene transfer • Wide host range • Low immunogenicity • Vector proteins not expressed in host • Well studied system *Lentivirus* • As for above, plus can infect non-dividing cells *Spumavirus* • As for above, plus large insert size	• Risk of insertional mutagenesis / activation of oncogenes • Difficult to target • Difficult to obtain high titre from packaging cells • Only infects dividing cells (except Lentivirus and Spumavirus)
Adenovirus	• Infects both dividing and quiescent cells • Insertional mutagenesis unlikely • High transduction efficiency • High titre easily achieved • Relatively stable (amenable to down-stream processing)	• Expression of therapeutic gene only transient • Viral proteins expressed • Potentially immunogenic • Common human virus (causes disease)
Adeno-Associated Virus (AAV)	• Can induce gene expression for years • Infects non-dividing cells • Relatively easy regulation of inserted genes • No immunity detected • Site specific integration (though this has been lost in current vectors)	• Mechanism for transduction unclear • Unknown effects of subsequent infection of patient with adeno- or herpes virus • Small insert size (4kb)
Herpes Virus (namely, herpes simplex virus, HSV)	• Large insert size (20-30kb) • High titres • Episomal, but latent infection can be life-long • Neuron specificity (HSV)	• Difficult to avoid cytotoxicity • Wide tissue tropism • Lower transduction efficiency • Potential to generate infectious HSV in humans

Table I

Summary of the main advantages and disadvantages of using various virus types for the development of vectors for gene transfer in humans.

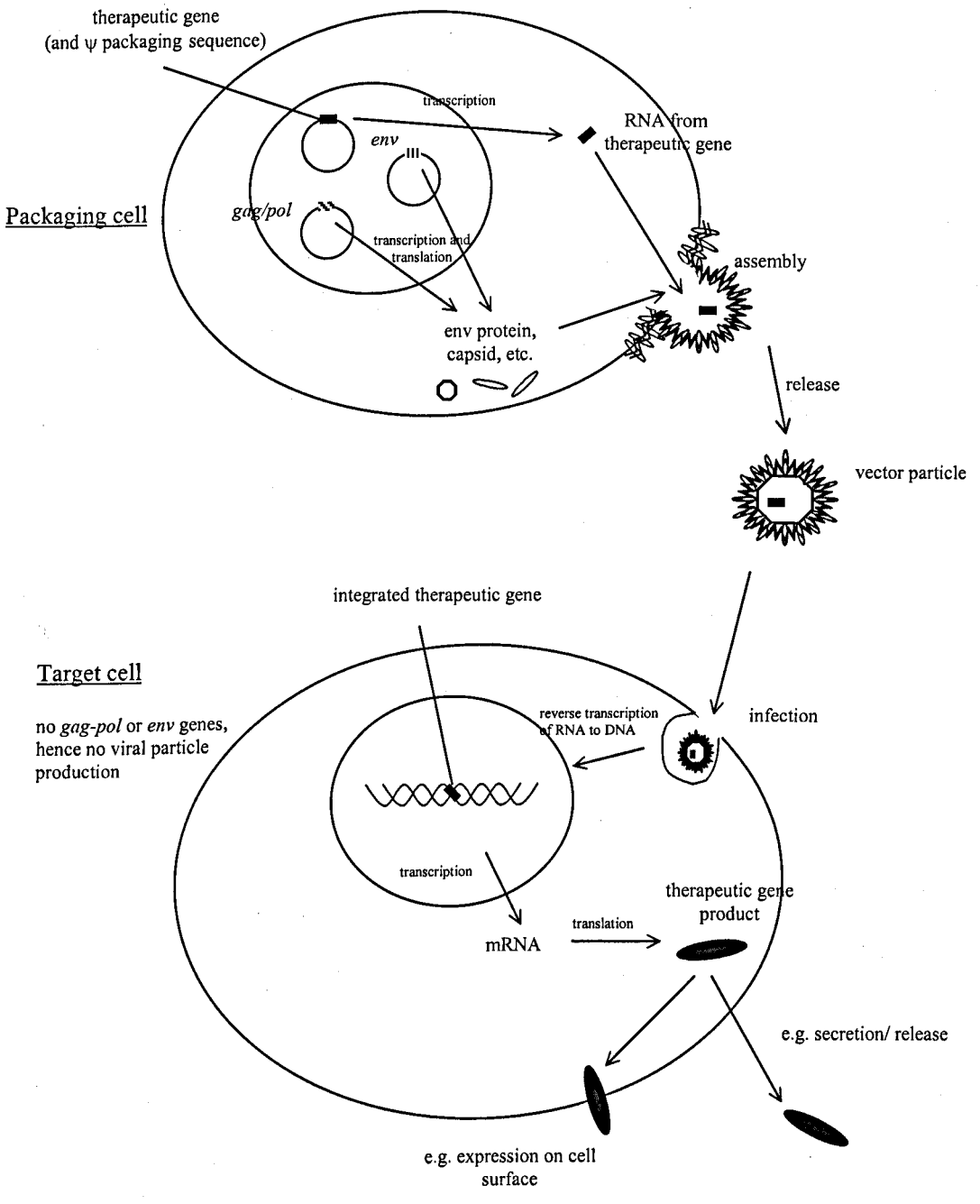


Figure 1.3

Therapeutic gene delivery using retroviral vectors produced by packaging cells (not to scale). Particles released by packaging cells are infectious, but replication incompetent.

Vector Safety

In recent years, packaging cells have been further modified to reduce the risks of producing replication competent virus particles (potentially caused by recombination between vector DNA and helper DNA or endogenous viral sequences). One approach has involved the use of separate plasmids for the insertion of the *gag-pol* and *env* genes (Markowitz at al., 1988; Morgenstern and Land, 1990; Cosset at al., 1995), as illustrated in Figure 1.3. The reverse transcriptase, and integrase enzymes are derived from a *gag/pol* fusion protein, hence the *gag* and *pol* genes cannot be separated and must be inserted into the packaging cells on the same plasmid. Still, the use of three separate plasmids (for *gag-pol*, *env* and the retroviral vector) means that three homologous recombination events must occur before replication competent virus particles can be constructed. Obviously the probability of this occurring is low, hence the safety of these packaging cells for vector production is significantly improved (Miller and Buttimore, 1986; Cosset et al., 1995). The risk of homologous recombination in a number of packaging cell lines is exacerbated by the fact that the *pol* and *env* coding sequences overlap in the wild-type genome, such that the *pol* gene ends after the *env* gene starts. Hence there will always be homologous sequences between the *gag-pol* transcriptional unit and the *env* transcriptional unit in packaging cells using helper genes from the same virus (Miller, 1990). One way to circumvent this problem is to use a *gag-pol* gene from one virus type and an *env* gene from another virus type (which has little sequence similarity). The risk of recombination between two such inserts is extremely low and this is a good strategy for producing safer packaging cell lines, provided the two inserts are compatible and do not result in reduced titres. Another problem particular to MoMLV is the overlap of the *gag* gene and the ψ packaging sequence, making it impossible to avoid some homology between the *gag* insert and the vector insert (which contains the ψ sequence).

This situation again increases the risk of homologous recombination occurring (Miller, 1990). The presence of endogenous retroviral sequences in the packaging cell line also increases the risks of recombination. Packaging cells should therefore be screened for the presence of such sequences (Miller and Buttimore, 1986; Rigg et al., 1996).

The production of replication competent retroviruses is also a risk after retroviral vector gene transfer *in vivo,* where there is a subsequent infection of the transduced cells by a wild-type retrovirus. So-called 'self-inactivating' vectors have therefore been developed, where the LTRs of the recombinant vector are inactivated upon integration into the target cell genome. This should prevent the LTRs being packaged in the event of a subsequent wild-type infection and hence avoid the spread of recombinant virus to other non-target cells (Andreadis et al., 1999). The safety of retroviral vectors is also undergoing continual improvement by minimising the viral sequences retained in the vectors or in the helper constructs of the packaging cells (Miller and Rosman, 1989; Cosset et al., 1995; Morgan, 1995). In a very recent paper, Yu et al., (2000) reported the development of a series of retroviral vectors that are absent of any retroviral coding sequences. This was achieved without compromising the vector titre. These vectors are claimed to be safer than other current vectors, which still contain one or more coding sequences from viral genes.

Another potential hazard involved with retroviral vector-mediated gene delivery is the reportedly high error rate in reverse transcription (Kurian et al., 2000). An error in reverse transcription of a therapeutic gene could result in the transduction of target cells with a mutant gene.

Vector Tropism

The packaging cell determines the tropism of the vector, where the *env* gene is contained within a separate plasmid and could theoretically be derived from a number of virus types. For example, the Gibbon Ape Leukaemia Virus (GALV) envelope, which has an increased affinity for human cell receptors, has been incorporated into a number of retroviral vector packaging cells (Glimm et al. 1998; Chuah et al., 2000).

The tropism of MMLV vectors is often referred to as ecotropic, xenotropic or amphotropic/polytropic. Ecotropic vectors are targeted to receptors unique to murine/rat cells, whereas xenotropic vectors are targeted to receptors on most cell species except murine. Finally amphotropic vectors can target different cell receptors in both murine non-murine species.

Modifications of the viral envelope proteins can alter the viral vector tropism and this has been exploited to target specific cell types (Cunliffe et al., 1995; Valsesia-Wittmann et al., 1996; Palú et al., 1999). Such targeted delivery of a therapeutic gene is most desirable for gene therapy applications. The delivery of genes to non–target cells is not only wasteful, but can also lead to adverse side effects (such as cell death of 'normal' cells or the inappropriate/ectopic production of a gene product). Alternative methods for viral vector targeting include the use of antibody-mediated binding or cross-linkers to either restrict or redirect the host cell range (Peng and Russell, 1999). Furthermore, so-called 'pantropic' retroviral vectors have been developed which use VSV-G, an envelope glycoprotein, to mediate viral entry through lipid binding and plasma membrane fusion. These vectors negate the need for specific cell receptors and hence will transduce *any* dividing cell type

(Burns et al., 1993). These vectors are obviously not licensed for human use (due to their indiscriminate transduction abilities), but can be used in the construction of cell lines for research purposes. Vector targeting can also be controlled at the level of gene transcription/expression, where 'switches' can be used to turn inserted gene expression on, or off, in certain cell types or under certain environmental stimuli (Coutre et al., 1994; Cunliffe et al., 1995; Miller and Whelan, 1997; Dutton, 1999).

It has also been postulated that viral envelopes are involved in the inactivation of retroviral vectors by complement components in human serum (Takeuchi et al., 1994; Cunliffe et al., 1995). This is a problem in gene therapy applications where the aim is to infect the target cells *in vivo*. However, the use of *env* genes from certain retroviral species, such as the cat endogenous virus, can afford resistance to such inactivation by human complement (Cosset, 1995). A recent paper by Mason et al., (1999) suggested that the role of envelope proteins in serum inactivation is not due to the binding of complement to these proteins, as has been previously hypothesised. This group proposed that the envelope proteins 'mask' epitopes on the viral membrane from complement attack, thereby conferring resistance to inactivation by serum. This theory may also explain why the level of serum resistance varies between different types of vector, since different envelope proteins will have different three-dimensional conformations, and vector particles may have varying amount of envelope proteins on their surface (depending on the packaging cell system).

Due to the important role of the *env* construct of a packaging cell in vector tropism (including fusion and gene entry) and serum resistance, a number of groups are investigating

the potential to improve gene transfer through modifications of the viral envelope proteins (Gordon and Anderson, 1994; Ragheb and Anderson, 1994a,b).

1.4 Status and Challenges in Retroviral Vector Production

Retroviral vectors can be harvested from the supernatants of packaging cell cultures, where vector particles are constitutively released. The relatively low titre of such retroviral supernatants (Braas et al, 1996) can lead to their increased production costs. Improving the conditions for optimised titre is therefore essential for cost-effective production of retroviral vectors for gene therapy applications. Obviously, optimisation of the culture conditions of the packaging cells can only increase vector yields to the maximum productivity of the packaging cell system itself. However, cell culture conditions during production can also have a significant impact on the vector stock quality and consistency.

The maximum titre of retroviral vector supernatants (produced by packaging cells) reported in the literature is typically 10^5-10^7 colony forming units per millilitre, cfu/ml, (Martinez, 1995; Morgan, 1995; Rigg et al., 1996; Glimm et al., 1998). Though many reported titres have been much lower (Olsen and Sechelski, 1995; Shen et al, 1996). While such titres may be high enough for some gene therapy applications (Eglitis et al., 1995), certain applications such as the transfer of genes *in vivo* and the transfer of genes to haemopoietic cells *in vitro* require significantly higher titres. Most efforts to increase titres have focused on modifications of the packaging cells or viral vectors and titres up to ten-fold higher have been achieved (Sakoda et al., 1999).

Andreadis et al. (1999) assessed the typical dose of retroviral vectors likely to be required in a typical gene therapy treatment and gave a conservative estimate of the annual production requirements in the U.S. alone as 10^7-10^{10} L. These figures were based on vector stock concentrations of 10^6-10^7 cfu/ml. Braas et al. (1996) also attempted to predict future requirements, with single doses ranging from 10^{11} to 10^{14} particles. While sensible predictions of market requirements are obviously very difficult at present, these early indications suggest an urgent need to develop cost-effective methods, which can reproducibly supply large volumes of high titre retroviral stocks. Yet, surprisingly few reports have attempted to examine the factors affecting retroviral vector production in packaging cell cultures in order to develop such methods. A small number of groups have assessed a limited number of parameters, using murine packaging cell lines and these findings are reviewed below.

Temperature

Kotani et al. (1994) investigated the production of retroviral vectors in twenty-two different packaging systems, derived from the PA317 murine packaging cell line. These studies revealed a 5- to 15-fold increase in vector titre from supernatants harvested from packaging cells cultured at 32°C, compared to cultures at 37°C. This group postulated that the increase in vector titre at this lower temperature could be a result of: (1) reduced metabolic activity/growth of the cells, causing their increased production of retroviral vectors and/or (2) increased stability of the retroviral vector activity at 32°C, resulting in an accumulation of active particles at the time of harvesting. The former hypothesis is highly unlikely, as a number of studies in this thesis and elsewhere indicate *decreased* vector production during decreased metabolic activity of packaging cells (Forestell et al., 1995; Shen et al., 1996).

Furthermore, Le Doux et al. (1999) have used a simple mathematical model to analyse the production and decay of retroviral vectors produced by packaging cells at both these temperatures. This group demonstrated that the vector production per cell is a weak function of temperature, whereas the decay rate of retroviral vectors is a strong function of temperature. Hence at the lower culture temperature of 32°C, there is a decrease in vector decay rate that is greater than the decrease in vector production. Hence an overall increase in vector titre is observed at 32°C.

Increased retroviral vector titres at 32°C compared to 37°C were also reported by Kaptein et al. (1997). This group found a 5- to 10-fold increase in vector concentration during culture of POPA/neo-4 murine packaging cells at 32°C. They also reported an increase in vector half-life from 9h to 39h at storage temperatures of 37°C and 32°C respectively (further demonstrating a relationship between increased titre and reduced vector decay). Interestingly, the increased vector stability was only observed within a narrow window, as the vector half-life was not significantly increased at 30°C or 34°C. No explanation for such an unusual finding was given.

Lee et al. (1996) also tested the effects of reduced culture temperatures on retroviral vector production. This group observed only a 2-fold increase in vector titre produced by PA317/LN and ψCRIP/MFG murine packaging cell lines at 32°C, compared to 37°C.

Forestell et al. (1995) reported similar titres in 32°C and 37°C cultures of PA317 packaging cells during the *first six hours* of culture. This finding further indicates that the increased titres at 32°C, seen at later time-points, are due to the accumulation of active particles and

not increased production. According to this argument, the titre of a 37°C culture will not be significantly lower than that of a 32°C culture until a significant number of active particles have thermally decayed at the higher temperature. The half-life of retroviral particles at 37°C has been reported to be in the order of 5-8 hours (Lee et al., 1996; Kaptein et al., 1997; Le Doux et al., 1999). Hence during the initial six hours of culture, titres would not be expected to significantly differ at 37°C compared to 32°C.

Serum

Reports to date on the effects of serum on retroviral vector production and/or activity have been conflicting. This may well be a result of different packaging systems used in each of the reported studies. Shen et al. (1996) showed increasing cell growth and retroviral vector titres in PMFG/ψCRIP murine packaging cell cultures when the serum concentration of the culture medium was increased from 1% to 5% or 10%. A further increase in serum concentration from 10% to 20% only slightly improved the retroviral vector titres. It was postulated that a strong promotion of vector production by cell growth was the reason for increased titres with increasing serum concentration.

Conversely, Lee et al. (1996) demonstrated no effect of decreasing serum concentrations from 20% to 1% in culture medium on the retroviral vector titre of ψCRIP cell cultures. A component in serum was deemed to be essential for cell growth and vector production, as complete removal of serum from the culture medium caused a significant reduction in vector titre. This group suggested that 1% serum would in fact be sufficient for the vector production by this cell line. However production in this low serum concentration was not

conducted for more than 24h, hence the long-term effects of low serum levels on cell growth and productivity was not tested.

Gerin et al., (1999a) have recently demonstrated a dose-dependent *negative* effect of serum on the titre of retroviral vectors produced by the human packaging cell line, FLYRD18. This effect was demonstrated *not* to be attributable to greater adherence of the retroviral particles to the culture surfaces in the presence of higher serum concentrations or extra-cellular inactivation of the vectors by serum components. It was therefore hypothesised that the negative effect of serum was at the level of virus production/assembly or maturation. Further studies revealed pre-treatment of serum-containing medium with trypsin reduced the negative effect of this medium on vector titre. This data suggested a possible role of protease inhibitors present in serum, however the mechanism of such inhibition was not elucidated.

It is possible that a similar negative effect of serum on virus production occurred with the packaging cell system used by Lee et al. (1996). If so, decreasing the serum concentration could *increase* vector production by reducing the negative effects of serum, but at the same time cell growth and/or metabolic activity could be decreased, thereby *decreasing* vector production via a separate mechanism. Hence the two effects of serum could mask each other, leading to no significant difference in vector production over a range of serum concentrations, as observed by Lee et al., (1996).

Aside from the reported negative effects of serum on retroviral vector titres, the presence of serum in vector stocks is undesirable due to the risks of potential contamination (such as

proteins, hormones, endotoxins, prions, and pathogenic organisms). The removal of serum components from vector stocks through down-stream processing will not only increase the costs of production, but will most likely result in reduced vector titres due to the time taken for such processes and the short half-life of retroviral vectors. As a raw material for vector production, serum can also present problems with batch to batch variation and cost. Serum-free medium is therefore a desirable alternative, however only a few reports demonstrate the successful use of serum-free medium for retroviral vector production.

Glimm et al., (1998) demonstrated successful production of GALV-pseudotyped retroviral vectors from PG13/LN packaging cells, using defined serum-free media. Similar titres to those obtained in serum-containing medium were achieved over a 24h-culture period. However, the survival, growth and virus production of these cells during longer culture periods was not reported.

Gerin et al., (1999b) demonstrated similar vector production by FLYRD18 packaging cells cultured in a commercially available serum-free medium, compared to basal medium supplemented with low serum concentrations. Vector titres increased throughout the 6-day culture period, however longer-term cultures in serum-free medium were not conducted.

Additives

A number of groups have attempted to obtain higher retroviral vector titres through the addition of activators/enhancers before harvest. For example, Pagès et al. (1995) undertook a number of studies to determine the effect of different activators (known to enhance the expression of MoMLV genes) on their ability to modulate the retroviral transcripts of two

different ψCRIP murine packaging cell lines. Dexamethasone, TPA (phorbol 12-myristate 13-acetate) and sodium butyrate have all been previously shown to enhance the transcription of retroviruses. These factors were therefore added alone, or in combination, to the packaging cells before harvesting the viral vectors from the culture supernatant in order to assess their effect on the expression of retroviral vectors. A five-fold increase in vector production was observed after the addition of sodium butyrate (5mM) and, in combination with dexamethasone (1μm), a 10-fold increase was measured. The addition of TPA alone (0.1μm) increased vector production 1.5-fold and in the co-presence of dexamethasone, production was increased 7-fold. However the addition of dexamethasone alone did not improve vector production.

The increase in vector production in the presence of these additives was believed to be a result of upregulation of LTR transcripts (as indicated by northern blot analysis of the cell cultures stimulated by these additives). LTRs play a central role in the control of viral gene expression. In the ψCRIP packaging cell line, the structural viral RNAs are under the control of the MoMLV LTR.

Soneoka et al. (1995) also demonstrated an increase in retroviral vector titre during sodium butyrate treatment, after transient co-transfection of a human cell line with three plasmid components. The three plasmids consisted of a Murine Leukaemia Virus-based retroviral vector and two packaging components (*gag-pol* and *env*) expressed from CMV promoters. Since sodium butyrate has been shown to activate a number of promoters, including the CMV promoter, the addition of this chemical prior to vector harvest was employed in an attempt to improve vector titres. A 10-fold increase in vector titre was observed after

treatment with sodium butyrate (10mM) for 12h and the titre peaked 48 hours post-transfection.

Sodium butyrate treatment has been reported to increase retroviral vector titres 20- to 1000-fold in range of retroviral packaging cell lines tested by Olsen and Sechelski (1995). It is worth noting that this group measured a 50-fold increase in LTR-driven RNA transcripts, but only a 4-fold increase in SV40 promoter driven RNA transcripts in the same cell line. Hence, while sodium butyrate has been reported to increase the level of RNA transcription under the control of a number of promoters, the actual responsiveness is dependent on promoter type. It has also been reported that the responsiveness is dependent on the packaging cell system where location of the promoters is important. The exceptionally large increase in vector production in some packaging cell systems observed by this group after sodium butyrate addition, may also be due to the low starting titres without this additive (10^3 – 10^4 cfu/ml). That is, low levels of transcription of some RNA components may well have been a limiting factor in active particle production. The other groups reported a higher titre in their systems before the addition of sodium butyrate hence, where transcriptional activity is already relatively high, exceptionally large increases after sodium butyrate addition may not be possible. Indeed, Cruz et al., (2000a) reported only a slight increase in vector titre in PA317 cell cultures, after the addition of sodium butyrate. This group demonstrated relatively high titres in the absence of this additive, hence vector production may be near maximum levels for this cell line without the addition of sodium butyrate.

The optimal effects of sodium butyrate were reported by Olsen and Sechelski (1995) to occur 20 to 24 hours after its addition. Prolonged exposure of the cells to sodium butyrate (up to

48 hours) resulted in a reduced vector titre (possibly due to cytotoxicity of this additive). Hence the addition of sodium butyrate is only really suitable for batch production of retroviral vectors and is unlikely to be useful during continuous production. Furthermore, the effects of its presence in the final preparation of a vector stock are unknown and removal may be difficult. The pre-treatment of cells with sodium butyrate, followed by harvesting of vectors during a culture period in medium without sodium butyrate may be a preferred method (as used by Soneoka et al.). The efficacy of such a method would have to be tested for each system.

Scale-Up

Attempts by Kotani et al. (1994) to scale-up retroviral vector production to larger volumes have involved the use of a CellCube™ (Costar). An increased vector titre was measured in the CellCube™ cultures, supposedly as a result of higher cell numbers within this system, which provides a greater surface area for cell growth. The vector titre per cell was not given, hence actual productivity of the cells within this apparatus could not be compared to standard monolayer cultures in T-flasks. Vector production was maintained in the CellCube™ cultures for up to twenty-three days, which is the longest production period reported, prior to the studies within this thesis (and a recent paper by Pan and Whitley, 2000). It is desirable to increase the length of production time of retroviral vectors in order to limit operating 'down time' and hence reduce production costs. Longer production periods also results in fewer batches requiring costly quality assurance assays. However, for some packaging cell systems, the length of a production phase may be limited by a reduction in cell productivity with time.

More recently, Pan and Whitley (1999) have shown promising results with a hollow-fibre system for retroviral vector production (though these systems have been reported to be more suitable for anchorage-independent cell types). Microcarrier culture is often the choice for large-scale production of anchorage dependent cells, however, Kang et al. (2000) reported a 10-fold reduction in vector titre during the microcarrier culture of packaging cells in spinner flasks, compared to monolayer cultures. In comparison, this group obtained reasonable vector titres (slightly lower than monolayer) using a packed bed system.

The most common system for larger scale retroviral vector production is currently roller bottle culture (Andreadis et al., 1999). The advantages and disadvantages of a number of culture systems that may be considered for the production of retroviral vectors are summarised in Table II.

Vector Concentration and Storage

Further attempts to increase the vector titres by the above groups have involved concentration processes. A tangential flow filtration system was demonstrated by Kotani et al. (1994) to increase titres by 15- to 24-fold with 91-96% recovery. Lee et al. (1996) also demonstrated successful ultra-filtration of their retroviral vector stocks, reporting a 16-fold increase in titre with only a 10% loss.

In an attempt to develop a method for long term storage of retroviral vector stocks, samples were lyophilised (Kotani et al., 1994). It was found that supplementation of vector supernatants with glucose, or sorbitol, and gelatin was necessary for any recovery of vector activity after lyophilisation. Using these supplements, a 64-83% recovery of active vector

particles was achieved. However, Lee et al., (1996) reported a 60% loss in vector activity after lyophilisation.

Retroviral vectors can also be stored in liquid nitrogen or in -80°C freezers, in order to prevent their decay. Kaptein et al. (1997) studied the effect of multiple freeze-thaw cycles on retroviral vector activity. They reported an initial activity loss of approximately 60% during the first few freeze-thaw cycles. However, very little effect on vector titre was observed during subsequent multiple freeze-thaw cycles (up to 14). Lee et al. (1996) also demonstrated sensitivity of retroviral vectors to freeze-thaw cycles, reporting a 30-50% loss of vector activity after just one cycle. However, this group found no apparent sub-population of resilient vectors, as a further five cycles resulted in only 10-20% of the infectious vector particles remaining.

1.5 Aims

The aim of this research was to determine the effects of various culture parameters on the production of retroviral vectors by a human packaging cell line, in an attempt to optimise conditions for large-scale production. Physical and chemical factors such as temperature, dissolved oxygen levels, pH, and nutrient levels were all assessed. The effect of the cell cycle of the packaging cells on vector release was also examined in detail. This systematic approach also included fundamental characterisation of the packaging cells and their released vector particles. Such information has practical implications in the manufacturing of vectors from these cells for achieving optimal culture and harvest conditions. It is evident from the current literature that no such systematic study on a wide range of parameters has been conducted to date. By determining which factors have a critical effect on vector production,

culture conditions of the packaging cells can be modified and controlled in order to optimise vector production.

The apparent absence of a suitable process for large-scale production of retroviral vectors has been postulated to hinder future gene therapy applications (Birch, 1997; Andreadis et al., 1999). At present, laboratory methods used for the production of relatively large volumes of retroviral vector stocks (for clinical trials) typically involve roller bottles (Andreadis et al, 1999). Such methods are both costly and labour-intensive in large scale. A further aim of this research was to assess alternative culture systems, such as a packed bed, for their suitability as a scalable method for retroviral vector production *in vitro*.

Most reports to date, which have examined the effects of a few culture parameters on retroviral vector production, have used murine packaging cell lines. The studies within this thesis have been conducted using a human packaging cell line (FLYRD18) since it offers a number of benefits for human gene therapy applications (Gerin et. al., 1999). This cell line was constructed to overcome a number of the potential problems with murine packaging cell lines (Cosset et. al., 1995), namely, the inactivation of the released retroviral vectors by complement in human serum (Takeuchi et. al., 1996). Furthermore, as with a number of recent packaging cell lines, the *gag-pol* and *env* genes have been inserted on separate expression cassettes and the viral sequences minimised to reduce the risk of producing replication-competent retrovirus particles. This cell line was therefore deemed suitable for the studies within this thesis.

Culture System	Advantages	Disadvantages
T-Flask	Simple Long history of use, hence well characterised	Difficult to scale-up Labour intensive No continuous control of culture parameters No perfusion of culture medium
Roller Bottle	Large surface area to volume ratio (relative to T-flask)	Still labour intensive in large scale No continuous control of culture parameters No perfusion of culture medium
Cell-Cube™	Larger surface area to volume ratio Culture medium can be perfused	Expensive Limited scale-up
Microcarrier suspension culture	Greatest surface area to volume ratio Culture medium can be perfused Culture parameters can be continuously controlled	Cells exposed to shear forces Some cell types show reduced growth or vector production (compared to monolayer culture)
Packed Bed/ Fluidised Bed	Culture medium can be perfused Culture parameters can be continuously controlled Low shear forces	Mass transfer limitation may occur Requires development

Table II

Advantages and disadvantages of various culture systems for the production of retroviral vectors. Increased surface area to volume ratio allows the potential for intensification of vector production. Continuous control of culture parameters will enable optimal conditions for production to be maintained throughout the culture period. Furthermore, perfusion of the culture medium allows the collection of supernatants at a lower temperature.

1.6 Accuracy of Retroviral Vector Titres

The concentration of infectious retroviral vectors is usually measured indirectly through the infection of target cells (using a so-called 'infection assay'). Such a method was used throughout the studies within this thesis. The values obtained from such infection assays are dependent on a large number of factors, apart from the actual vector concentration. Therefore the vector titres cannot be taken as absolute values, but allow a comparison of *relative* vector concentrations achieved under various conditions.

The inherent variability in the titre (measured as colony forming units, CFU, derived from target cells cultured with vector-containing supernatants) is a result of many contributory factors. This is because the ability of a vector to successfully infect a target cell, such that the cell will go on to express the inserted genes, is dependent on a number of parameters. These include: the metabolic state of the target cell culture, concentration of the vector stock, target cell density, ratio of vector particles to target cells, composition of infection medium, time of exposure of target cells to virus and target cell type (Morgan et al., 1995; Palsson and Andreadis, 1997; Gerin et al., 1999a; Emery et al., 2000). Therefore the titres quoted in any study can only be used as an indication of increased or decreased virus production/stability as a result of various culture conditions. Where retroviral vector stocks are produced for clinical applications, the actual titres quoted should be those measured using a method applicable to the protocol used for transduction of patient cells.

It is highly likely that a number of the infection assays, used to determine the titre of a retroviral stock, significantly under-estimate the actual vector concentration. This is evident from the work of many groups who have optimised the conditions for target cell transduction

and obtained increased vector titre values. For example, Kotani et al. (1994) demonstrated a 4- to 18-fold increase in measured vector titre after centrifugation of vector supernatants covering target cell monolayers, compared to target cells exposed to the vector supernatant during static conditions. Palsson and Andreadis (1997) estimated the gravitational settling velocity of retroviral particles to be in the order of microns per hour. Therefore, under static conditions, such particles are governed by Brownian motion in the culture medium and hence their interaction with a target cell monolayer is random. Due to their short half-life, retroviral particles are quite likely to decay before they actually come into contact with a target cell. The use of centrifugal forces to bring more of these particles into contact with the target cells in a shorter period of time can therefore increase the chances of infection. Obviously such methods for increasing the transduction of target cells are only suitable for *ex vivo* delivery of genes. However it does demonstrate how infection assays that do not utilise this centrifugation procedure can significantly under-estimate the actual titre of a vector supernatant.

The contact of a virus particle with a target cell will not guarantee virus binding to that cell. However by increasing the frequency of particle collision with a target cell, the chances of binding and successful infection are increased. This has been the theory behind a number of methods for improved target cell transduction. For example, fluid flow has been demonstrated to increase the transduction efficiency of retroviral stocks. Mixing of the vector supernatants while in contact with the target cells can allow particles remote from the target cell monolayer to come into contact with the cells. However this does not overcome the effects of Brownian motion at the boundary layer between the cells and the suspension. Fluid flow *through* a monolayer of target cells, inoculated onto a porous substratum, allows

continuous transport of the particles to the target cells and this method has been shown to yield significantly improved vector titres (Palsson and Andreadis, 1997).

Other attempts to increase the transduction of target cells have involved the co-localisation of retroviral vector particles and target cells on fibronectin fragments (Hanenburg et al., 1996; Mortitz et al., 1996), where cells and virus particles can be brought together to increase the chances of infection.

The use of polycations such as Polybrene™ (hexadimethrine bromide) has been widely accepted to improve transduction efficiencies of retroviral stocks (Toyoshima and Vogt, 1969; Coelen et al., 1983). Like the target cells, retroviral vector particles have a negatively charged surface (since their outer membrane is taken from the packaging cells from which they bud off). Hence there is an electrostatic repulsion between the target cells and vector particles. The addition of positively charged Polybrene™ to a suspension of vector particles is believed to reduce the electrostatic repulsion between the particles and target cells, and thereby improve the contact between the two. Interestingly, the optimal concentration of Polybrene™ for high transduction efficiencies has been shown to be dependent on serum concentration (Andreadis and Palsson, 1997). Other groups have favoured the use of DEAE-Dextran (Lee et al., 1996), which is believed to mediate the *adhesion* of virus particles to cells possibly through the reduction in osmotic pressure in the region between the bound virus and the cell. However, this polycation is somewhat toxic to some cell types and is therefore not suitable for all target cell transductions.

The above studies illustrate the arbitrary nature of a vector titre measured by the infection of target cells, since it is highly unlikely that all active particles will be detected. However, as mentioned earlier, these titres do serve a purpose when demonstrating an increase or decrease in vector production and/or stability as a result of modified culture methods. Infection assays were therefore used throughout the studies described in this thesis. In addition, the transduction *efficiency*, as determined by measuring the *percentage* of target cells successfully infected by a retroviral vector stock, was assessed in a select number of preparations. Forestell et al. (1995) found no correlation between end point titres and infection efficiency measurements. It is therefore pertinent to determine the transduction efficiency (in addition to the vector titre) of a retroviral vector preparation, which has been produced under modified conditions.

Le Doux et al. (1999) has also observed a lack of correlation between transduction efficiency and end point titre. This group increased the titre of a retroviral stock by ultra-filtration, however this did not result in an increase in the transduction efficiency of that stock. Both Forestell et al., (1995) and Le Doux et al., (1999) attributed this phenomenon to an increase in inhibitory factors with increasing vector titre. Recently Seppen et al., (2000) also demonstrated the presence of inhibitors which co-purify with retroviral preparations. A number of inhibitors could be present in the supernatant of a packaging cell culture. For example, free envelope proteins present in a retroviral vector preparation (shed from the vector particles themselves, or released by the packaging cells) can compete with the retroviral vectors for binding sites on the target cells and thereby reduce the efficiency with which the vectors bind. If only some of the envelope proteins are shed from a particle, that particle may still be able to bind, hence titre measurements may not be affected.

So-called 'empty' particles, which contain no RNA and hence are inactive, may also be released by packaging cells. These non-transducing retroviral particles can also compete with active particles for binding. While it would be expected (for most supernatants) that the number of retroviral receptors per cell is in excess of the number of vector particles to which they are exposed, competition for binding sites could occur in *concentrated* vector preparations. Vector titres are usually measured using *diluted* suspensions, hence such competition for binding sites is unlikely to have a measurable effect during titre measurements. In contrast, transduction efficiency measurements are conducted using *neat* vector preparations (where the receptor to particle ratio is much lower), hence competing particles may reduce transduction efficiencies. This could explain the poor correlation between titre and transduction efficiency reported by Forestell et al., (1995).

Earlier studies by Le Doux et al., (1998) also demonstrated the presence of proteoglycans (released by packaging cells) to have a negative effect on vector transduction efficiency. Such inhibitory factors will be co-concentrated with vectors during standard concentration procedures such as ultra-filtration and centrifugation. Since the measurement of vector titres is conducted using diluted preparations, these proteoglycans may well be diluted to levels that are ineffective and hence do not cause a reduction in titre. However the concentration of proteoglycans will be at a maximum during transduction efficiency measurements, where vector preparations are used neat, hence inhibitors such as proteoglycans only exert an effect on transduction efficiencies. Again this may lead to a poor correlation between the titre and transduction efficiency of retroviral preparations.

The presence of transduction inhibitors may also explain why Forestell et al., (1995) observed no increase in transduction efficiency while vector titres were observed to increase with time. This group postulated that with increasing production times, inhibitory factor concentrations also increased. This is a reasonable assumption as proteoglycans and envelope proteins will be continuously released by the packaging cells (and thereby accumulate), furthermore, envelope protein shedding and particle inactivation it likely to increase with incubation time. This theory can be used to explain the findings of this group, where a maximum transduction efficiency, of vector preparations in confluent roller bottle cultures, was achieved just 3-5 h after the start of production (addition of fresh medium), whereas the titre progressively increased for 30 h. For this reason, the optimal harvest time for these cultures was concluded to be 3-5 h after medium replacement.

Other groups have reported optimal harvest times at 48 h (Kotani et al., 1994), 3 days post-confluence (Lee et al. 1996) or during sub-confluence of packaging cell cultures (Shen et al, 1996). These findings were based on measured retroviral vector *titres*. Optimal harvest times based on transduction efficiencies may correlate better between different research groups, though it is likely that it will dependent on packaging cell type and culture density.

Chapter 2:

General Materials and Methods

This section describes the materials and methods that are common to most studies throughout this thesis. Details of individual experiments are given, where appropriate, in each chapter.

2.1 Cell Lines

FLYRD18/LNC-hB7:

The FLYRD18 retroviral packaging cell line contains the retroviral helper genes *gag-pol* and *env* on two separate expression cassettes. This cell line was constructed by Cosset et al. (1995) using the HT1080 human fibrosarcoma cell line. The *gag* and *pol* genes originate from Moloney murine leukaemia virus (MoMLV) and the *env* gene originates from the cat endogenous virus, RD114.

FLYRD18 cells were used by M. Gilligan and P. Searle of the Cancer Studies Institute, University of Birmingham, to construct the FLYRD18/LNC-*hB7* cell line. In brief, a third plasmid was used to insert the human CD80/B7 gene and the G418/neomycin resistance gene, from Tn5 (Miller and Rosman, 1989, Freeman et al., 1989, Linsley and Ledbetter, 1993, Daly and Gilligan, personal communication) under the control of the internal cytomegalovirus (CMV) promoter. The human CD80/B7 gene encodes the human B7 protein, designated CD80. This protein is a member of the immunoglobulin superfamily and is expressed on the surface of antigen presenting cells. B7 protein is the natural ligand for

CD28 receptors on the surface of T-cells and is involved in their activation. The FLYRD18/LNC-hB7 cell line was originally constructed for research purposes, aimed at invoking immunogenicity of cancer cells. A linear map showing the organisation of the retroviral vector and the helper functions in the FLYRD18/LNC-hB7 cell line is given in Figure 2.1.

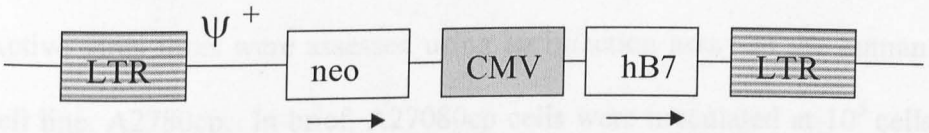
A2780cp:

Cisplatin resistant human ovarian carcinoma cells, A2780cp, (Khokhar et. al., 1992) were used as target cells to measure retroviral vector titre in all the infection assays (also courtesy of P. Searle, Cancer Studies Institute, University of Birmingham).

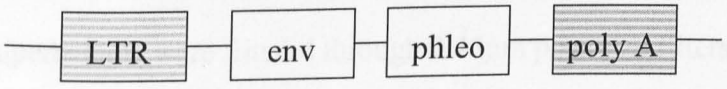
2.2 Monolayer Cell Culture

Both the producer and target cell lines were maintained in monolayer cultures using standard tissue culture flasks (T-flasks). Cells were cultured in Dulbecco's Modified Eagles Medium and Ham's F-12 Nutrient Mix (DMEM:F-12,1:1) plus 5% (v/v) Foetal Calf Serum, (FCS, Life Technologies, UK). FLYRD18/LNC-*hB7* cells were inoculated at 2×10^4 cells /cm^2 (5×10^4 cells/ml) and passaged once confluent (every 3-4 days) in fresh medium. A2780cp target cells were inoculated at 1×10^4 cells/cm^2 (2.5×10^4 cells/ml) and maintained as above.

LNC-hB7-1 vector:



env construct (Cosset et al., 1995):



gag-pol construct (Cosset et al., 1995):

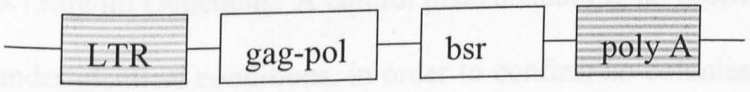


Figure 2.1

Linear maps of the LNC-hB7 vector and helper constructs in FLYRD18/LNC-hB7 packaging cells. The gag-pol-bsr and env-phleo expression cassettes were transferred sequentially into the parental HT1080 cells. Clones with stable integration of the constructs in genomic DNA and expressing high levels of the encoded products were selected using the antibiotics blasticidin and phleomycin, respectively (Cosset et al., 1995) to generate the FLY RD18 clone of packaging cells. Production of bsr and phleo proteins depends upon reinitiation of translation, shortly after the stop codon of the first open reading frame. The neomycin resistance gene in the LNC-hB7 construct is expressed from the upstream LTR of chromosomally integrated provirus and confers resistance to G418 in eukaryotic cells. The B7 protein is expressed from the internal CMV immediate early promoter.

ψ^+ : packaging signal, CMV: cytomegalovirus promoter, polyA; polyadenylation signal.

2.3 Measurement of Vector Titre by an Infection Assay

Active virus titres were assessed using an infection assay of the human ovarian carcinoma cell line, A2780cp. In brief, A27080cp cells were inoculated at 10^5 cells per well in 6-well culture plates (Nunc) and cultured in 2ml DMEM:F-12 plus 5% FCS (v/v) overnight. The spent medium from each well was replaced with 1ml diluted virus supernatant, $1:10^2$ to $1:10^4$, in DMEM:F-12 (no serum) + 8μg/ml Polybrene™ (Sigma, UK). Undiluted virus supernatants were filtered through 0.45μm pore size filters (to remove any suspended cells or cell debris) and stored in liquid nitrogen prior to assay. Diluted virus supernatants (two wells for each dilution) were left in the wells at 37°C for 8h before adding 2ml DMEM:F-12 + 5% FCS (v/v) + 1.125mg/ml Geneticin (G418) per well, to give a final concentration of 0.75mg/ml Geneticin. A control plate containing no retrovirus supernatant was also cultured under identical conditions, in order to confirm no colonies would survive in the presence of Geneticin (0.75 mg/ml). All plates were incubated at 37°C for 10 days, with a complete medium change in each well after 5 days (using fresh DMEM:F12 + 5% (v/v) FCS + 0.75mg/ml Geneticin). On day 10 the medium was removed and each well rinsed with 1-2ml phosphate buffered saline (PBS). After removing the PBS, 1ml crystal violet solution (0.3% (w/v) crystal violet, 70% (v/v) methanol in H_2O) was added to each well and left for 15 minutes. The crystal violet solution was removed and each well was rinsed with water. The number of stained colonies in each well was counted and the number of colony forming units (CFU) per ml of neat virus supernatant calculated (by multiplying the number of CFU by the dilution factor). Figure 2.3 illustrates a typical colony of transfected A2780cp cells stained with crystal violet.

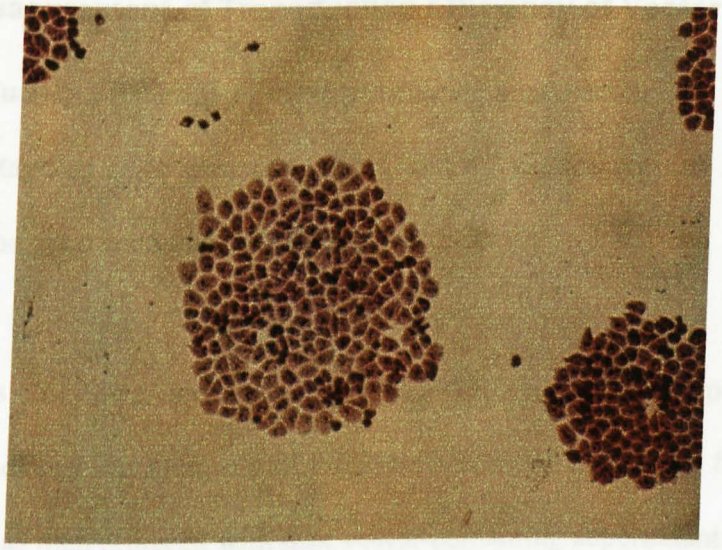


Figure 2.3

Photomicrograph of a typical colony of A2780cp cells transfected with retroviral vectors from FLYRD18/LNC-hB7 and selected in Geneticin-containing medium (0.75 mg/ml G418) for 10 days, before staining with crystal violet. Magnification × 100.

2.4 Measurement of Transduction Efficiency of Vector Preparations

The transduction efficiency of vector-containing supernatants was determined by measuring the percentage of cells expressing the CD80/B7 surface antigen after exposure to vectors (carrying the gene encoding for CD80/B7).

Target cells (A2780cp) were inoculated at a density of 10^5 cells per well in 6-well culture plates (Nunc) and cultured in 2ml DMEM:F-12 + 5% FCS (v/v) for 24h. The medium in each well was then replaced with neat vector-containing supernatant (1ml) plus DMEM:F-12 + 5% FCS (v/v) + 8 μg/ml Polybrene™ and the plates left for a further 6-8h at 37°C. After this exposure to the vector preparation, DMEM:F-12 + 5% FCS (2ml) was added to each well and the plates cultured for 42h. Cells from each well were then detached by trypsin digestion and counted using a haemocytometer. Cells were washed in PBS before adding 20μl per 10^6 cells of an FITC-conjugated anti-human CD80/B7-1 antibody (BB1, Pharmingen International). Cells were incubated with this antibody at 4°C for 60 minutes, washed in PBS and then fixed in 1% paraformaldehyde for 30 minutes. Fixed cell preparations were washed again in PBS before analysing by flow cytometry. An EPICS Elite Flow Cytometer (Coulter Electronics, UK) with an argon laser (488nm emission) was used to measure the fluorescence intensity per cell. Control cells were treated as above, but incubated in medium *without* vectors for 6-8h (fluorescence levels in these cells were negligible). A further control was prepared where cells were exposed to the vector preparation, but subsequently labelled using an FITC-conjugated goat anti-murine IgG antibody. This irrelevant antibody was used to check for non-specific binding of FITC-conjugated antibodies to the target cells (fluorescence levels in these cells were also

negligible). The expression of CD80/B7-1 on the surface of the target cells was detected by increased fluorescence (positive antibody labelling) relative to the controls.

2.5 Cell Cycle Analysis using Flow Cytometry

Cells were fixed in cold 70% (v/v) ethanol (1×10^6 cells per sample) and stored at -20°C until analysed. Immediately before analysis, cells were washed in phosphate buffered saline (PBS) then resuspended in RNase (250 μg/ml in PBS; Sigma, UK) and incubated at 37°C for 20 minutes. The DNA stain, Propidium Iodide (PI), was then added to give a final concentration of 50 μg/ml and the samples incubated for a further 10 minutes at room temperature. An EPICS Elite Flow Cytometer (Coulter Electronics, UK) with an argon laser (488 nm emission) was used to measure the relative DNA content of each cell. The forward scatter (FS) was collected using a 488 nm band pass filter and the PI fluorescence collected using a 488nm long pass filter, followed by a 635 nm band pass filter. The FS integral, side scatter (SS) signal integral, PI fluorescence signal integral and the PI fluorescence signal peaks were all recorded. Any doublets or aggregates were 'gated out' using the PI fluorescence signal vs. PI fluorescence signal peak plots, hence only single cells were analysed. The relative PI fluorescence signal integral (relating to DNA content) in each cell was plotted as a frequency histogram and the proportion of cells in G1, S and G2/M-phases of the cell cycle was determined using Multicycle software (Phoenix Flow Systems, San Diego, USA).

2.6 Determination of Cell Numbers using an MTT Assay

Where cell numbers could not be counted directly (for example, where cells were attached to microcarriers), the ability of mitochondrial enzymes within viable cells to convert MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) to an insoluble coloured product (formazan) was exploited to estimate the cell number (Mosmann, 1983; Gerlier and Thomasset, 1986; Pagé et al., 1988; Al-Rubeai et al., 1990; Nomura et al., 1996).

In brief, a sample of carriers was added to PBS (1ml) plus 100µl freshly prepared MTT solution, 5 mg/ml in PBS, (Sigma, UK). All samples were incubated at 37°C for 3h to allow the conversion of MTT to formazan. After this incubation period the formazan crystals were dissolved by the addition of acidic isopropanol (1ml of 0.04N HCl in isopropanol). Samples were shaken at room temperature for 30 minutes, or until the carriers turned from blue to white (indicating all the formazan product had been released from the carriers into solution). The relative concentration of formazan in each sample was determined by measuring the absorbance using a Pharmacia LKB Ultrospec III spectrophotometer at 570nm.

In order to convert the formazan concentration (absorbance) of each sample to cell number, a calibration curve was constructed using FLYRD18/LNC-hB7 cells. A suspension of known cell number (determined by haemocytometer counts) was used to prepare pellets with a range of cell numbers (duplicate for each concentration). PBS (1ml) and 5mg/ml MTT solution (100µl) was added to resuspend each pellet, before incubating at 37°C for 3 h. The formazan concentration of each sample was then measured as described above. The resulting calibration curve is shown in Figure 2.6.

The MTT assay has the advantage of distinguishing viable from non-viable cells, however due to its dependence on the metabolic activity of each cell, determination of actual cell number may not be reliable. Where the activity of cells within a culture is not constant (as can be the case in batch cultures), the amount of formazan produced per cell will not be constant (Yamaji and Fukuda, 1992). In addition, the metabolic activity of cells varies throughout the cell cycle, hence the MTT assay is effected by the cell cycle. Hence estimated cell numbers determined by MTT conversion to formazan should be used with caution and taken only as an indication of increasing or decreasing cell density/activity or as a comparison of cell number between cultures from the same batch of cells.

2.7 Calculation of Cell Growth Rate (μ)

In a number of studies, the cell growth rate (μ) of FLYRD18/LNC-hB7 cells was compared between different culture conditions. Cell growth rates were calculated using the following specific rate formula (Leelavatcharamas, 1997):

$$\mu = \frac{1}{(\chi_2+\chi_1)/2} \times \frac{\chi_2-\chi_1}{t_2-t_1}$$

Where χ_1 is the number of cells at the start of the culture period (at time t_1) and χ_2 is the total cell number at the end of culture (at time t_2).

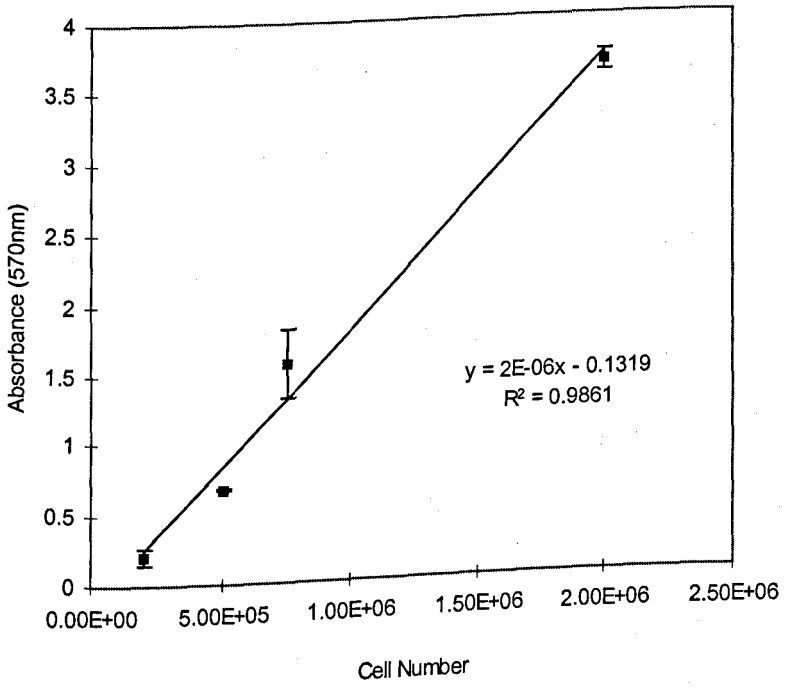


Figure 2.6

MTT calibration curve for determining FLYRD18/LNC-hB7 cell number. Known cell numbers incubated with MTT for 3 h and absorbance measured at 570 nm. Error bars represent observed range, n=2.

2.8 Cell Counts

Cell numbers from monolayer culture were estimated by counting replicate samples on an improved Neubauer haemocytometer under phase contrast microscopy. Viable cells were distinguished from non-viable cells by exclusion of the vital dye, Trypan Blue. Cells cultured in monolayers on tissue grade plastic were detached by trypsin digestion before counting. In brief, the spent culture medium was removed from each flask and the attached cells were gently washed with sterile phosphate buffered saline (PBS), to remove excess serum proteins. The PBS was then replaced with 1% v/v trypsin-EDTA (Life Technologies, Scotland), pre-warmed to 37°C. Cells were incubated in the trypsin solution at 37°C for approximately one minute, or until the cells were observed to start 'rounding up' and detach from the culture surface (cells were visualised using an inverted light microscope). Once all the cells had detached, fresh medium containing 5% FCS was added to the flasks to 'inactivate' the trypsin solution. The cells and medium were gently mixed to obtain a single cell suspension before centrifuging. The supernatant was removed and the cell pellets were resuspended in a known volume of medium. Replicate samples (100µl) of this cell suspension were mixed with 0.5% v/v Trypan Blue solution (100µl) and transferred to the haemocytometer for counting.

2.9 ImmobaSil™ Microcarriers

A number of studies in Chapters 3 and 5 involve the use of microcarriers. Unless otherwise stated, the microcarriers used were ImmobaSil-FS™ or ImmobaSil-G™ (Ashby Scientific Ltd, U.K.). These microcarriers are composed of silicone rubber, engineered to give a

sponge-like structure with interconnecting pores (see Figure 2.9). The average pore size is in the order of 50 × 150μm. The properties of –FS and –G types are summarised below.

Type	Shape	Approximate dimensions	Recommended use
-FS	discs	0.8mm diameter, 0.25mm depth	Spinners /stirred tanks
-G	rods	0.8mm diameter, 1mm length	Packed/fluidised beds

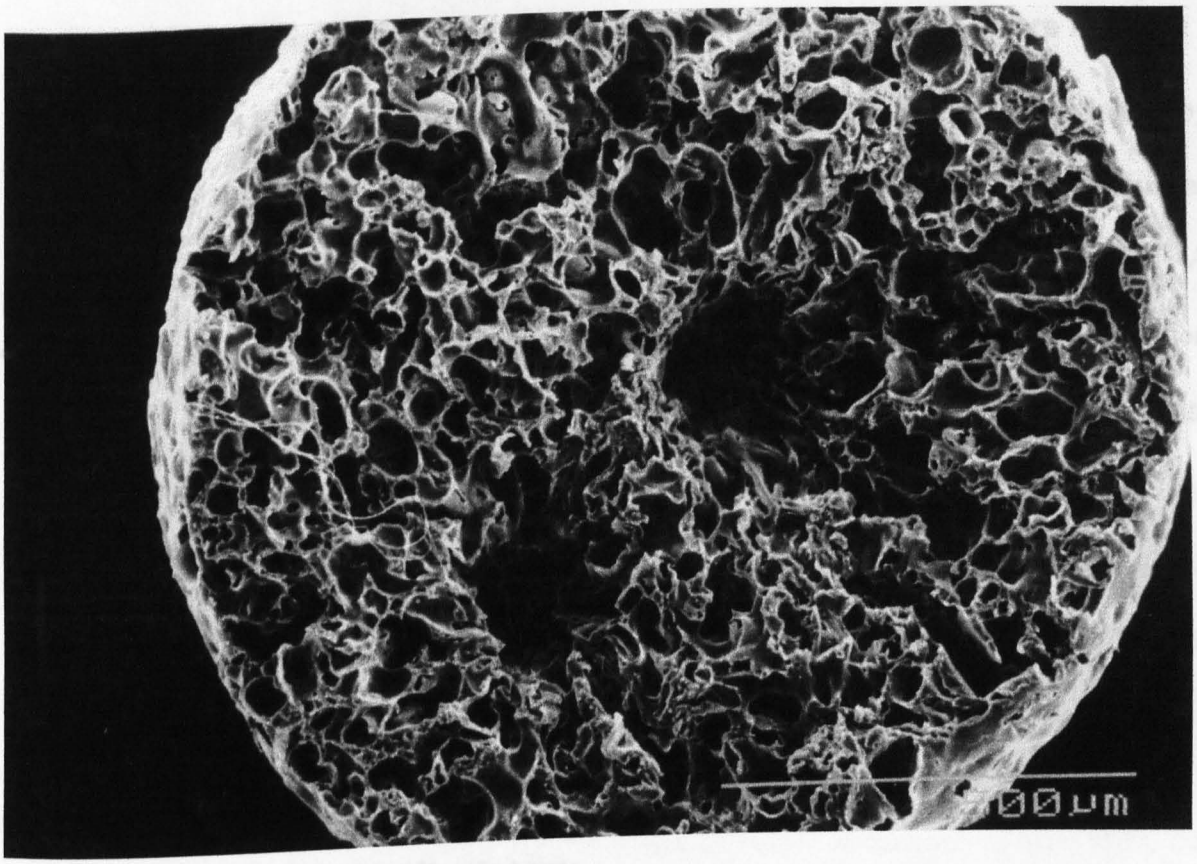


Figure 2.9

Scanning electron micrograph of an ImmobaSil™ microcarrier (without cells). Bar represents 500µm.

2.10 Measurement of Glucose and Lactate Concentration in Culture Medium

The glucose consumption and lactate production in various cell cultures was determined by measuring the concentration of each in fresh and spent medium. Glucose concentration was measured using the Refloflux II reflectance photometer (Boeringer Mannheim, U.K.). A sample of the culture medium (50μl) was added to a test strip and the glucose concentration determined by colourimetric analysis as per manufacturer's instructions. Lactate concentration was measured using the Biolyser Rapid Analysis System (Eastman Kodak Co., U.S.A). A sample of the culture medium (10μl) was added to a slide containing all the appropriate reagents (within a semi-permeable membrane) and the concentration of lactate determined as per manufacturer's instructions.

2.11 Scanning Electron Microscopy

In order to observe attached cells within the ImmobaSil™ microcarriers during culture, samples were taken periodically and fixed for scanning electron microscopy (SEM). In brief, samples were removed from culture, rinsed gently in PBS and fixed in 2.5% phosphate buffered gluteraldehyde (freshly prepared), overnight at 4°C. Samples were then dehydrated by immersion in a series of ethanol solutions of increasing concentration. Samples were soaked in each solution for approximately one hour before transferring to the next. Eventually samples were soaked in absolute ethanol and kept at 4°C until observing by SEM.

Chapter 3:

Fundamental Characterisation of FLYRD18/LNC-hB7 Cells and Vector Particles

3.0 Introduction

Prior to any modifications of the typical culture conditions of FLYRD18/LNC-hB7 cells, the fundamental characteristics of this cell line and their released vector particles were determined. These studies included assessment of the behaviour of these cells during batch culture, determination of cellular oxygen uptake rate, effects of cell cycle on vector production, maintenance of vector production, ability of the cells to adhere to microcarriers and effects of agitation. The stability of the vector particles was also assessed under various conditions. In addition, the method used to estimate vector concentration was tested for reproducibility in order to assess its suitability for future studies. Finally, the typical transduction efficiency of the vector-containing culture supernatants was determined.

The results from these initial studies were used to determine the appropriate handling procedures of the cells and vector preparations in future experiments. These studies also allowed a basic understanding of some of the parameters likely to affect vector production.

Cell Cycle

Included in these studies was an investigation into the effects of cell proliferation and cell cycle phase on the vector production by FLYRD18/LNC-hB7 cells. A wealth of literature exists concerning the determination of *target* cell cycle effects on infection by retroviral particles (Fritsch and Temin, 1977; Harel et al., 1981; Springett et al., 1989; Andreadis et al.,

1998; Sladek and Jacobberger, 1998). Such studies have demonstrated that target cells must be undergoing mitosis to allow successful infection by most retroviruses (Miller et al., 1990b; Roe et al., 1993; Lewis and Emerman, 1994; Andreadis et al., 1997) excluding lentiviruses (Lewis and Emerman, 1994). However, the effect of cell cycle of the packaging cells used for retroviral vector production has received no attention to date. While the effect of cell cycle of the target cells is important with respect to clinical applications (i.e. optimisation of gene transfer protocols), the cell cycle of packaging cells holds equal importance with respect to the production of viral vectors for gene transfer. If the cell cycle phase of packaging cells were to have an effect on vector production, this would have a great impact on the optimal culture conditions for maximising vector titres.

In cell lines used for the production of recombinant proteins, under different promoters, the relationship between cell cycle phase and product expression has proven to be highly variable (Lloyd et al., 1999a,b). A number of groups have reported maximal product expression during the Synthetic (S) phase (Matherly et al., 1989; Gu et al., 1993, 1996). While other researchers, using different cell systems, have found maximal expression during Gap-1 (G_1) phase (Al-Rubeai & Emery, 1990; Kubbies and Stockinger, 1990; Al-Rubeai et al., 1992; Park and Ryu, 1994) or Gap-2/Mitotic (G_2/M) phases (Aggeler et al., 1982). Furthermore, many cell lines have shown aphasic production (Feder et al., 1989; Matherly et al., 1989). The relationship between cell cycle phase of FLYRD18/LNC-hB7 packaging cells and vector production was therefore investigated.

In addition, the effect of cell proliferation rate on vector production was assessed. Work by Shen et al. (1996) suggested that retroviral vector production by the amphotropic ψCRIP

murine packaging cell was dependent on cell proliferation, however this was not conclusively proven. The studies within this chapter employed a number of different methods to manipulate the proliferation of FLYRD18/LNC-hB7 cells, in order to determine whether vector production by this cell line was dependent on cell proliferation/cell cycle progression.

Microcarrier Culture

Methods for the large-scale culture of anchorage-dependent cell lines (beyond roller bottle scale) almost exclusively involve the use of carriers for cell immobilisation (Adamson and Schmidli, 1986; Scott, 1987; Looby and Griffiths, 1990; Hu and Peshwa, 1991; Hu and Aunins, 1997). Microcarrier culture of mammalian cells *in vitro* was first introduced by van Wezel (1967). Since then a wide range of cell types have been successfully cultured on various different microcarriers (Crespi et al., 1981; Yamaji et al., 1989; Chengzu, 1994a,b; Ong et al., 1994; Kennard and Piret, 1995; Alves et al., 1996; Yamaji and Fukuda, 1997; Berry et al., 1999). The immobilisation of cells on such carriers provides an increase in the surface area to volume ratio during culture (especially with porous microcarriers), hence this is an attractive method for the scale-up of cell culture. The success of large-scale microcarrier cell culture has been demonstrated in a number of different systems, including stirred tanks, air-lift fermenters, fluidised beds and packed beds (Yamaji and Kukuda, 1991; Cosgrove et al., 1995; Kong et al., 1999; Pörtner et al., 1999). The ability of FLYRD18/LNC-hB7 cells to be cultured on microcarriers was therefore investigated to aid the future development of a scalable method for vector production by this cell line.

The importance of cell attachment to carriers *in vitro* has been reported extensively (Hu and Wang, 1985; Hu et al., 1985; Forestell et al., 1992; Ohlson et al., 1994; Ng et al., 1996). Therefore the first step in the development of a carrier culture of the FLYRD18/LNC-hB7 cell line was to study the attachment of these cells to carriers under different conditions.

3.1 Materials and Methods

3.1.1 Batch Culture of FLYRD18/LNC-hB7 Cells

The behaviour of the FLYRD18/LNC-hB7 cell line in typical batch culture (with respect to cell growth and vector production) was assessed over a 10-day period. Monolayer cultures were established in T-25 flasks using DMEM:F-12 + 5% FCS (as described in Chapter 2) at 1×10^5 cells/ml and the culture medium in each flask was left unchanged for the entire culture period. The supernatant in duplicate flasks was sampled daily for measurement of glucose concentration, lactate concentration and vector titre. After sampling, cells were detached from the flasks by trypsin digestion and counted with the aid of a haemocytometer. Total cell number and percentage viability were both determined. Cell samples were also fixed for cell cycle analysis using flow cytometry (as described in Chapter 2).

3.1.2 Effect of 'Conditioned' Medium on Vector Production

Monolayer cultures of FLYRD18 cells (parent cell line of FLYRD18/LNC-hB7 cells) were established as described in Chapter 2. These cells do not contain the retroviral vector carrying the neomycin resistance gene and the human CD80/B7 gene and hence only produce 'empty' particles. The spent medium from these cultures (left unchanged) was collected from replicate flasks each day, for 10 days, before discarding the cultures. The

spent medium samples ('cell-conditioned' medium) were stored at 4°C until the last sample was collected on day 10. FLYRD18/LNC-hB7 cell cultures were established as before and cultured for 24h in the collected media samples (conditioned for 1 to 10 days) or fresh medium. Supernatants from each culture were then sampled for vector titre measurements in order to test the effects of cell-conditioned medium on vector production.

3.1.3 Re-selection of Vector-Producing Cells in G418

Vectors released by the FLYRD18/LNC-hB7 cell line contain the neomycin resistance gene (conferring resistance to Geneticin, G418, antibiotic). Any cells that do not contain this gene will die in the presence of sufficient concentrations of G418. FLYRD18/LNC-hB7 cell monolayer cultures were therefore established (as described in Chapter 2) in the presence of 0, 0.25, 0.50 or 0.75 mg/ml G418 for one week in order to check that *all* cells within the population did indeed contain the vector carrying the neomycin resistance gene. The culture medium was changed for fresh (with or without G418) on day 4 and 6. On day 7 the cell numbers and vector titres in each culture were compared.

3.1.4 Relationship between Cell Proliferation, Cell Cycle Phase and Vector Production

3.1.4.1 Effect of Thymidine-Arrested Cell Growth on Vector Production

Replicate monolayer cultures of FLYRD18/LNC-hB7 cells (as described in Chapter 2) were grown to sub-confluence in DMEM:F-12 + 5% FCS. The culture medium in each culture was then changed for fresh medium either with or without 4mM thymidine (Sigma, U.K.)

and samples of the supernatants were taken on days 1, 2 and 3 for measurement of the vector titre.

A second study using a range of thymidine concentrations was also conducted. Cells were cultured on 6-well tissue culture plates (1.8×10^5 cells per well) in DMEM:F-12 + 5% FCS for 24h before replacing the medium in each well (2ml) with fresh medium containing 0.5, 1.0, 2.0, or 4.0mM thymidine (triplicate wells for each concentration). After a further 24h the supernatant from each well was taken for subsequent vector titre measurements. The cell number and viability in each well was also determined after releasing the cells by trypsin digestion and counting with the aid of a haemocytometer (viability was determined by Trypan Blue exclusion). The released cells were then fixed in cold 70% ethanol and stored at -20°C for later analysis of the cell cycle distribution by flow cytometry (as described in Chapter 2).

3.1.4.2 Relationship between Cell Cycle Phase and Vector Production

Cells in different phases of the cell cycle were separated by centrifugal elutriation and the vector production in cell cultures enriched for specific cell cycle phases was compared. It was necessary to take samples for vector titre measurement soon after separation of the cell fractions, before a significant loss in cell cycle synchrony was incurred. To ensure that virus titres could in fact be measured after just a few hours in culture, (using the cell concentrations employed in this study), a set of preliminary monolayer cultures were set up and the supernatant sampled 2, 4, 6 and 8 h after inoculation of cells. Results from this study confirmed the ability to detect vector production after just 2 h in culture. Therefore the titre

in cell fractions separated by centrifugal elutriation was measured 2 h after inoculation of each cell fraction into T-flasks.

To prepare each cell fraction, FLYRD18/LNC-hB7 cells were detached from monolayer by trypsin digestion, spun down and pooled in 10ml cold PBS (2×10^8 cells in total). This concentrated cell suspension was then added to the sample port of a Beckman J-6M/E centrifugal elutriator. During centrifugation, PBS was pumped through and 50ml samples were collected at increasing pump speeds (16 fractions collected in total). The collected fractions were kept on ice until the last fraction was eluted. Cells from each fraction were then taken for cell size determination using the Coulter Multisizer II (cells suspended in Isoton II) and cell cycle analysis by flow cytometry (cells fixed in cold 70% v/v ethanol).

Results from this experiment allowed determination of the pump speeds required to elute FLYRD18/LNC-hB7 cells in each phase of the cell cycle. The experiment was then repeated under aseptic conditions with the exception that only three fractions were collected: pump speed 2 - 3 for cells in G_1, pump speed 3.75 - 4.75 for cells in S and pump speed 5.5 – 6.5 for cells in G_2 (pump speeds are arbitrary units). The cells in each fraction were then spun, resuspended in fresh medium (DMEM:F-12 + 5% FCS), counted, and plated down in triplicate flasks. Non-ellutriated cells were used as a control. After 2 hours the culture supernatant was harvested for virus titre measurements (this short culture period allowed the production of enough virus particles to be detected, whilst minimising the loss of cell cycle phase homogeneity during cell cycle progression). Cell samples were also fixed before and after the 2-hour culture period for cell cycle analysis, in order to confirm fractions contained

cells enriched for a particular phase of the cell cycle and to assess cell progression through the cell cycle during the 2-hour culture period.

3.1.4.3 Effect of Demecolcine-Arrested Cell Growth on Vector Production

Cells were inoculated into T-flasks, as before, and incubated for 4 hours to allow cell attachment. The medium in each flask was then replaced for fresh, with or without 100 nM Demecolcine. On days 1 and 2, the supernatant from each well was sampled for vector titre measurements and the cells counted and fixed for cell cycle analysis on day 2.

3.1.4.4 Effect of Glucose Concentration on Cell Growth and Vector Production

In an attempt to compare cell growth rate with the virus productivity per cell, FLYRD18/LNC-hB7 cells were cultured in a range of glucose concentrations. The glucose concentration in the DMEM:F-12 medium used in all other experiments is 3.5 g/L. In this study, the packaging cells were cultured in medium containing 0, 0.5, 1.0, 2.0 or 3.0 g/L glucose for up to 3 days and the cell number and virus titre measured on day 3. The virus titre was calculated *per cell* in order to account for varying cell yields in the different glucose concentrations. The cell growth rate (μ) in each culture was also determined, using the specific rate formula given in Section 2.7 (Leelavatcharamas, 1997).

3.1.5 Attachment of FLYRD18/LNC-hB7 Cells to Microcarriers

The ability to attach FLYRD18/LNC-hB7 cells to microcarriers and maintain cell viability was assessed. These studies were conducted using ImmobaSil-FS™ carriers (Ashby Scientific Ltd., U.K.) in Bellco™ spinner flasks (100ml volume). Each spinner flask was coated with Sigmacote™ silicone solution (Sigma, U.K.) to prevent cell attachment to the

glass walls of these culture vessels. After rinsing in Sigmacote™, the vessels were left to dry before autoclaving at 121°C for 15 minutes.

Earlier studies (using an alternative cell line) revealed intermittent stirring, rather than continuous stirring, of the ImmobaSil-FS™ microcarriers allowed a greater percentage of cells to attach to these carriers. This method was therefore used in the following studies. A sample of carriers (5ml settled volume) was added to each spinner flask with 50ml PBS and the whole system sterilised in an autoclave at 121°C for 15 minutes. The sterile carriers in each flask were then rinsed in culture medium before adding 30×10^6 FLYRD18/LNC-hB7 cells in 50ml fresh culture medium (to give a final cell density of 6×10^5 cells/ml). These cell suspensions were mixed with the carriers then left static at 37°C for up to 5 hours. During this attachment period, the cells and carriers were mixed for 30 seconds every 30 minutes (so-called 'intermittent' agitation). A small sample of supernatant was removed from the spinner flasks every hour in order to determine the number of cells remaining in suspension (unattached cells). This data was used to estimate the number of cells attached to the carriers at each time point.

An extra sample of ImmobaSil-FS™ carriers was soaked in foetal calf serum for 30 minutes at 37°C before assessing the attachment of FLYRD18/LNC-hB7 cells as described above. This study was conducted in order to determine whether the coating of ImmobaSil-FS™ carriers with serum components (such as fibronectin) could enhance the attachment rate of FLYRD18/LNC-hB7 cells.

After 5 h, a sample of carriers was taken from each flask for an MTT assay, as described in Chapter 2. This data was used to estimate the number of cells attached to the carriers, in order to confirm that the loss of cells from suspension was due to attachment to the carriers. After the MTT assay was complete, the number of carriers in each sample was counted in order to determine the mean absorbance per carrier. This absorbance was converted to cell number, using the calibration curve shown in Figure 2.6 (Chapter 2). The total number of ImmobaSil-FS™ carriers in a settled volume of 5ml (added to each flask) is approximately 6620. Therefore, in order to determine the total number of cells attached to the carriers after 5 hours, the estimated number of cells per carrier was multiplied by 6620.

3.1.6 Effect of Agitation on FLYRD18/LNC-hB7 Cell Cultures

The above cell attachment procedure was repeated with non-treated ImmobaSil-FS™ carriers (using the same amount of carriers, cells and medium). After the 5h attachment period, the culture volume was made up to 100ml with DMEM:F-12 + 5% FCS and the cultures were stirred at 42 rpm. After overnight stirring of the cultures another MTT assay was performed on a sample of carriers, in order to ascertain whether the cells had remained viable on the beads during such culture conditions.

Due to the inability to maintain cell viability on the ImmobaSil-FS™ carriers during stirring at 42 rpm, a study on the effects of agitation on FLYRD18/LNC-hB7 cell cultures was conducted. A sample of ImmobaSil-FS™ carriers was inoculated with FLYRD18/LNC-hB7 cells as before and left static at 37°C overnight. The cell-seeded carriers were then spun at 11 rpm and samples of the supernatant taken every 15 minutes for cell counting, in order to

determine whether cells detached from the carriers at this slow agitation speed. Samples of the inoculated carriers were also taken periodically for an MTT assay, in order to assess whether cells attached to the carriers remained viable during such agitation. The stir speed was progressively increased with periodic sampling of the supernatant (for cell counts) and carriers (for MTT assays). A control culture containing the same cell number per ml, *without* carriers, was stirred and sampled under identical conditions.

In a further study, the ability of ImmobaSil-FS™ carriers to support FLYRD18/LNC-hB7 cell viability during non-agitated culture was assessed. This experiment was conducted in order to confirm whether the loss of cells attached to the carriers in stirred cultures was due to agitation effects or an adverse effect of the carriers themselves. ImmobaSil-FS™ carriers were once again inoculated with FLYRD18/LNC-hB7 cells as before, and cultured under static conditions overnight. The inoculated carriers were then divided into two vessels and one was returned to static culture while the other was stirred at 42 rpm. Both cultures were monitored for cell viability by sampling the carriers for an MTT assay. Where viable cells could be detected on the carriers, cultures were maintained for one week with replacement of the exhausted medium for fresh (50%) every 2 days. A sample of the culture supernatant was taken on Day 6 for measurement of the retroviral vector titre.

3.1.7 Oxygen Uptake Rate of FLYRD18/LNC-hB7 Cells

It is apparent from the above studies that large-scale culture of FLYRD18/LNC-hB7 cells is likely to only be successful under conditions of low shear forces. For this reason, the suitability of a packed bed system (with low shear forces) for the production of retroviral vectors by the FLYRD18/LNC-hB7 cell line was investigated (Chapter 5). In order achieve

optimal cell growth in a packed bed system, the delivery of oxygen and various nutrients must be sufficient to support the required cell number. Hence the delivery of oxygen/nutrients in the packed bed should be compared to the uptake rates of these components by the cells to be cultured in this system. As part of this aim, the oxygen uptake rate (OUR) of FLYRD18/LNC-hB7 cells was determined for cells attached to ImmobaSil-G™ carriers (used to immobilise the cells in packed bed cultures) and cells in suspension (detached from tissue culture plastic by trypsin digestion).

Oxygen uptake rates (OUR) of FLYRD18/B7 cells were measured using a respirometer (Digital Oxygen System Model 10, Ranks Brothers Ltd.) which incorporates a Clarke type electrode (platinum and silver chloride electrodes). Oxygen diffuses from the cell suspension across a thin Teflon membrane to the platinum electrode (Figure 3.1.7).

A known number of FLYRD18/LNC-hB7 cells attached to ImmobaSil-G™ microcarriers, or in suspension, were placed in the chamber of the respirometer in 5ml DMEM:F-12 + 5% FCS. Samples were taken straight from incubation at 37°C and the chamber itself was maintained at 37°C by means of a water jacket re-circulating to a heated water-bath. Immediately before addition of the sample, the electrodes were calibrated. Saturated sodium citrate was used as a 'zero' reading and culture medium, warmed to 37°C and purged with air, was used to represent 100% dissolved oxygen. After addition of the sample, the drop in dissolved oxygen in the culture medium was traced with a chart recorder and the slope used to determine the OUR of the cells, as described below. Control slopes were obtained by adding 5ml of culture medium only to the chamber (warmed to 37°C and purged with air) and recording any drop in dissolved oxygen levels.

An accurate determination of exact cell numbers attached to the ImmobaSil-G™ microcarriers is difficult. Therefore, in order to determine the amount of oxygen consumed per cell per minute, cells were allowed to attach to the microcarriers for only 3 hours (with intermittent agitation) before measuring the OUR. This allowed the cell number to be estimated by the number of cells lost from suspension during the 3-hour inoculation period (during this time no significant increase in cell number is expected). As a control, cells were also inoculated onto tissue culture plastic and left for 3 hours before releasing and measuring the oxygen uptake rate. For this measurement the cell number could accurately be determined by cell counts, hence the uptake rates from both groups could be compared to check that they were in the same order of magnitude. In addition, the OUR of cells cultured on tissue culture plastic for 2, 4 and 7 days was determined in order to assess the variation in oxygen consumption over time in culture. This could not be performed with the cells attached to the microcarriers, due to the difficulty in determining precise cell numbers, as mentioned previously.

For each sample, readings were taken until the drop in dissolved oxygen level was observed to plateau. The slope was taken as the straightest part of the curve. As this straight line starts to level out, the oxygen concentration is considered to be below the 'critical oxygen concentration' where the OUR is reduced (Stanbury et al. 1995; Preissmann et al., 1997), hence this part of the curve was not used to calculate the OUR. The gradient of the slope and that from medium only were determined and the 'medium-only gradient' subtracted from the 'sample gradient' to give the actual drop in dissolved oxygen due to uptake by the cells. Where 0.2125 mmol/L is assumed to be the saturated oxygen concentration in culture media (100% dissolved oxygen), the oxygen consumed (mol/ml/min) could be determined by

multiplying the percentage oxygen consumption per minute (slope) by 0.2125 mmol/L. For a known cell number per ml, the oxygen uptake rate could be determined as mol/cell/min. An example of this calculation is given in Figure 3.1.7.

3.1.8 Stability of Vector Particles

The activity of retrovirus particles is notoriously unstable. The loss of activity, that is, the loss of ability to infect target cells, has been shown to be temperature dependent (Lee et. al. 1996; Kaptein et. al. 1997). The typical half-life of retrovirus particles at 37°C is approximately 8 hours, hence virus-containing supernatants in cultures of packaging cells must be harvested frequently in order to obtain the maximum yield. This leads to low titres of the harvested stocks. The half-life of retrovirus particles released by the FLYRD18/LNC-hB7 cell line was measured at various temperatures in order to (1) determine the optimal harvest time for 37°C cultures and (2) determine whether alternative culture temperatures or harvest techniques (e.g. harvest onto ice) could improve active retrovirus titres.

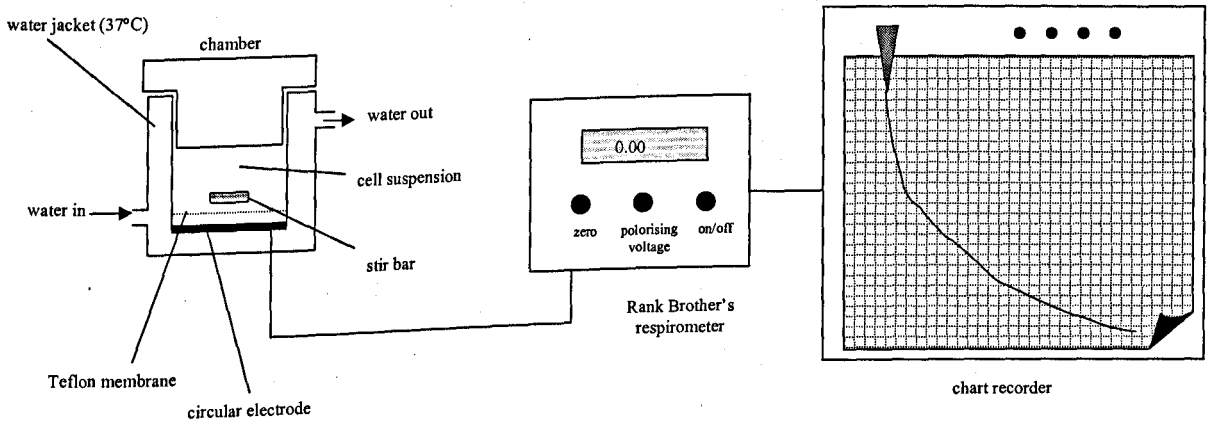
Due to the instability of retroviral particles, long-term storage is usually carried out in liquid nitrogen or freezers at -80°C. The effects of freezing and subsequent thawing of retroviral vector stocks are likely to be detrimental to their activity. The activity loss with repeat freeze-thaw cycles was assessed in this section in order to determine whether stocks could be repeatedly frozen and thawed for research purposes.

3.1.8.1 Vector Half-life at Various Temperatures

A stock of retrovirus-containing supernatant, harvested from a monolayer culture of FLYRD18/LNC-hB7 cells (as described in Chapter 2), was passed through a 0.45μm filter and divided into 1ml samples. Replicate samples were stored at 37°C, 32°C or on ice for up to 72 hours. At various time points samples were removed from their respective storage conditions and frozen in liquid nitrogen. Control samples were placed in liquid nitrogen immediately after removal from culture and filtering. After all samples had been stored in liquid nitrogen for at least 24 h the active virus titre was measured simultaneously using the infection assay previously described in Chapter 2.

3.1.8.2 Effect of Freeze-Thaw Cycles on Vector Stability

A stock of virus-containing supernatant was collected from a monolayer culture of FLYRD18/LNC-hB7 cells (established as described in Chapter 2). This supernatant was passed through a 0.45μm filter and divided into separate cryovials. One set of vials were placed in liquid nitrogen and kept under these conditions until analysed. The other set was frozen in liquid nitrogen overnight, then fully thawed before returning to liquid nitrogen 30 minutes later. The next day both sets were thawed and the vector titre measured by an infection assay (described in Chapter 2).



Example calculation

For 5×10^5 cells/ml, and a decrease in dissolved oxygen from 80% to 40% over 56 minutes:

Gradient = (0.80-0.40) / 56 = 7.143×10^{-3} per min

Where gradient of medium only (control) is 1.5×10^{-3}, the gradient due to cellular oxygen consumption is:

$7.143 \times 10^{-3} - 1.5 \times 10^{-3} = 5.643 \times 10^{-3}$ per min

Assuming the saturated oxygen concentration of medium to be 0.2125 mmol/L, oxygen consumption rate is:

$(5.643 \times 10^{-3}) \times 0.2125 = 1.199 \times 10^{-3}$ mmol/min/L $\equiv 1.199 \times 10^{-9}$ mol/min/ml

Divide by cell concentration to give OUR:

1.199×10^{-9} mol/min/ml $\div 5 \times 10^5$ cells/ml = $\underline{2.398 \times 10^{-15}}$ mol/cell/min

Figure 3.1.7

Schematic of respirometer used for measuring oxygen consumption of cells (not to scale) and an example of the calculation for determining OUR.

3.1.9 Reproducibility of Infection Assay

The concentration of active vector particles in the culture supernatant of FLYRD18/LNC-hB7 cell cultures is determined indirectly by the infection of target cells (as described in Chapter 2). The titre measured in such an infection assay is dependent upon a number of parameters and is prone to significant variation (as discussed in Chapter 1). The actual extent of this variation should be assessed before comparisons are made between the titres in various studies. Variation in the titre of a single batch of vector supernatant was measured over several months, in order to assess both the inter-assay variation in titre and the effect of long-term storage in liquid nitrogen.

A monolayer culture of FLYRD18/LNC-hB7 cells was established as described in Chapter 2. Once the cell monolayer reached confluence, the medium was replaced with fresh (50ml) and the cells incubated for a further 24h. The culture supernatant was then removed, passed through a 0.45μm filter and divided into fifty cryovials (1ml per vial) which were stored in liquid nitrogen. In each subsequent infection assay that was performed, one of these vials was thawed and included in the set of samples for vector titre measurement. The results from this single batch of supernatant allowed comparisons between assays.

3.1.10 Determination of Transduction Efficiency of Vector Preparations

A number of groups have reported a lack of correlation between the end point titre and transduction efficiency of retroviral vector preparations (Le Doux et al., 1999; Forestell et al., 1995). These findings are reviewed in Chapter 1. The typical transduction efficiency of retroviral vectors released by the FLYRD18/LNC-hB7 cell line during monolayer culture was therefore determined. Such data can be used to assess the effects of different culture

conditions (e.g. in packed bed culture) on this parameter (in addition to end point titre measurements).

The supernatant from monolayer cultures of FLYRD18/LNC-hB7 cells was collected and stored in liquid nitrogen before determination of the transduction efficiency (hereafter called 'stored' samples). Alternatively, samples of supernatant were taken and measured immediately, without freezing (hereafter called 'fresh' samples). As described in Chapter 2, the transduction efficiency of vector preparations was determined by measuring the percentage of successfully transduced target cells, detected by labelling with an FITC-conjugated anti-human B7 antibody. Target cells were exposed to 'fresh' or 'stored' vector preparations from monolayer cultures for 8 hours and cultured for a further 40 hours before labelling with antibody (method described in detail in section 2.4). The end point titre of the stored sample was also determined by an infection assay (described in section 2.3).

3.2 Results

3.2.1 Batch Culture of FLYRD18/LNC-hB7 Cells

Cell numbers increased in monolayer batch culture until day 7, after which a gradual decrease in cell number was observed. The percentage viability remained at 100% for the first 2 days and then decreased daily, dropping to 69% by day 10. The growth curve of this typical batch culture is illustrated in Figure 3.2.1.1.

The glucose consumption of FLYRD18/LNC-hB7 cells in batch culture was surprisingly low. The glucose concentration in the spent medium only dropped from 14 mmol/L to 10 mmol/L over 10.5 days. Significant lactate production was only evident for the first 2 days of culture. The mean daily glucose and lactate concentrations in the supernatant are shown in Figure 3.2.1.2.

Cell cycle analysis revealed a change in distribution of cells in G_1 (Gap1), S (Synthetic) and G_2 (Gap2) phases of the cell cycle with time in batch culture (Figure 3.2.1.3). That is, the percentage of cells in the S-phase decreased with time and accumulation in the G_1-phase was observed. This is typical for cell growth arrest in batch culture, due to serum and nutrient deprivation (Schliermann et al., 1987; Ray et al., 1989; Adams, 1990; Al-Rubeai et al., 1991; Lloyd and Al-Rubeai, 1999; Holmes and Al-Rubeai, 2000).

Mean daily vector titres, like the cell numbers, increased until day 7 and then decreased (Figure 3.2.1.4). This increase in vector titre from day 1 through to day 7 was greater than the increase in cell number between these two time points. The mean vector titre *per cell*

was calculated by dividing the daily vector titre by the corresponding cell number. These values increased from 8.6×10^{-3} cfu/cell on day 2 to 2.1×10^{-2} cfu/cell on day 7.

3.2.2 Effect of 'Conditioned' Medium on Vector Production

Exposure of FLYRD18/LNC-hB7 cell cultures to 'conditioned' (spent) medium for 24h caused a significant decrease in vector titre, compared to cultures in fresh medium. Medium that was conditioned for longer periods (three or more days) had a greater negative effect on vector titre than medium conditioned for 1 or 2 days. Figure 3.2.2. illustrates the decrease in vector titre with increasing 'age' of the spent medium.

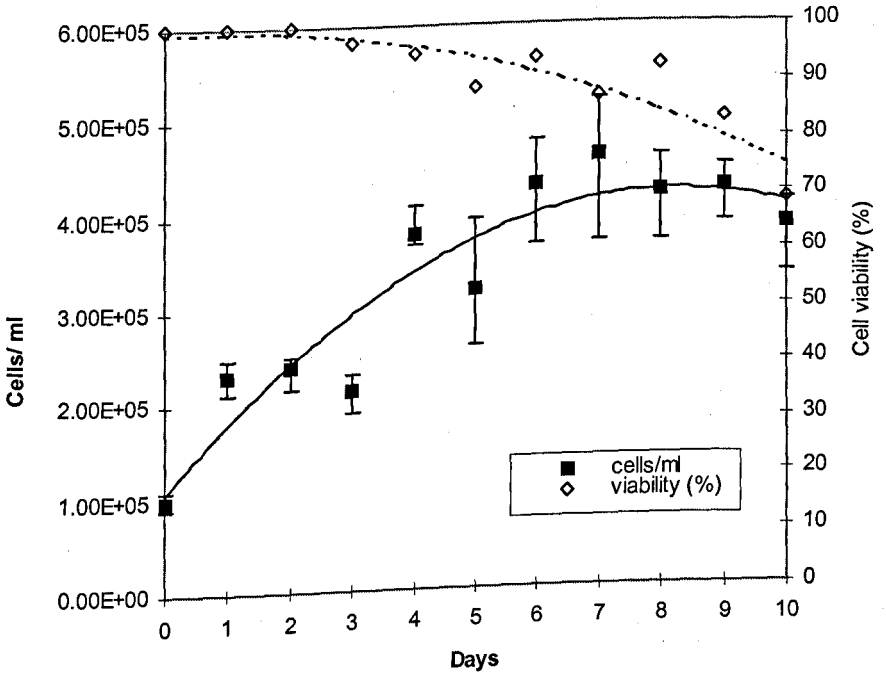


Figure 3.2.1.1

Mean cell number per ml and percentage viability during a 10-day batch culture of FLYRD18/LNC-hB7 cells in monolayer. Error bars represent observed range, n=2.

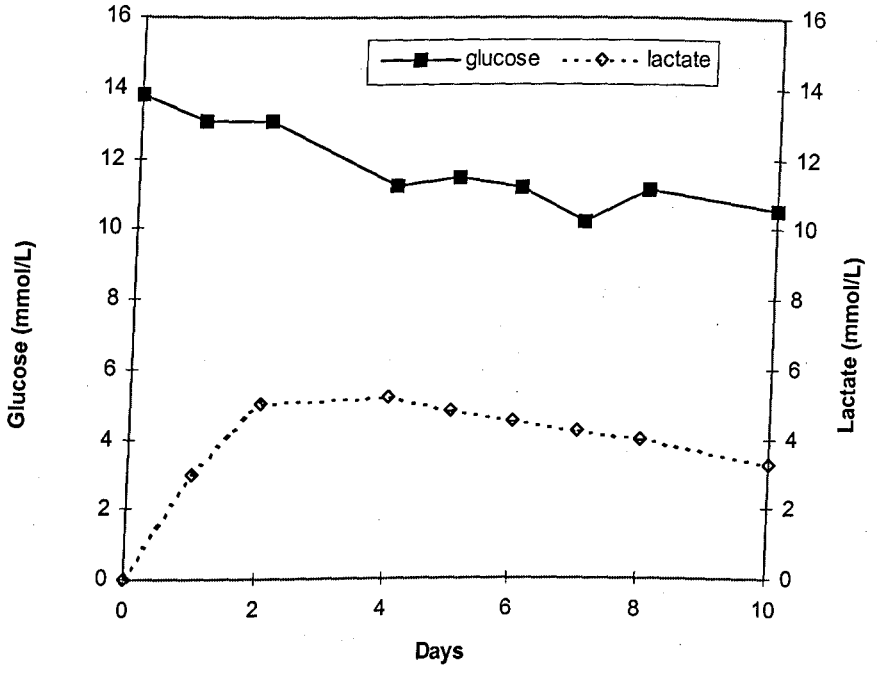


Figure 3.2.1.2

Glucose consumption and lactate production during a 10-day batch culture of FLYRD18/LNC-hB7 cells in monolayer.

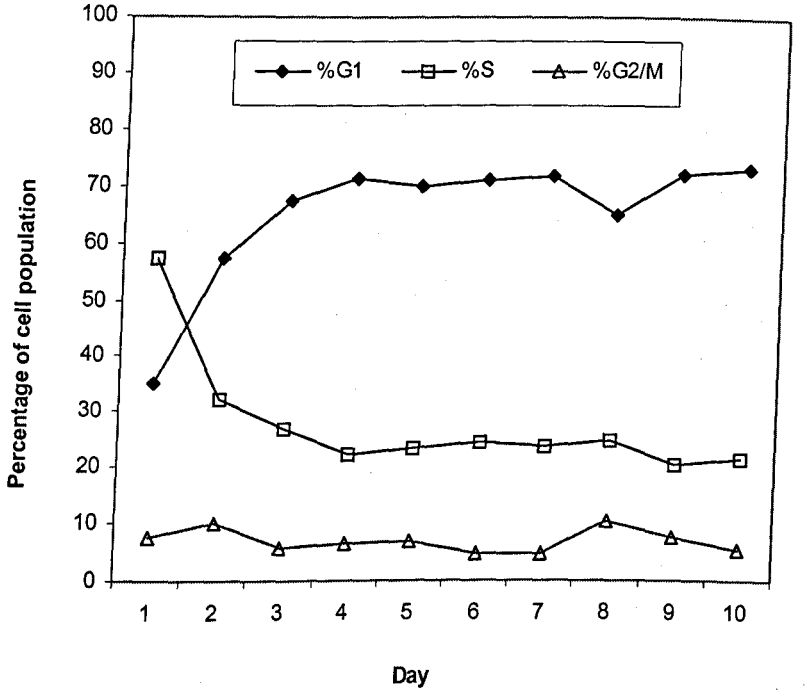


Figure 3.2.1.3

Cell cycle distribution of FLYRD18/LNC-hB7 cells over 10-day batch culture.

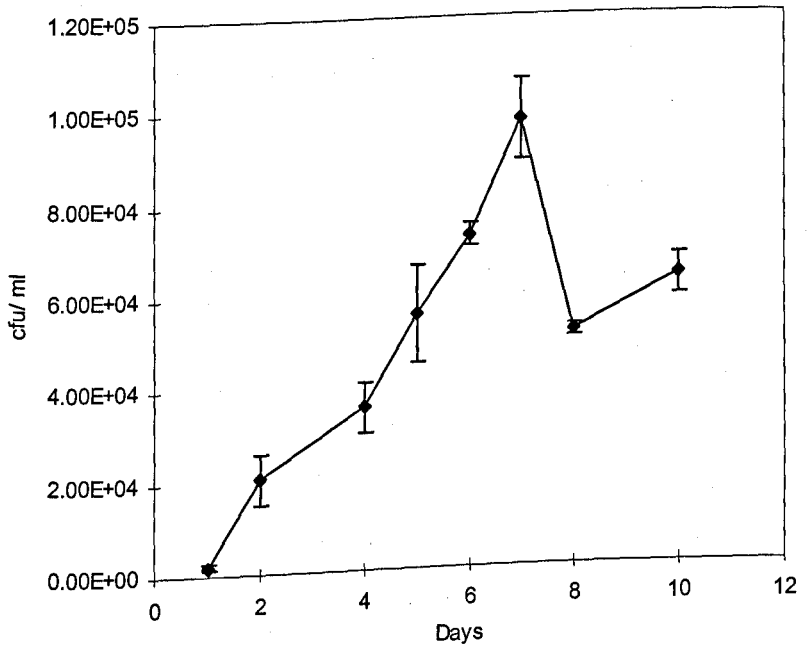


Figure 3.2.1.4

Mean daily vector titres during 10-day batch cultures of FLYRD18/LNC-hB7 cells in monolayer. Error bars represent observed range, n=2.

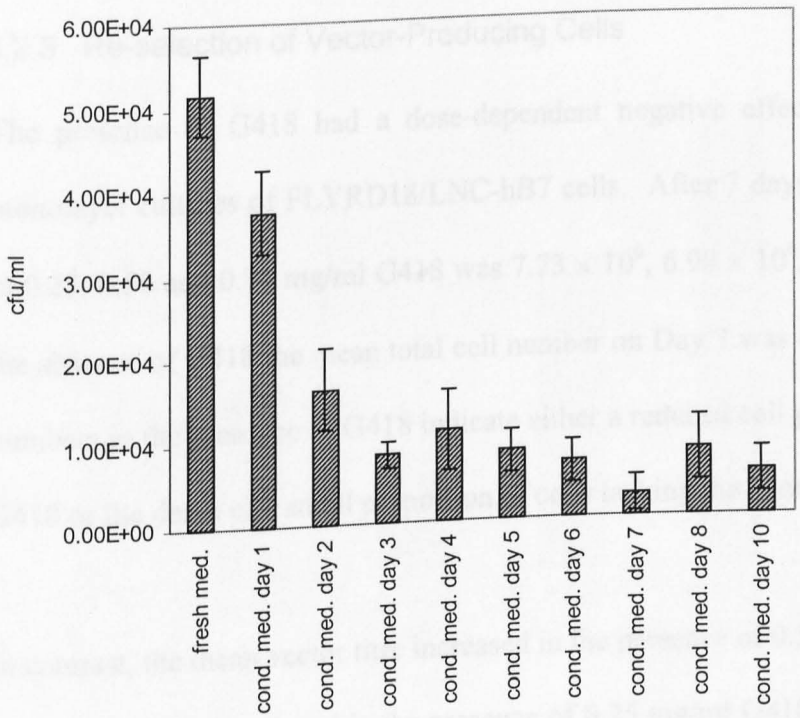


Figure 3.2.2

Mean vector titre in FLYRD18/LNC-hB7 cell cultures after 24h exposure to medium conditioned by FLYRD18 parent cells for 1 to 10 days or fresh medium. Error bars represent standard deviation, n=6.

3.2.3 Re-selection of Vector-Producing Cells

The presence of G418 had a dose-dependent negative effect on the total cell yield in monolayer cultures of FLYRD18/LNC-hB7 cells. After 7 days, the mean total cell number in 0.25, 0.50 and 0.75 mg/ml G418 was 7.73×10^6, 6.90×10^6, 6.37×10^6, respectively. In the absence of G418 the mean total cell number on Day 7 was 8.16×10^6. The reduced cell numbers in the presence of G418 indicate either a reduced cell growth rate in the presence of G418 or the death of a small proportion of cells lacking the neomycin resistance gene.

In contrast, the mean vector titre increased in the presence of 0.50 and 0.75 mg/ml G418 (but titres were slightly reduced in the presence of 0.25 mg/ml G418). The mean vector titre per cell was calculated by dividing each vector titre by the corresponding cell numbers at each concentration of G418. The vector titre per cell in increasing concentrations of G418, compared to that measured in the absence of G418, is plotted in Figure 3.2.3. In the higher concentrations of G418, the cell specific vector titre (cfu/cell) was significantly increased. This data suggests that the reduced cell number in the presence of G418 is likely to be a result of the death of cells without the neomycin resistance gene, rather than reduced cell growth rates (since vector productivity is increased). The cultures in 0.50 and 0.75 mg/ml G418 probably show a greater vector titre per cell due to the selection of cells containing the vector.

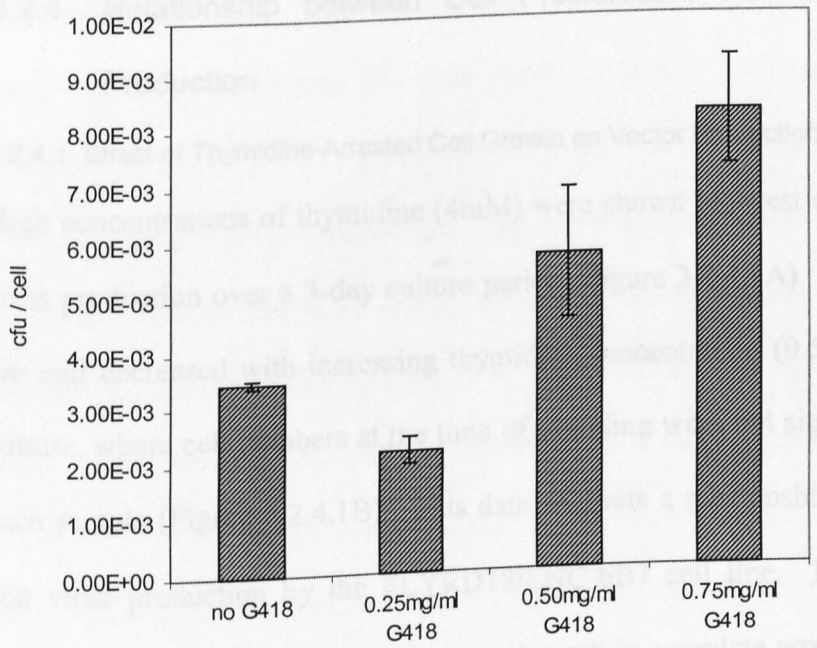


Figure 3.2.3

Cell specific vector titres, produced over 24h, after 7 days culture of FLYRD18/LNC-hB7 cells in G418, compared to cultures without G418. Error bars represent observed range, n=2.

3.2.4 Relationship between Cell Proliferation, Cell Cycle Phase and Vector Production

3.2.4.1 Effect of Thymidine-Arrested Cell Growth on Vector Production

High concentrations of thymidine (4mM) were shown to arrest cell proliferation and reduce virus production over a 3-day culture period (Figure 3.2.4.1A). Furthermore, the virus titre *per cell* decreased with increasing thymidine concentration (0.5 - 4 mM) during overnight culture, where cell numbers at the time of sampling were not significantly different between each sample (Figure 3.2.4.1B). This data suggests a relationship between cell proliferation and virus production by the FLYRD18/LNC-hB7 cell line. It should be noted that the concentrations of thymidine used may not result in complete arrest of the cells as it has been reported that between 5 and 10mM may be required to completely arrest the growth of some cell types (Adams, 1990). Hence some virus production is still observed in the thymidine-arrested cultures, but at significantly reduced levels.

Flow cytometric analysis of each culture showed an accompanying decrease in percentage of cells in the S-phase, (Figure 3.2.4.1C). This is expected, as high concentrations of thymidine inhibit DNA synthesis and hence entry into the S-phase, thereby arresting cells at the G_1/S boundary (Fantes and Brooks, 1993). The relationship between cell cycle phase and vector production was therefore investigated in order to determine whether the decreased titres in the presence of thymidine were due to the altered cell cycle distribution, or reduced cell proliferation rates.

3.2.4.2 Relationship between Cell Cycle Phase and Vector Production

To ensure that virus titres could be measured after a few hours culture, using the cell concentrations employed in this study, a set of preliminary cultures were set up and the

supernatant sampled 2, 4, 6 and 8h after inoculation of cells in the plates. In addition, a sample of supernatant from the same batch of freshly suspended cells was taken prior to inoculation, to use as a 0 h sample (at the same cell concentration as those inoculated onto plates). Figure 3.2.4.2A shows that titres were indeed measurable after just 2 h in culture (titres increased from 2 to 8 h post-inoculation). Since no virus was found in the sample not inoculated onto plates (0 h sample), this confirmed that the values for the 2-8h time points were due to new virus production in these cultures and not residual virus from the previous passage. In addition, the cell cycle distribution before and after culture for 2, 4, 6 and 8 hours was assessed by flow cytometry. Figure 3.2.4.2B shows that the cell cycle distribution did not change significantly for the first eight hours after inoculation. During the first four hours, a small increase in the percentage of S-phase and a small decrease in the percentage of G_1-phase cells was observed, i.e. the cells did not arrest and accumulate in G_1 for the first 6 hours of culture, as observed by Andreadis et al., (1997). Here we show no such perturbation of cell cycle progression in the FLYRD18/LNC-hB7 cell line as a result of cell detachment. This data showed that culture supernatants could be taken for virus measurements after 2 hours culture.

Centrifugal elutriation was used to separate cells in different phases of the cell cycle. Figures 3.2.4.2C and 3.2.4.2D illustrate the cell diameter and cell cycle distribution, respectively, in sixteen fractions collected from the centrifugal elutriator with increasing pump speeds. As expected, the mean size of eluted cells increased with increasing pump speed. Cell cycle analysis confirmed that cells predominantly in G_1 were eluted at the lower pump speeds, while mainly cells in G_2/M were eluted at the higher pump speeds. Cells in the S-phase were eluted in between these two ranges. From this data the optimal pump

speeds for eluting cell fractions enriched for G_1, S and G_2/M were determined (namely 2-3, 3.5-4.5 and 5.5-6.5, respectively). After collecting cells at these three pump speeds, the cell cycle distribution was again determined by flow cytometry. The cells from each fraction were then cultured for 2h at identical concentrations, as previously described, before collecting the supernatant and releasing the cells to once again determine the cell cycle distribution. The cell cycle distributions for each of these three fractions (before and after 2 hours' culture) are given in Table 3.2.4.2. Figure 3.2.4.2E shows no significant difference in the vector titre taken from cell cycle enriched cultures two hours after inoculation. This data suggests that there is in fact no correlation between cell cycle *phase* and virus production. If, for example, virus particles were produced predominantly in the S-phase (and virus production was negligible in the G_1-phase), a 2-fold increase in virus titre would be expected in the culture from Fraction 2 (43% cells in S-phase), compared to Fraction 1 (22% cells in S-phase).

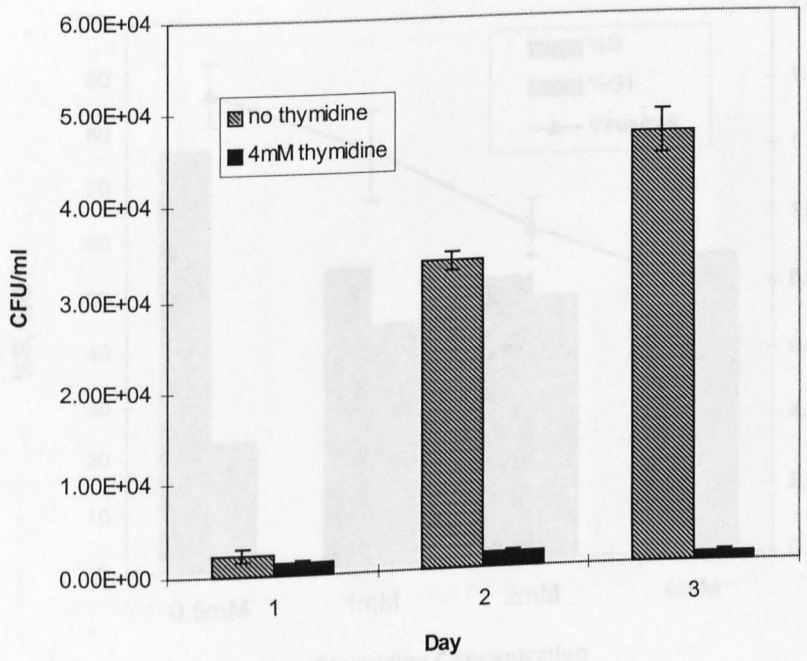


Figure 3.2.4.1A

Daily vector titres measured in growing and thymidine-arrested FLYRD18/LNC-hB7 cultures. Medium was replaced every day. Error bars represent observed range, n=2.

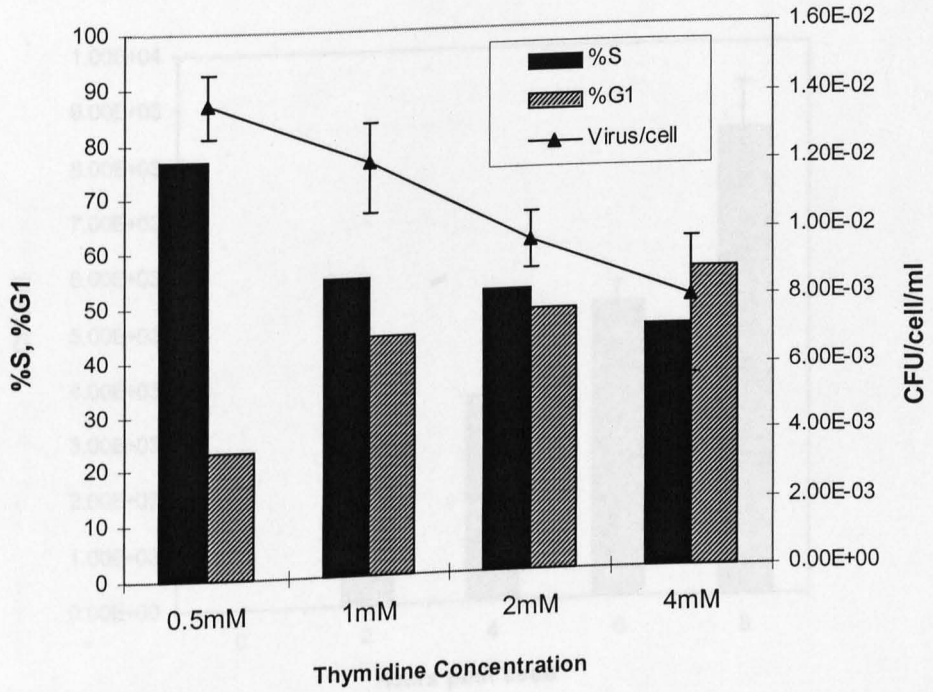


Figure 3.2.4.1B

Vector titres *per cell* and cell cycle distribution after 24h incubation of FLYRD18/LNC-hB7 cells in increasing thymidine concentrations. Error bars represent observed range, n=3.

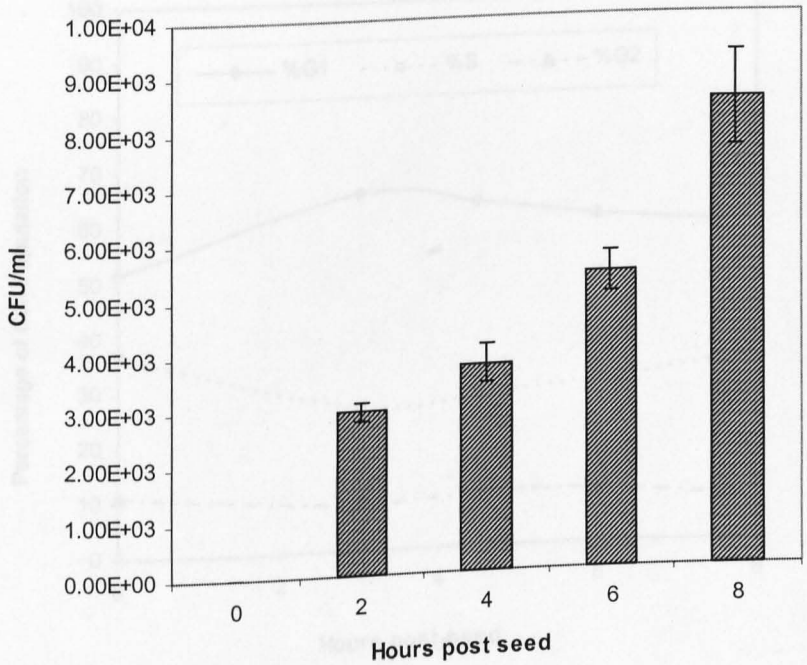


Figure 3.2.4.2A

Vector titre produced by FLYRD18/LNC-hB7 cells 0 - 8h post-seed. Vector levels are low, but detectable, after just 2h. These levels are not due to residual vector from previous passage, as no vectors were detectable at 0h. Error bars represent observed range, n=2.

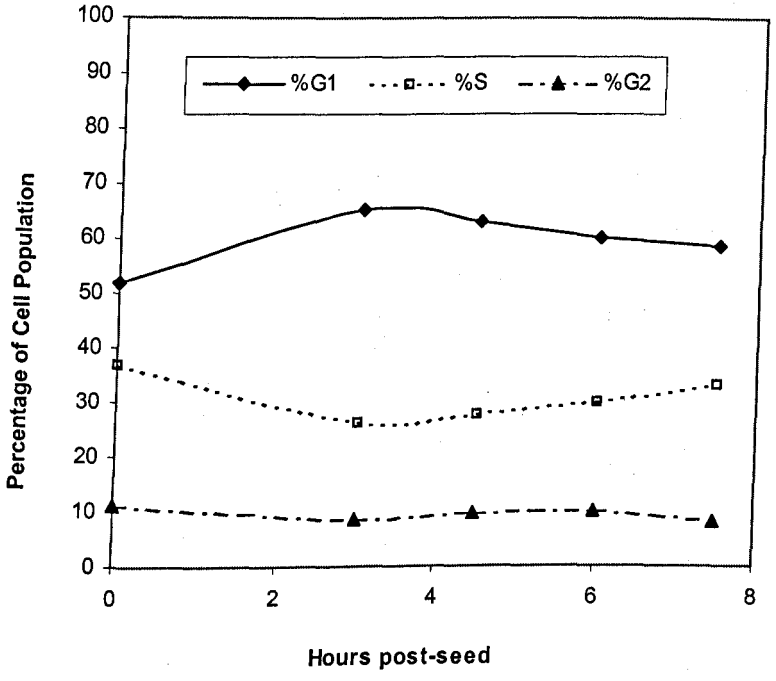


Figure 3.2.4.2B

Cell cycle distribution of FLYRD18/LNC-hB7 cells 0 - 8h post-seed. Cell cycle progression does *not* appear to be inhibited during the hours immediately after seeding.

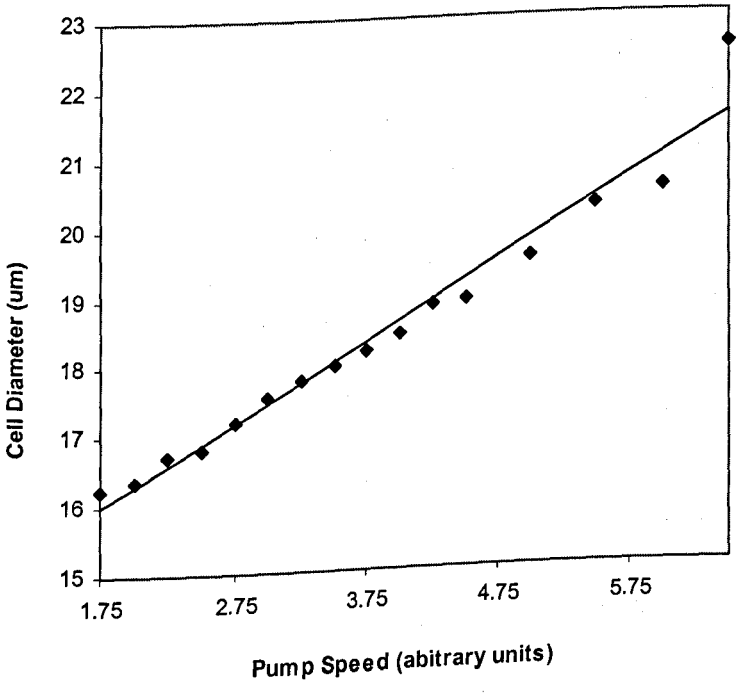


Figure 3.2.4.2C

Mean cell diameter of sixteen fractions (FLYRD18/LNC-hB7 cells) collected from centrifugal elutriator, at increasing pump speeds. Cell size measured using Coulter Multisizer II.

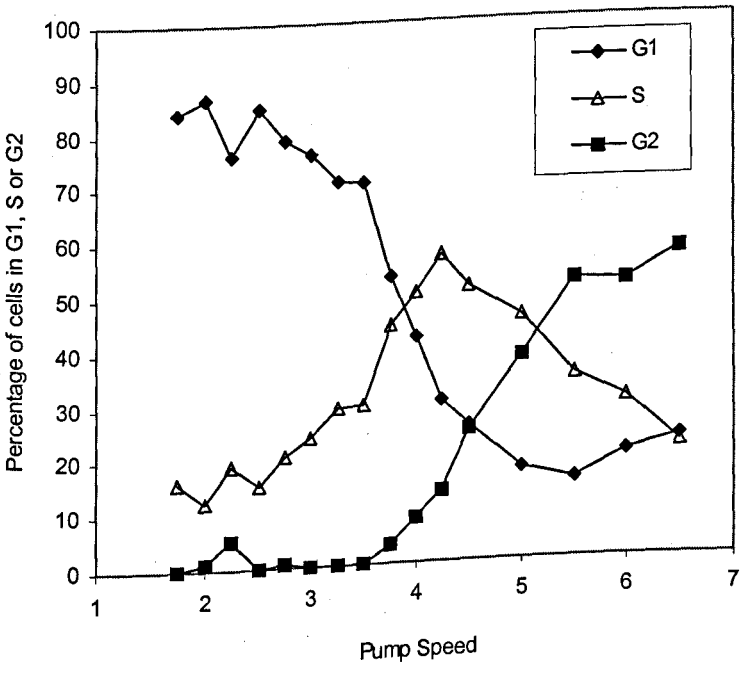


Figure 3.2.4.2D

Separation of FLYRD18/LNC-hB7 cells according to their position in the cell cycle: Cell cycle distribution of sixteen cell fractions collected from centrifugal elutriator, at increasing pump speeds.

	Before 2h culture:			After 2h culture:		
Fraction Number	**% G_1**	**% S**	**% G_2/M**	**% G_1**	**% S**	**%G_2/M**
1	**75**	21.8	3.3	**81.2**	17.9	0.9
2	44.2	**43.2**	13.6	35.9	**38.1**	26
3	46	22.5	**31.4**	18.3	20.8	**60.9**

Table 3.2.4.2

Cell-cycle distribution of cell fractions collected from centrifugal elutriator (measured by flow cytometry), before and after 2-h culture period. Fractions 1, 2 and 3 were enriched for cells in G_1, S and G_2/M respectively. Bold figures indicate percentage of cells in phase for which fraction was enriched.

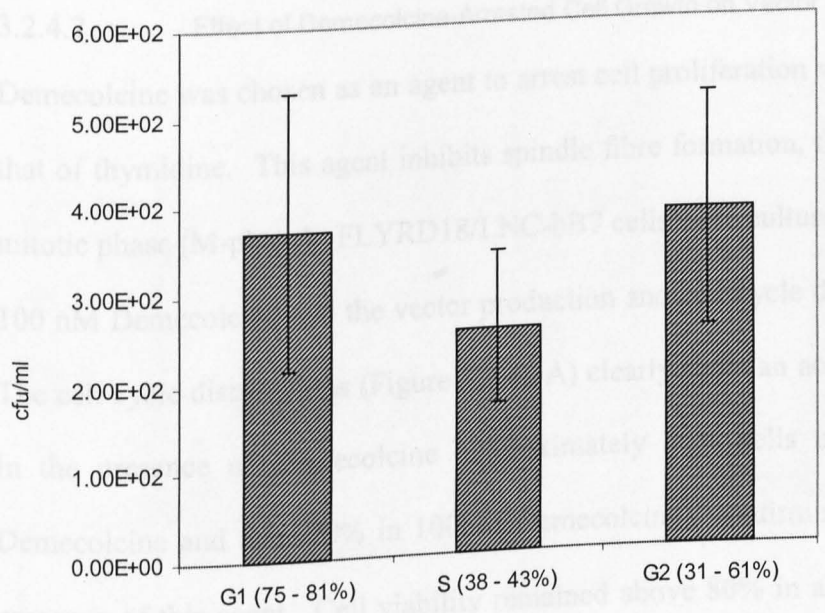


Figure 3.2.4.2E

Effect of cell cycle phase on vector production: Vector titres (2h post seed) from three cell fractions enriched for cells in G_1, S or G_2 phase of the cell cycle (separated by centrifugal elutriation). Error bars represent standard deviation, n=6.

3.2.4.2 Effect of Demecolcine-Arrested Cell Growth on Vector Production

Demecolcine was chosen as an agent to arrest cell proliferation via a different mechanism to that of thymidine. This agent inhibits spindle fibre formation, thereby arresting cells in the mitotic phase (M-phase). FLYRD18/LNC-hB7 cells were cultured overnight with or without 100 nM Demecolcine and the vector production and cell cycle distributions were compared. The cell cycle distributions (Figure 3.2.4.3A) clearly show an accumulation of cells in G_2/M in the presence of Demecolcine (approximately 30% cells in G_2/M in the absence of Demecolcine and over 70% in 100nM Demecolcine), confirming the arrest of cells in the presence of this agent. Cell viability remained above 80% in all cultures. Figure 3.2.4.3B shows a decrease in daily virus titres when the cells are arrested in Demecolcine. Virus titres were also calculated *per cell*, as cell numbers were lower in the Demecolcine cultures due to cell growth arrest (5×10^5 cells with Demecolcine, 28×10^5 cells without). The cell specific titres were significantly reduced in the presence of Demecolcine (Figure 3.2.4.3C), further suggesting a correlation between cell proliferation and virus production. It should be noted that flow cytometry cannot distinguish between cells in G_2 and cells in M, as both possess duplicate amounts of DNA. Therefore, not *all* the cells in G_2/M peak will be arrested after Demecolcine addition, only those which have reached metaphase. Furthermore, cells in M that have reached anaphase or beyond, at the time of Demecolcine addition, will continue to pass through the cell cycle. In addition, those cells in G_1 and S (approximately 30%) are not immediately arrested by Demecolcine addition. For all these reasons, a significant amount of vector per cell was measured in the Demecolcine-arrested cultures. However, the titre per cell is significantly reduced compared to the non-arrested cultures, indicating little or no vector production by the non-cycling cells.

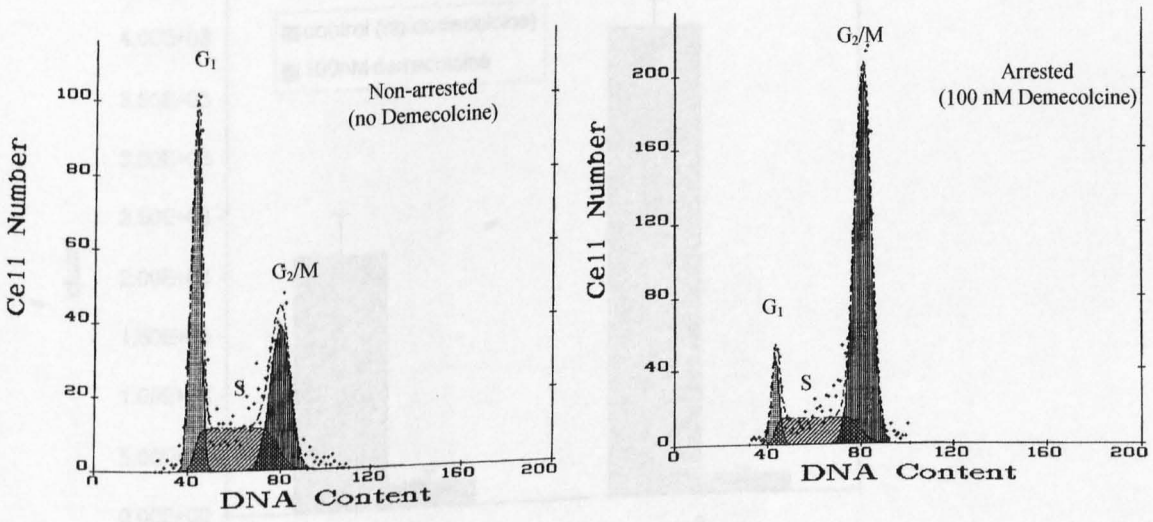


Figure 3.2.4.3A

Cell cycle distribution of non-arrested and Demecolcine-arrested FLYRD18/LNC-hB7 cells (determined using flow cytometry).

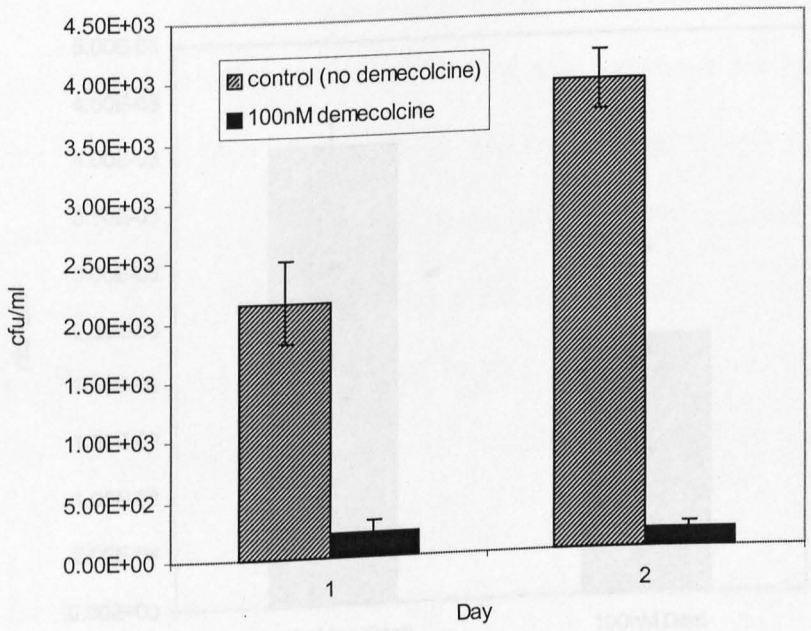


Figure 3.2.4.3B

Daily virus titre in FLYRD18/LNC-hB7 cell cultures with and without 100nM Demecolcine. Error bars represent observed range, n=2.

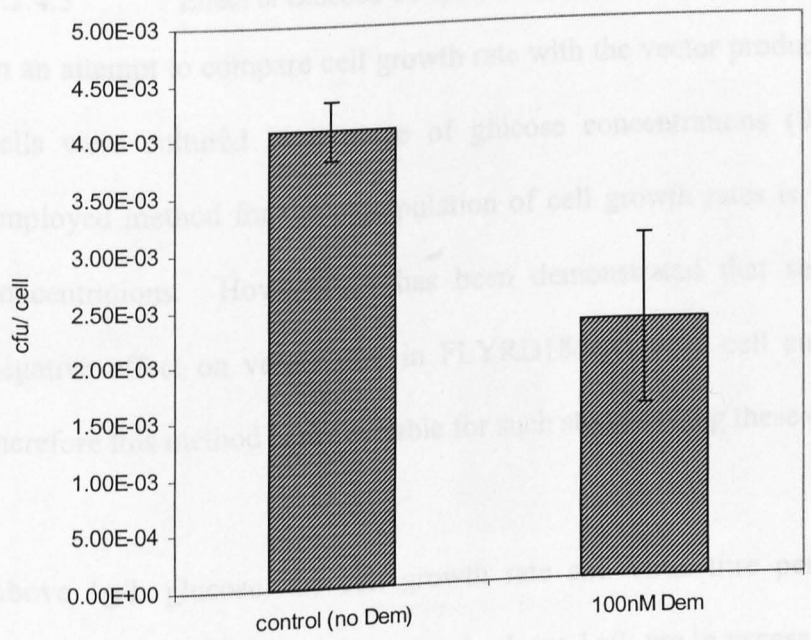


Figure 3.2.4.3C

Cell specific virus titres (day 2) with and without 100nM Demecolcine. Error bars represent observed range, n=2.

3.2.4.3 Effect of Glucose Concentration on Cell growth and Vector Production

In an attempt to compare cell growth rate with the vector productivity, FLYRD18/LNC-hB7 cells were cultured in a range of glucose concentrations (0.5-3.0 g/L). A frequently employed method for the manipulation of cell growth rates is the use of increasing serum concentrations. However, it has been demonstrated that serum has a dose-dependent negative effect on vector titre in FLYRD18/LNC-hB7 cell cultures (Gerin et al. 1999a), therefore this method is not suitable for such studies using these cells.

Above 1g/L glucose, the cell growth rate and virus titre per cell was not significantly different, indicating that glucose levels above 1g/L are in excess of the requirements of these cells at the densities used. From 0 to 1 g/L glucose the cell numbers increased and were accompanied by a significant increase in vector titre *per cell* (Figure 3.2.4.4). Hence, increased glucose concentrations increased both the cell growth rate (μ) and virus productivity, within this range.

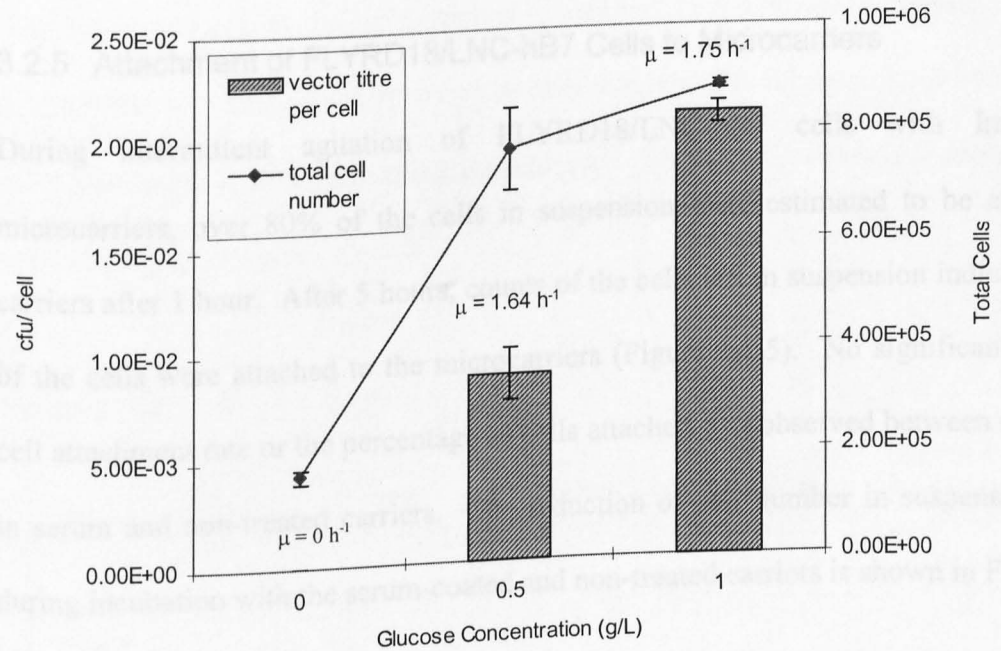


Figure 3.2.4.4

Cell number and virus titre *per cell* after 3 days culture in medium containing 0, 0.5 or 1g/L glucose. Error bars represent standard deviation, n=4. Specific cell growth rates (μ) are given for each glucose concentration.

3.2.5 Attachment of FLYRD18/LNC-hB7 Cells to Microcarriers

During intermittent agitation of FLYRD18/LNC-hB7 cells with ImmobaSil-FS™ microcarriers, over 80% of the cells in suspension were estimated to be attached to the carriers after 1 hour. After 5 hours, counts of the cells left in suspension indicated over 95% of the cells were attached to the microcarriers (Figure 3.2.5). No significant difference in cell attachment rate or the percentage of cells attached was observed between carriers coated in serum and non-treated carriers. The reduction of cell number in suspension with time, during incubation with the serum-coated and non-treated carriers is shown in Figure 3.2.5.

An MTT assay was conducted on a sample of carriers from each flask, 5 hours post-seed. This data was used to estimate the total number of cells attached to the carriers (to confirm that the reduced cell number in suspension was due to attachment of the cells to the carriers). It should be noted, as discussed in Chapter 2, that the conversion of MTT to the coloured formazan product is dependent on the mitochondrial content of the cells (and hence the cellular metabolic activity). For this reason, the use of the calibration curve to convert absorbance (concentration of formazan) to cell number may not always provide accurate cell numbers. For instance, the metabolic activity of cells recently attached to microcarriers could be different to that of the cells used to construct the calibration curve. Hence the data given by this assay can only be used to confirm that the majority of cells lost from suspension had attached to the carriers and *not* as a measure of absolute cell number on the carriers.

The approximate total cell number attached to the non-treated and serum-coated carriers was estimated to be 24.75×10^6 and 27.30×10^6 respectively, using the MTT assay. The

calculation of these figures, as described in section 2.6, is outlined in Table 3.2.5. Since 95% of the cells were lost from suspension in both samples after 5 hours, the estimated number of cells attached to the carriers, using cell counts, is 28.5×10^6, which correlates well with the MTT data.

Table 3.2.5

Sample	Abs (570nm) 5h post-seed	Abs per carrier	Conversion to cell number (using calibration curve, Figure 2.6)	× 6620 carriers: total number of attached cells
Non-treated (27 carriers)	0.07	2.59×10^{-3}	3.7×10^3	24.75×10^6
Serum-treated (40 carriers)	0.198	4.95×10^{-3}	4.1×10^3	27.30×10^6

3.2.6 Effect of Agitation of FLYRD18/LNC-hB7 Cell Cultures

After overnight stirring of ImmobaSil-FS™ carriers seeded with FLYRD18/LNC-hB7 cells at 42rpm, an MTT assay failed to reveal the presence of any viable cells attached to the carriers. Likewise, no cells (viable or dead) were detected in the supernatant after overnight stirring of the cultures.

A study on the effects of agitation on the survival of FLYRD18/LNC-hB7 cells attached to ImmobaSil-FS™ carriers revealed a loss of cell viability on the carriers with increasing stir speeds. After 1 hour at 11 rpm no cells were released into the supernatant (as determined by cells counts of the supernatant every 15 minutes). Neither did the conversion of MTT to formazan appear to be affected, indicating the presence of viable cells still attached to the

carriers. At 11 rpm the carriers were not completely suspended, but the medium above was mixed. The agitation rate was therefore increased to 24 rpm. After 1.5 hours at this speed, no cells were released into the supernatant and again the MTT conversion by cells attached to the carriers appeared unaffected. The stir speed was then increased to 38 rpm. Once again no cells were released into the supernatant, even after 3.5 hours at this speed. However after just 1.5 hours at 38 rpm, a lack of MTT conversion was observed on some of the carriers sampled. After 3.5 hours at this speed, only 3 out of the 17 carriers sampled showed any signs of MTT conversion to coloured formazan. The stir speed of 38rpm is similar to that believed to have caused cell death due to agitation in the previous experiment. The agitation was therefore maintained at this level overnight and samples taken the following day, by which time no MTT conversion could be detected in any of the carriers sampled.

The control suspension culture (no carriers) was stirred at the same speeds and for the same periods as the ImmobaSil-FS™ culture. During the progressive increases in agitation rate, the cell viability remained above 90%, however the cell number reduced by approximately 50%. This finding suggests that cells may have been lost from culture due to bursting. After overnight agitation at 38 rpm the cell viability was reduced to only 27%. These results demonstrate the inability of this cell line to survive in stirred suspension culture. Table 3.2.6.1 summarises the above findings.

A comparison of stirred and static cultures of FLYRD18/LNC-hB7 cells attached to ImmobaSil-FS™ carriers revealed a dramatic difference in cell survival. After 24 hours stirring at 42rpm, the inoculated carriers failed to show any conversion of MTT to formazan,

indicating the absence of viable cells on these carriers. Hence, as observed previously, the attached cells were assumed to have died due to the effects of agitation. This culture was therefore discarded. In contrast, the inoculated carriers cultured in *static* conditions showed a good MTT response after the same period of culture, which corresponded to approximately 8×10^3 cells per carrier. This culture was therefore maintained for one week with replacement of the exhausted medium (50%) for fresh every 2 days. Table 3.2.6.2 shows the MTT data from carrier samples during this culture period.

An infection assay of the culture supernatant on day 6 revealed a titre of 8.1×10^3 cfu/ml, demonstrating the ability of the FLYRD18/LNC-hB7 cells to produce retroviral vectors when cultured on ImmobaSil-FS™ carriers.

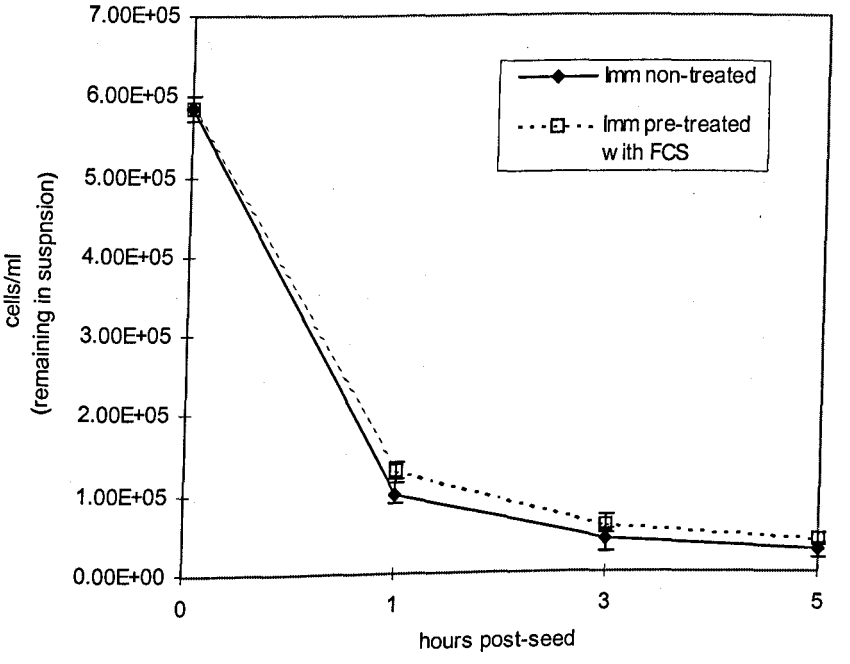


Figure 3.2.5

Attachment of FLYRD18/LNC-hB7 cells to ImmobaSil-FS™ carriers (serum-coated or non-treated) as indicated by a reduction in the number of suspended cells with time. Cells and carriers were incubated at 37°C and mixed intermittently (every 30 minutes). Error bars represent observed range, n=4.

	ImmobaSil-FS™ culture		Suspension culture	
Stir speed/ time	**Cells in supernatant**	**Conversion of MTT to formazan (in carriers)**	**Cell number ($\times 10^5$)**	**Cell viability (%)**
Start of culture	None	yes	0.56	100
11rpm, 0.25h	None	n.d.	0.43	100
11rpm, 0.75h	None	Yes	0.50	100
11rpm, 1h 24rpm, 0.5h	None	Yes	0.36	96.9
11rpm, 1h 24rpm, 1h	None	Yes	0.29	96
11rpm, 1h 24rpm, 1.5h	None	n.d.	0.42	100
11rpm, 1h 24rpm, 1.5h 38rpm, 0.5h	None	Yes	0.30	100
11rpm, 1h 24rpm, 1.5h 38rpm, 1h	None	n.d.	0.38	91
11rpm, 1h 24rpm, 1.5h 38rpm, 1.5h	None	Yes (but not all carriers)	0.40	94.4
11rpm, 1h 24rpm, 1.5h 38rpm, 2.5h	None	Yes (but not all carriers	0.33	90
11rpm, 1h 24rpm, 1.5h 38rpm, 3.5h	None	Only 3 out of 17 carriers	0.26	91
11rpm, 1h 24rpm, 1.5h 38rpm, overnight	None	No	0.24	27

Table 3.2.6.1

Stirred cultures of FLYRD18/LNC-hB7 cells attached to ImmobaSil-FS™ carriers or in suspension. Effect of increasing stir speeds on cell number and viability. (n.d. not done).

Day	Number of carriers in sample	Absorbance (after culture with MTT)	Estimated number of cells per carrier (using calibration curve, Fig 2.6)
1	16	0.123	7.97×10^3
2	13	0.102	8.99×10^3
5	40	0.255	4.84×10^3
7	23	0.205	7.32×10^3

Table 3.2.6.2

Survival of FLYRD18/LNC-hB7 cells (as indicated by an MTT assay of sample carriers) during *static* culture on ImmobaSil-FS™ carriers.

3.2.7 Oxygen Uptake Rate of FLYRD18/LNC-hB7 Cells

The oxygen uptake rate (OUR) of FLYRD18/LNC-hB7 cells in suspension (immediately after detachment from tissue culture plastic) was higher at 3 hours post-seed than at 2 days post-seed, (possibly the result of high oxygen demands during cell attachment). The OUR was observed to increase from day 2 to day 4 when cells were in the exponential phase of growth and decrease by day 7 (as cells reached late exponential/stationary phase). The OUR of cells attached to ImmobaSil-G™ microcarriers 3 hours post-seed was lower than that of the suspended cells (released from tissue culture plastic after the same culture period). Table 3.2.7 shows the OUR of FLYRD18/LNC-hB7 cells measured in each of these conditions.

3.2.8 Stability of Vector Particles

3.2.8.1 Vector Half-life at Various Temperatures

As expected, the decay rate of vector activity decreased with decreasing storage temperature. The active virus titre, for samples stored at 0°C, 32°C and 37°C, was plotted against time (see Figure 3.2.8.1). An exponential curve fit was used to determine the half-life of the active virus particles at each temperature. At 37°C, 32°C and on ice the half-life of the active retrovirus particles was calculated to be 6.9h, 11.0h and 64.3h respectively.

3.2.8.2 Effect of Freeze-Thaw Cycle on Vector Stability

A slight drop in active virus titre was measured in the samples which underwent two freeze-thaw cycles, as compared to the samples from one freeze-thaw cycle, though this difference was not statistically significant (see Figure 3.2.8.2).

Time (post seed)	Oxygen Uptake Rate (mol/cell/min) × 10^{-15}: Suspended	Attached to ImmobaSil-G™ carriers
3h	3.40	1.55
2 days	0.75	n.d.
4 days	5.20	n.d.
7 days	2.00	n.d.

Table 3.2.7

Oxygen uptake rate of FLYRD18/LNC-hB7 cells attached to ImmobaSil-G™ carriers or released into suspension after culture on tissue culture grade plastic.

(n.d., not done).

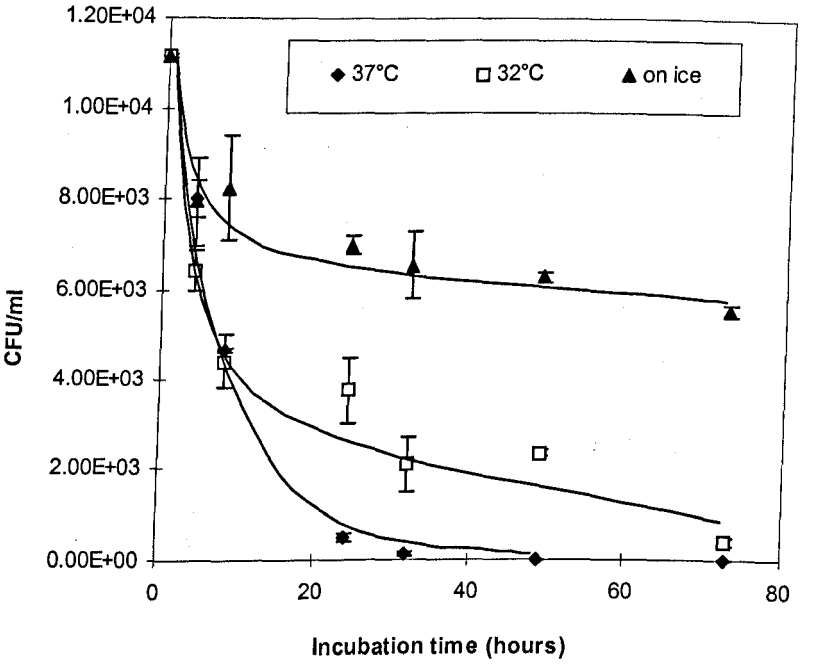


Figure 3.2.8.1

Active vector titre after incubation at 37°C, 32°C or on ice for up to 73 hours. Error bars represent observed range, n=2.

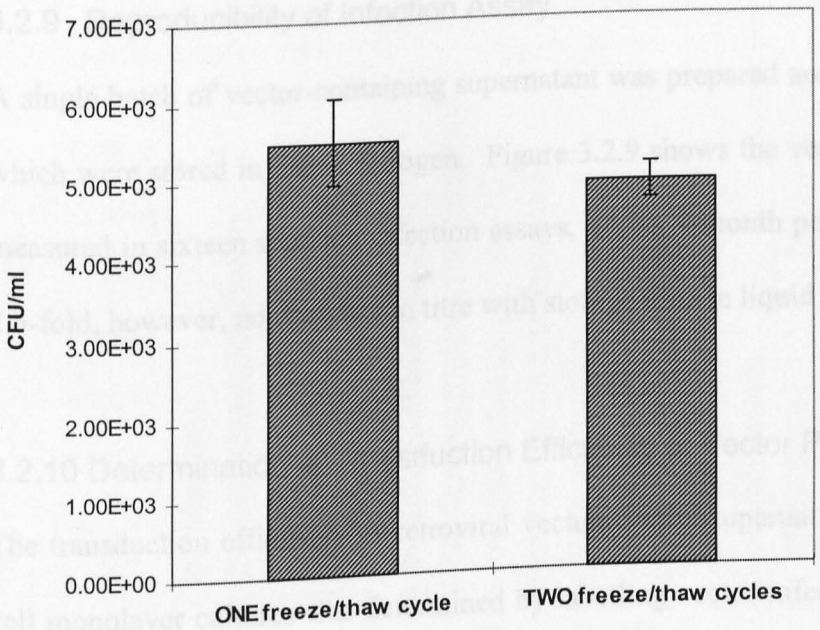


Figure 3.3.8.2

Mean vector titre after one or two successive freeze-thaw cycles. Error bars represent observed range, n=2.

3.2.9 Reproducibility of Infection Assay

A single batch of vector-containing supernatant was prepared and divided into 1ml aliquots, which were stored in liquid nitrogen. Figure 3.2.9 shows the vector titre in this supernatant measured in sixteen separate infection assays, over a 9-month period. The titre varied up to 2.5-fold, however, no decrease in titre with storage time in liquid nitrogen was observed.

3.2.10 Determination of Transduction Efficiency of Vector Particles

The transduction efficiency of retroviral vectors in the supernatant of FLYRD18/LNC-hB7 cell monolayer cultures was determined by labelling vector-infected target cells with FITC-conjugated anti-human B7 antibody. The distribution of fluorescence intensity of these cells, compared to target cells not exposed to the vector preparation, is shown in Figure 3.2.10 (using 'fresh' and 'stored' vector preparations). For both samples, the fluorescence intensity increased in *nearly all* the cells exposed to vectors, compared to control cultures not exposed to vectors. This finding demonstrates the high transduction efficiency of retroviral vectors released by FLYRD18/LNC-hB7 cells cultured in monolayer at 37°C. The end point titre of the 'stored' sample, as measured by an infection assay, was 1×10^5 cfu/ml.

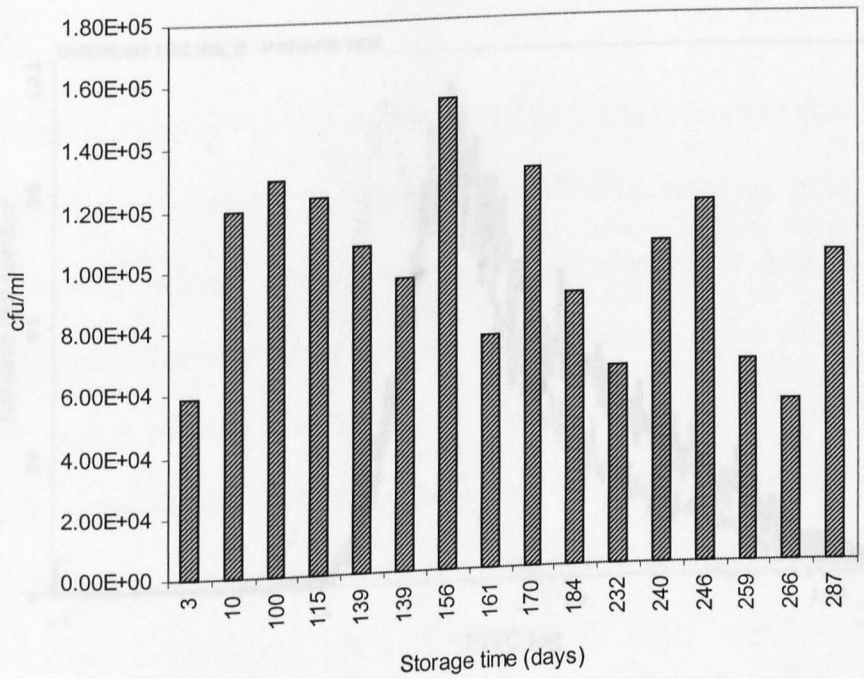


Figure 3.2.9

Vector titre in a single batch of supernatant measured in sixteen *separate* assays. The storage period of each sample, in liquid nitrogen, is indicated on the *x*-axis.

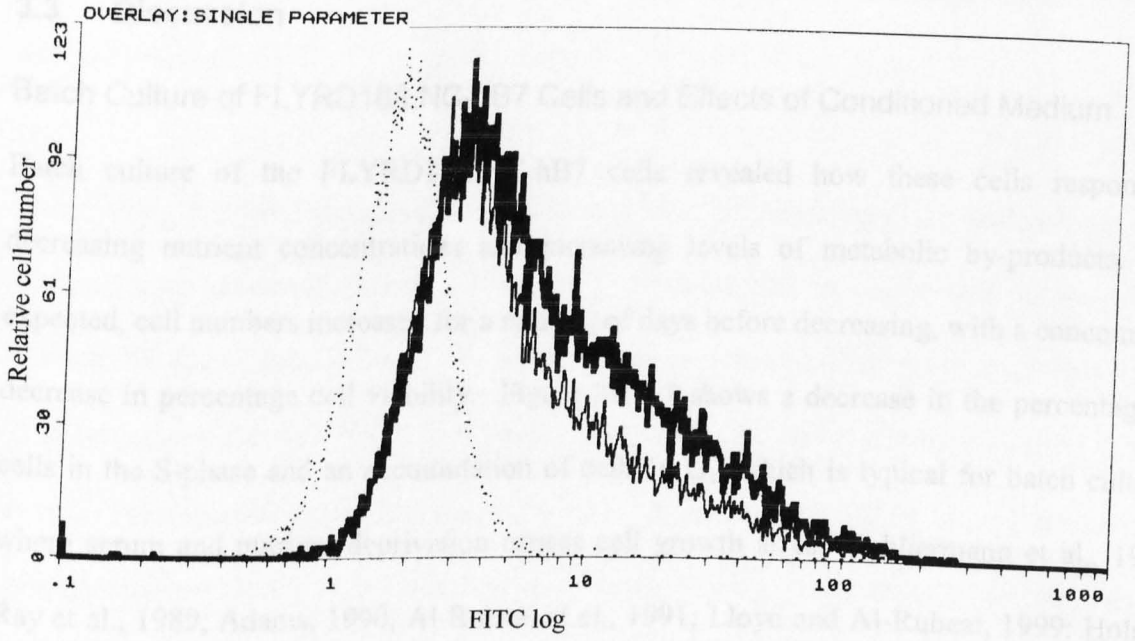


Figure 3.2.10

Distribution of fluorescence intensity of A2780cp cells labelled with FITC-conjugated anti-human B7 antibody (measured by flow cytometry). Comparison between cells exposed to 'fresh' or 'stored' vector preparations for 8 h and then cultured for 48 h (thick solid line and thin solid line respectively), and control cultures not exposed to vector (dotted line).

3.3 Discussion

Batch Culture of FLYRD18/LNC-hB7 Cells and Effects of Conditioned Medium

Batch culture of the FLYRD18/LNC-hB7 cells revealed how these cells respond to decreasing nutrient concentrations and increasing levels of metabolic by-products. As expected, cell numbers increased for a number of days before decreasing, with a concomitant decrease in percentage cell viability. Figure 3.2.1.3 shows a decrease in the percentage of cells in the S-phase and an accumulation of cells in G_1, which is typical for batch cultures where serum and nutrient deprivation causes cell growth arrest (Schliermann et al., 1987; Ray et al., 1989; Adams, 1990; Al-Rubeai et al., 1991; Lloyd and Al-Rubeai, 1999; Holmes and Al-Rubeai, 2000). Surprisingly, this occurred after only 3 days in culture. Glucose levels were not limiting at this time point and lactate concentrations had not reached toxic levels, hence another factor, or factors, may have limited cell growth at this stage. Microscopic observations revealed the establishment of a confluent monolayer by day 2, hence available surface area may be a limiting factor for cell growth in these cultures after this time point. However medium components not measured, such as glutamine concentration, cannot be ruled out as contributory factors in reducing cell growth and survival.

The oxidation of glutamine to CO_2 is an essential source of energy for cells in culture. Zielke et al., (1984) reported that this oxidation of glutamine provides 30-50% of the cells' energy source, in the presence of sufficient glucose. However, as glucose concentrations decrease, the rate of glutamine oxidation increases. Over the 10-day batch culture, increased glutamine consumption rates, along with its decomposition (glutamine also decomposes at

37°C, with a loss of approximately 2-3% per day), may have caused glutamine levels to become limiting.

Vector titres followed the same trend as cell growth. Interestingly, the *cell specific* titres (cfu/cell) also increased from day 2 to day 7. This increase in vector titre per cell is not likely to be due to accumulation of vectors in the supernatant, due to the short half-life of these vector particles at 37°C (determined to be 6.9h, see 3.2.8). It was hypothesised that the increased cell specific vector titre may be due to a change in the composition of the medium, during culture with the FLYRD18/LNC-hB7 cells. During batch culture, factors may be released by the cells themselves, which have a stimulatory effect on vector production. An increase in concentration of these factors during the 10-day culture period could explain the increasing cell specific vector titre from days 2 - 7.

This hypothesis of 'cell-conditioned' medium was tested in a further study where cells were cultured in such medium for 24h. In order to avoid the inclusion of active vector particles in the cell-conditioned medium, the FLYRD18 parent cell line, which only releases 'empty' particles, was used to prepare the cell-conditioned medium. Results showed a *negative* effect of cell-conditioned medium on vector titre. Furthermore, the length of time the medium was exposed to the FLYRD18 cells (during conditioning) correlated with the reduction in vector titre. This data suggests that medium conditioning during batch culture of the FLYRD18/LNC-hB7 cells was *not* the cause of the increased cell specific vector titres.

The decrease in vector titre in the presence of 'cell-conditioned' medium is likely to be due to depletion of nutrients and/or increased metabolic by-product concentrations in the medium

(during previous culture with the parental cell line). The FLYRD18 parental cell line also produces a large number of 'empty' particles, which may compete with the active vector particles during infection of the target cells. Inhibition of active particle infection by such empty particles would lead to a decrease in measured vector titre. Hence this may also explain the decrease in vector titre with increasing 'age' of the conditioned medium. A more suitable cell line for production of the cell-conditioned medium may have been the human fibrosarcoma cell line, HT1080, from which the FLYRD18 cell line was constructed. HT1080 cells do not contain any proviral sequences and hence do not produce empty particles.

A more probable explanation for the increasing vector titre per cell observed during the first few days of batch culture, is the higher cell growth rate during this exponential phase of growth. Where cells are in the exponential phase of growth, their physiological state is at an optimum (including mitochondrial activity). This may result in increased vector production rates. Indeed, a study of the relationship between cell proliferation and vector titre (section 3.1.4) indicated vector production to be related to cell proliferation.

The increasing cell density during batch culture could also affect cell specific vector titre. When the cells became more tightly packed (as they reached confluence) their morphology changed from an elongated, bipolar shape to a more polygonal shape. A relationship between cell morphology and cell function/physiology has been reported for a number of cell lines (Curtis, 1997; Tighe, 1994; Folkman and Moscona, 1978). Furthermore, Lee et al. (1996) reported an optimal retroviral vector production by ΨCRIP cells only after a confluent monolayer of cells was established.

The degradation of factors in the culture medium, which may inhibit virus production, could also play a role in the increasing cell specific vector titre during batch culture of FLYRD18/LNC-hB7 cells. Studies in Chapter 4 and those by Gerin et al. (1999a) show FCS to have a dose-dependent negative effect on the vector titre of FLYRD18/B7 cell cultures during early time points. However, after prolonged culture at 37°C this inhibition is lost. Hence during a 10-day batch culture it is possible that such inhibitory factors in the serum are gradually degraded, thus eliciting a daily increase in virus production per cell until all such factors are degraded.

If the above hypothesis were true, it would be expected that the '10-day old' cell-conditioned medium used in this study would contain the lowest concentration of inhibitory factors and hence yield the highest virus titres (which was not the case). However, if increased concentrations of 'empty' particles in the conditioned medium do indeed reduce the measured virus titres, such benefits of low serum inhibitor concentrations may be masked.

'Re-Selection' of FLYRD18/LNC-hB7 Cells in G418

It was found that the incubation of FLYRD18/LNC-hB7 cells in Geneticin-containing medium could significantly increase the cell specific vector titre, compared to cultures without Geneticin. This data suggests that some of the FLYRD18/LNC-hB7 cells in our cultures did not contain the retroviral vector carrying the neomycin resistance gene and that culture in Geneticin for one week caused the death of these cells. The presence of cells without this vector can explain the apparently lower vector titre per cell before culture in Geneticin-containing medium. During construction of the FLYRD18/LNC-hB7 cell line, those cells possessing the retroviral vector were selected for in G418-containing medium.

However, it is possible that during multiple passages, this vector is lost from a small proportion of these cells. It is therefore recommended that these cells are periodically 're-selected' in Geneticin in order to ensure all cells in the population contain the retroviral vector.

Relationship between Cell Proliferation, Cell Cycle Phase and Vector Production

A common method for arresting cell growth is serum deprivation (Adams, 1990). However, it has been clearly demonstrated that serum has a dose-dependent negative effect on the virus titre in FLYRD18/LNC-hB7 cell cultures (Gerin et al., 1999a). Therefore, serum deprivation was deemed to be unsuitable for determining the effect of cell proliferation of this packaging cell line on virus production. For this reason, alternative methods for inhibiting or reducing cell growth were adopted. However, it is recognised that each method may also have secondary effects that could alter virus production rates. Therefore a range of methods, normally used for studies of cell cycle synchronisation and arrest, were employed. These methods all affect the cell proliferation rate/progression through the cell cycle via different mechanisms. This allowed discrimination between direct effects of cell proliferation and secondary effects of the inhibitors.

Cell specific vector titres in thymidine-arrested cultures were significantly reduced, compared to non-arrested cultures of FLYRD18/LNC-hB7 cells. This finding indicates a relationship between cell proliferation and vector production. However the decrease in vector titre was accompanied by a decrease in the percentage of cells in the S-phase of the cell cycle. This is because thymidine inhibits DNA synthesis, thereby inhibiting cell entry into the S-phase. At the time of this experiment, the effect of cell cycle phase on vector

production was unknown. Therefore, it could not be ruled out that the reduced vector production was due to a reduced percentage of cells in the S-phase, rather than reduced cell proliferation rates (*if* in fact virus production occurred predominantly during the S-phase rather than the G_1 or G_2/M phases). In order to ascertain which factors were primarily responsible for the decrease in virus production, the effects of cell cycle phase on virus production were assessed using centrifugal elutriation to separate cells in different phases of the cell cycle.

Centrifugal elutriation has been shown to have little or no secondary effects on cell productivity, hence this method was most suitable for determination of the effects of cell cycle phase on virus production (Adams, 1990; Holmes and Al-Rubeai, 2000). The centrifugal elutriator consists of a chamber in which cells of the same size or density are held in position when their velocity is counterbalanced by a medium flowing in the opposite direction. When this medium flow is increased (by increasing the pump speed), cells are eluted from the chamber, the size of the eluted cells increasing with increasing pump speed. Hence cells are separated by size (where their density remains constant), which in turn is related to their DNA content. The DNA content per cell changes according to their position in the cell cycle (e.g. cells in G_1 are nearly half the size and possess half the total DNA of cells in G_2). While other chemical methods exist (Adams, 1990; Fantes and Brooks, 1993; Holmes and Al-Rubeai, 2000), this physical method was chosen in order to avoid any adverse conditions which may perturb subsequent cell cycle progression. If cell proliferation is indeed essential for virus production, it is important to maintain progression through the cell cycle in order to measure the virus titre in each separated cell fraction. Centrifugal elutriation also avoids a number of artefacts possible with chemical methods for cell

synchronisation. For example, nutrient deprivation or the addition of cytomodulators can lead to unbalanced cell growth (Holmes and Al-Rubeai, 2000), the consequences of which on virus production are unknown. Furthermore, cell fractions separated by centrifugal elutriation are produced at the same time and hence can be plated down together and grown in synchrony for easy comparisons.

Cell fractions enriched for a particular cell cycle phase can lose synchrony within a few hours. Therefore, in order to measure virus titre produced in a particular cell cycle phase, samples from separated cell fractions need to be taken within this time period. However, a number of groups have reported temporary inhibition of cell cycle progression (up to 6 hours) following the detachment of some cell types from the culture substratum (Campisi and Medrano, 1983; Andreadis at al., 1997). As mentioned above, if cell cycle progression is essential for virus production, the cells need to be cycling immediately after inoculation in order to compare virus levels produced during the first few hours. A preliminary experiment showed that FLYRD18/LNC-hB7 cells underwent no such inhibition in cell cycle progression after cell detachment (Figure 3.2.4.2B) and that active vectors could be measured just a couple of hours after the cells were re-plated (Figure 3.2.4.2A).

No significant difference in the virus titre was observed between cultures enriched for cells in different phases of the cell cycle. Although each fraction was by no means pure, the predominance of virus production in one particular phase would certainly have lead to a significant difference in the titre measured from each cultured fraction. For example, in the second fraction the percentage of cells in the S-phase was double that of the other fractions (see Table 3.2.4.2), hence if virus particles were produced predominantly in the S-phase, a 2-

fold increase in virus titre would be expected in the culture from this fraction. The data in Figure 3.2.4.2E suggests there is in fact no correlation between cell cycle *phase* and virus production. Hence the decreased cell specific titre observed with *thymidine-arrested* cells was probably due to decreased cell proliferation rates, suggesting that cell cycle *progression* is indeed essential for virus production.

An alternative explanation for the decreased virus titre in the presence of high concentrations of thymidine, is a possible secondary effect of the thymidine itself. High concentrations of thymidine inhibit the synthesis of deoxyribonucleotide triphosphates, dNTPs, (through inhibition of the ribonucleotide reductase enzyme), thereby inhibiting DNA synthesis (Adams, 1990). Reduced dNTP pools *could* in itself have an effect on measured virus titres. It has been reported that increasing the dNTP concentration in virus-containing supernatants can increase the transduction efficiency of these virus stocks (Zhang et al., 1995). This is brought about by an increase in reverse transcription in the virus particles (due to increased dNTP availability). This in turn can lead to an increased infection efficiency in the target cells, as more of the viral RNA may be reverse transcribed into DNA for integration into the target cell genome. If the *reverse* is true, then decreased dNTP pools could lead to reduced reverse transcription within the virus particles, resulting in lower infection efficiencies and hence lower measured virus titres. Hence the reduced cell specific titres in the thymidine-arrested cell cultures could also be due to reduced dNTP pools. Further studies, using alternative methods for cell arrest were therefore investigated.

Demecolcine (Colcemid™) disrupts tubulin assembly, thereby inhibiting spindle fibre formation. As a result, cells cultured in Demecolcine cannot exit metaphase and are arrested

in the cell cycle at the M-phase (Fantes and Brooks, 1993). This agent was therefore chosen to arrest cell proliferation via a different mechanism to that of thymidine. The cell specific titres were significantly reduced in the presence of Demecolcine (Figure 3.2.4.3C), further suggesting a correlation between cell proliferation and virus production.

Finally, the growth rate of FLYRD18/LNC-hB7 cells was shown to increase with increasing glucose concentrations, within a range. The cell specific vector titre increased with these increasing growth rates (Figure 3.2.4.4). These data again suggest that there is a correlation between the growth rate of FLYRD18/LNC-hB7 cells and their vector production. However, as with all nutrient manipulations, (despite their recommendation for such studies), it must be borne in mind that altering nutrient levels can alter the transcriptional and translational machinery of the cells. These effects cannot be ruled out as a contributory factor in the reduced vector levels at lower cell growth rates.

In Chapter 4, a study on the effects of culture temperature on vector production demonstrated a significant inhibition in proliferation of FLYRD18/LNC-hB7 cells at 27°C. This was accompanied by significantly reduced cell-specific vector titres, despite an increase in vector half-life at this lower temperature (cell viability was unaffected). This study avoids the use of chemical inhibitors and nutrient deprivation and the findings once again suggest a correlation between cell proliferation and vector production.

It is practically impossible to separate the effects of proliferation and metabolic state of packaging cells on vector production. However, the above studies certainly demonstrate that

FLYRD18/LNC-hB7 cells that are highly proliferative, that is, cells with a high metabolic activity, are likely to produce higher retroviral vector titres.

Cell Attachment to Carriers and Effects of Agitation

FLYRD18/LNC-hB7 cells were found to attach readily to ImmobaSil-FS™ carriers, with or without pre-coating with serum. However, subsequent stirring of such cell-seeded carriers lead to the loss of attached viable cells (as indicated by an MTT assay of carrier samples).

It was postulated that the lack of viable cells on the carriers after overnight agitation could be due to the susceptibility of the FLYRD18/LNC-hB7 cell line to shear forces exerted during stirring of the carriers, or, the inability of the ImmobaSil-FS™ carriers to maintain cell viability during prolonged culture. Both these possibilities were tested in subsequent studies. Firstly, the effect of increasing agitation rates on the viability of cells attached to the carriers, was assessed. Culture at stir speeds necessary to completely suspend the cell-seeded carriers resulted in a rapid loss of viable cells on the carriers. No cells were detected in the supernatant of these cultures, indicating that the loss of viable cells on the carriers was not due to cell detachment during stirring. These findings suggest that cell death is brought about by shear forces experienced by the cells during stirring of the suspended carriers. Cells stirred in suspension (not attached to carriers) also failed to survive at these agitation rates (probably due to a combination of the shear force effects and the inability of these anchorage-dependent cells to survive in suspension for long periods).

In a further study, cell-seeded carriers were cultured under static conditions and compared to stirred cultures. Unlike the stirred cultures, viable cells were detected on carriers for up to

one week in the static cultures. Furthermore, retroviral vector production was still measurable after static culture of the FLYRD18/LNC-hB7 cells on these carriers. These results confirm that the loss of cells on the carriers in previous experiments was not due to any adverse effects of culturing the cells on ImmobaSil-FS™ carriers, but rather an effect of agitation during stirring.

The effects of agitation on cell growth and viability *in vitro* has been researched extensively (Thomas and Zhang, 1998; Al-Rubeai et al., 1995a, 1995b, Thomas et al., 1994; Oh et al., 1993; Zhang et al., 1993a, 1993b; Born et al., 1992; Dodge and Hu, 1986). Recently is has been recognised by a number of groups that cell death in stirred cultures is usually due to air sparging (as a result of the forces incurred during bubble bursting). Thomas and Zhang (1998) claim that agitation *without* sparging is unlikely to cause significant breakage of animal cells in stirred bioreactor cultures. This is because animal cells are significantly smaller than the smallest eddy likely to exist in a stirred culture (at typical stir speeds). Where eddies are larger than a suspended particle, they will tend to entrain it and the particles will be exposed to little or no shear forces. In contrast, eddies which are smaller than a particle cannot entrain it and hence will act on the particle surface and, in the case of animal cells, cause damage. In microcarrier cultures the particle size is significantly larger and hence cell-seeded carriers may not be entrained by the smallest eddies during stirring. For this reason, it is generally believed that cells attached to microcarriers are more susceptible to shear forces during stirring than cells in suspension culture. Gregoriades et al., (1998) studied the damage of cells attached to microcarriers as a result of local energy dissipation. In typical spinner cultures, a majority of the energy introduced by the impeller is dissipated in regions near the impeller, which are typically characterised by vortices. The

energy dissipation required to damage the attached cells was orders of magnitude lower than that reported to kill suspended cells. Furthermore, it has been postulated by Croughan et al. (1986) that unlike suspended cells, cells attached to carriers cannot freely rotate and hence cannot reduce the net forces and torque experienced when they are exposed to excessive hydrodynamic forces. It has also been suggested that carrier to carrier bombardment can cause cell damage, in stirred microcarrier cultures (Papoutsakis, 1991).

A number of robust cell lines can tolerate the forces exerted during stirred microcarrier culture (e.g. CHO cells). However, the inability to maintain viable FLYRD18/LNC-hB7 cell cultures attached to carriers, when stirred at speeds necessary to fully mix the carriers, suggests that suspended microcarrier cultures are unlikely to be a suitable method for the large-scale production of retroviral vectors by this cell line. High-density cultures in stirred tank fermenters are likely to require even higher agitation rates than those used in the studies described here. Hence, the death of FLYRD18/LNC-hB7 cells, due to their susceptibility to shear forces in such culture systems, is inevitable. Alternative culture systems for the large-scale production of retroviral vectors by FLYRD18/LNC-hB7 cells, which use the same carrier material but expose the cells to very low shear forces, were therefore assessed (Chapter 5).

Oxygen Uptake Rate (OUR)

Knowledge of the oxygen uptake rate (OUR) of a cell line is critically important in the selection and design of a bioreactor for their large-scale culture (Pugh, 1988; Yoon and Konstantinov, 1994). The oxygen transfer capability of a bioreactor should exceed, or at least meet, the OUR of the whole cell culture. The relationship between OUR of

FLYRD18/LNC-hB7 cells and the oxygen transfer rate of a packed bed system is assessed in Chapter 5. The determination of OUR of FLYRD18/LNC-hB7 cells was therefore essential for these studies as well as providing information on the physiological properties of these cells.

The OUR of FLYRD18/LNC-hB7 cells was measured whilst attached to ImmobaSil-G™ carriers (to mimic the conditions used in the packed bed cultures) as well as after detachment from monolayer culture (to allow assessment of the OUR over 7 days). The OUR was highest after just a few hours of culture. It has been reported that the oxygen requirement of cells increases during their attachment to a substratum (Rottem et al., 1992). This group suggested that such increased oxygen uptake rates are due to the high metabolic state of cells during their spreading onto the culture surface. This may explain the higher oxygen uptake rate at this early time point.

During culture of the cells, the OUR increased over the first few days, perhaps due to increasing cell proliferation rates during this exponential phase of growth. By the end of the culture period the oxygen uptake rates markedly decreased. Cell proliferation rates and vector production also started to decrease by this time in previous batch cultures of FLYRD18/LNC-hB7 cells (see 10-day batch culture, section 3.2.1). Hence a correlation between cell activity and oxygen consumption is likely to be the cause of this decrease in OUR (as the cells entered the stationary phase of growth). The maximum OUR was also observed on day 4 in monolayer cultures of rat hepatocytes (Balis et al., 1999). This group also reported a decrease in OUR from day 4 to 30% of maximal levels by day 15. These findings also correlate with those of Yoon and Konstantinov (1994) who reported a decrease

in specific oxygen uptake rate during a 10-day culture of an NS0 myeloma cell line. Furthermore, Radlett et al., (1972) demonstrated an increase in OUR during the exponential growth phase of BHK21 cells, followed by decreasing OURs as the cells entered the late exponential and stationary phases of growth (as observed for FLYRD18/LNC-hB7 cells). Finally, Kioukia (2000) showed a range in OUR, during different stages of batch culture of hybridomas and insect cells.

Wohlpart et al., (1990) reported a decrease in OUR of cultured cells with increasing cell densities. This could also play a role in the decrease in OUR observed in the above studies during prolonged culture. Wohlpart and co-workers also demonstrated a negative effect of high glucose levels on cell specific oxygen consumption, while glutamine was shown to increase cellular OUR. Such findings demonstrate the range of factors that can effect cellular oxygen consumption. As a further example, Kioukia et al., (1995) revealed a 40% increase in OUR in insect cells after infection with baculovirus. This group suggested that this increase was associated with viral multiplication and was not due to recombinant protein production.

The OURs of FLYRD18/LNC-hB7 shown in Table 3.2.7, are comparable to those reported for a number of mammalian cell types (Emery et al. 1995). The reported OUR of murine hybridoma cells, Chinese hamster ovary (CHO) cells and NS0 myeloma cells falls within the range 1.25×10^{-15} to 1.34×10^{-14} mol/cell/min (Hiller et al.,1991; Lovrecz and Gray, 1994; Yoon et al., 1994; Bonarius et al., 1995; Zupke et al., 1995; Gray et al., 1996; Jan et al., 1997). In comparison, the OUR of porcine and rat hepatocytes has been reported to be significantly higher, ranging from 1.88×10^{-11} to 5.40×10^{-11} mol/cell/min (Balis et al.,

1999), though Bratch (2000) demonstrated an OUR of 2.18×10^{-14} mol/cell/min in rat hepatocyte cultures. Hence the OUR of FLYRD18/LNC-hB7 cells (in the order of 10^{-15} mol/cell/min) is not considered to be high.

The oxygen uptake rate of FLYRD18/LNC-hB7 cells cultured on ImmobaSil-G™ carriers was lower (perhaps due to less cell spreading), but of the same order of magnitude, as that measured in cells released from tissue culture plastic. Bratch (2000) also reported a decreased OUR of hepatocytes cultured on carriers compared to hepatocytes released into suspension (2.12×10^{-15} and 2.18×10^{-14} mol/cell/min, respectively). The OUR of other attached cell types has been reported to be 3.54×10^{-15} mol/cell/min (Smith et al., 1996) and 1.5×10^{-15} mol/cell/min (Foy et al., 1994), hence the values for FLYRD18/LNC-hB7 cells attached to ImmobaSil™ carriers are fairly typical.

This data can be used to give an approximation of the oxygen requirements of FLYRD18/LNC-hB7 cells, in order to check the oxygen transfer rates in the packed bed system are sufficient to support cell proliferation, and hence virus production, over long term cultures.

Retroviral Vector Stability

The half-life of active retrovirus particles produced by the FLYRD18/LNC-hB7 cell line correlates well with the half-life quoted for a number of murine and human retroviral vectors, at 37°C (see section 1.4). Reducing the storage temperature led to an increased half-life, suggesting the stability of the virus particle activity is indeed temperature dependent.

The virus half-life was increased almost 10-fold when stored on ice as compared to 37°C. It would therefore seem probable that an increase in daily virus titre could be obtained if the supernatant from cultures was continually harvested onto ice instead of remaining in the culture at 37°C. This was investigated in a later study (Chapter 5).

An increase in half-life was also measured when the virus supernatant was stored at 32°C as compared to 37°C. Therefore culture of FLYRD18/LNC-hB7 cells at this reduced temperature may also be a useful method for obtaining increased titres. However, reduced culture temperatures are likely to reduce cell proliferation rates, leading to reduced cell numbers. Furthermore, retroviral vector production may be reduced at this lower temperature. A study was therefore conducted to determine the effect of this reduced culture temperature on subsequent virus titres and cell proliferation rates (Chapter 4).

In order to compare the vector titre of various cultures, samples must be measured at the same time due to the inherent variation of the infection assay (see Introduction). It is therefore necessary to store samples in liquid nitrogen until all samples are collected and ready for analysis. In most circumstances samples can be thawed once for analysis then discarded. However, in the event of a failed assay, or where samples are re-frozen for later co-analysis with another set of samples, multiple freeze-thaw cycles may be necessary. For this reason, the effect of two successive freeze-thaw cycles on active vector titre was assessed. In this study, an additional freeze-thaw cycle did not markedly decrease the mean vector titre of duplicate samples. Therefore vector stocks may be re-frozen after analysis in case further analysis is required, provided that samples are not thawed for longer than 30 minutes (longest time tested). If samples are thawed for a second time they should be

discarded after analysis, as the effect of further freeze-thaw cycles is unknown for this cell line.

Reproducibility of Infection Assay

Figure 3.2.9 illustrates the inter-assay variation in measured vector titre using an infection assay. Due to this variation, a comparison of the vector titres between different cultures should be made by measuring each sample in the *same* assay. Where this is not possible (e.g. too many samples) the incorporation of a sample of *known* vector titre can be used to determine the inter-assay variation. This data can allow the adjustment of vector titres from one assay to compare to those from another assay. This has been done in a number of studies in Chapter 5 where samples from multiple packed bed cultures could not all be measured in the same infection assay.

The vector titre did not significantly decrease in samples stored in liquid nitrogen for over nine months (Figure 3.2.9). This finding demonstrates the suitability of liquid nitrogen for the long-term storage of vector preparations.

Transduction Efficiency of Vector Preparations

The successful transduction of target cells, after exposure to vector preparations, was determined by labelling with a fluorescent anti-human B7 antibody. The increase in fluorescence intensity of these cells, relative to control target cell cultures *not* exposed to vectors, was detected by flow cytometry. This increase in fluorescence was shown not to be due to non-specific binding of FITC-conjugated antibodies to the vector-exposed target cells. This was demonstrated by labelling a further culture of target cells with an irrelevant FITC-

conjugated antibody (namely, FITC-conjugated goat anti-mouse IgG) after exposure to the same vector preparations. The fluorescence intensity in these samples was the same as that of control samples labelled with the anti-B7 antibody (not exposed to vectors). Hence the increased fluorescence of the target cells in Figure 3.2.10 was due the direct labelling of human B7 protein expressed on their surface, after successful infection by the vectors. This data demonstrates the high transduction efficiency of vectors harvested from monolayer cultures of FLYRD18/LNC-hB7 cells at 37°C.

The transduction efficiency, unlike vector titre, has been shown by Forrestell et al., (1995) to remain fairly constant throughout culture (once a maximum is reached). This group postulated that this is due to the increase in transduction inhibitor concentration with increasing vector concentration (see section 1.6). Hence the transduction efficiency of a vector stock is an important parameter to measure when determining optimal harvest times, since the relationship between transduction efficiency and time is not the same as that between vector titre and time.

Chapter 4:

Influence of Various Physical and Chemical Factors on Vector Production by FLYRD18/LNC-hB7 Cells

4.0 Introduction

In order to determine the optimum culture conditions of FLYRD18/LNC-hB7 cells for the production of high titre retroviral vectors, the effects of a number of culture parameters were assessed. Cell growth and vector concentrations were measured during the culture of the packaging cells over a range of different temperatures, pH levels and dissolved oxygen concentrations. In addition, the effect of various serum concentrations and additives such as sodium butyrate, dexamethasone, peptone and fibroblast growth factor were assessed.

The dissolved oxygen level (dO_2) in culture medium has been reported to have a dramatic effect on cell productivity. For example, Klöppinger et al., (1990) demonstrated the effects of reduced dO_2 levels on the virus production and cell growth of Sf-9 cells infected with Ac-NPV baculovirus. This group reported a 50% decrease in virus production when the dO_2 level of the culture was reduced from 40% to 20%, thus illustrating the importance of dO_2 in certain culture systems. Other groups have reported *beneficial* effects of low oxygen levels. Lin et al., (1993) reported that under mild hypoxic conditions, the oxygen consumption rate of cells decreased, thereby allowing a 2-fold increase in the maximum cell concentration. This increase in cell production was obtainable only when glucose was supplemented to offset the increased glycolytic activity of the cells. Shi et al., (1993) also reported increased glucose and glutamine consumption rates and lactate production rates by hybridoma cells in oxygen-limiting conditions. However, extreme oxygen limitation or deprivation can reduce

cell viability (Mercille and Massie, 1994, Simpson et al., 1997). Simpson et al., (1997) reported complete cell death in hybridoma cell cultures after 32 hours of oxygen deprivation. High oxygen levels have been also shown to be detrimental to cell growth and viability (Emery et al., 1995; Simpson et al., 1997). An excess of 400% dO2 (based on the oxygen tension of air sparging) was demonstrated by Simpson et al., (1997) to cause complete death of hybridoma cell cultures after just 20h. These hyperoxic conditions are believed to cause toxic stress to the cells through the formation of free radicals and peroxides (Ellis, 1991; Simpson et al., 1997). In this chapter, the effects of dO_2 level on the growth and vector production of FLYRD18/LNC-hB7 RD18 cells was tested between 20% and 80% dO_2 in order to avoid any adverse effects of hypoxia or hyperoxia.

A wealth of literature exists on the effects of pH on mammalian cell growth, however nothing is known about the effects of pH on retrovirus production. Schmid et al., (1990) have reported the beneficial effects of low pH levels (pH 6.7) on monoclonal antibody production by hybridoma cells. In contrast, Harbour et al. (1989) showed a negative effect of low pH levels on monoclonal antibody production. These findings illustrate the variability of optimum pH values for growth and production of various cell lines. The effect of a range of pH levels on vector titre was therefore assessed in order to determine the optimum for vector production by the FLYRD18/LNC-hB7 cell line.

The reported effects of various additives such as serum, sodium butyrate and dexamethasone, during the culture and vector production by various retroviral packaging cells, has been reviewed in Chapter 1. The effects of serum concentration on vector titre have been conflicting (Lee et al., 1996; Shen et al., 1996; Gerin et al., 1999a) possibly due to the

different packaging cells and vectors used in each study. Likewise, the responsiveness of vector production to sodium butyrate and/or dexamethasone addition has been reported to vary greatly between different promoter types in various packaging cell systems (Olsen and Sechelski 1995; Pagès et al., 1995; Soneoka et al., 1995). Studies were therefore undertaken to determine the effects of such additives on the production of active vector particles by the FLYRD18/LNC-hB7 cell line.

It has been clearly demonstrated by Gerin et al. (1999a) that serum has a dose-dependent *negative* effect on vector production by the FLYRD18/LNC-hB7 cell line. The growth of FLYRD18/LNC-hB7 cells during long term culture in 5% serum is not significantly different to that in 10% serum. Therefore the studies throughout this thesis were conduced using 5% serum (as opposed to 10%, as previously used by other groups). In this chapter the effect of *lower* serum concentrations (1-2%) on cell growth and vector production was further investigated since previous reports only assessed vector production after 24h exposure to such low serum concentrations.

In order to confirm that the negative effects of serum on the vector titre of FLYRD18/LNC-hB7 cell cultures was not due to inactivation of the vector particles by serum components, the decay rate of vector stock activity was compared between stocks with and without serum. Since the decay rate is lower at 32°C, the effect of serum on vector stability was tested at both 32°C and 37°C, in order to confirm that the lower decay rate at 32°C is not due to a lower activity of potential inhibitory factors in serum. Furthermore, 'heat-inactivated' serum was compared to non-treated serum to further check that factors such as complement

components and antibodies (which are denatured by heat treatment) are not responsible for the lower titres in higher serum concentrations.

Two commercially available serum–free media were tested for their suitability for long term culture and vector production by the FLYRD18/LNC-hB7 cell line. Aim-V™ (Life technologies, Scotland) was selected as a suitable candidate as this medium has previously been demonstrated to support vector production by FLYRD18/LNC-hB7 cells at titres similar to that achieved in low serum concentrations, over a 24 hour culture period (Gerin et al., 1999a). Episerf™ (Life technologies, Scotland) was also chosen as this medium was developed by the manufacturer for the culture of adherent cells and has been claimed to promote the propagation of viruses (technical literature, Life Technologies). A further six different serum-free media under development by BioWhittaker Ltd. (not commercially available) were also assessed for their suitability to support the growth and vector production by FLYRD18/LNC-hB7 cells. These media, referred to as Hyb-F, Hyb-F/Version 2, Modified Hyb 2, LC-15, KF23 and Jab- 4F, were chosen for their ability to support the growth of HT1080 cells (personal communication, Lee Edwards, BioWhittaker U.K. Ltd.), as the FLYRD18 cell line was constructed from these fibrosarcoma cells (Cosset et al., 1995).

The addition of peptone to culture medium has been demonstrated to improve the growth and productivity of mammalian cells in batch and continuous culture (Jan et al., 1994). Meat peptone is a mixture of compounds derived from the digest of animal tissue and animal products and has been used as an inexpensive alternative to serum in animal cell culture medium. Jan et al. (1994) demonstrated an increased cell density and cell specific monoclonal antibody productivity in hybridoma cell cultures after the addition of meat or

protease peptone to the serum-containing culture medium. Earlier studies in this thesis indicate that increased proliferation rates of the FLYRD18/LNC-hB7 cell line can lead to increased vector titres. The effect of meat peptone on the growth and vector production by these cells was therefore investigated. In another attempt to increase cell growth rates (and hence vector production) fibroblast growth factor (FGF) was added to the culture medium of FLYRD18/LNC-hB7 cells and the vector titres were compared to cultures without this additive.

4.1 Materials and Methods

4.1.1 Culture of FLYRD18/LNC-hB7 Cells at Various Temperatures

Replicate monolayer cultures of FLYRD18/LNC-hB7 cells were established as described in Chapter 2. Sample flasks were incubated at 27°C, 32°C or 37°C for up to 8 days and the relative daily vector titres were compared. At the end of culture the cells were released from the flasks by trypsin digestion and counted using a haemocytometer. The vector concentration was divided by the total cell number in order to determine the mean vector titre *per cell* at each culture temperature.

4.1.2 Culture of FLYRD18/LNC-hB7 Cells at Various pH Levels

FLYRD18/LNC-hB7 cells were inoculated into T-flasks as described previously and left overnight. The culture medium from each flask was then replaced with medium adjusted to pH 6.0, 6.4, 6.6, 7.2 or 7.8 (triplicate flasks for each pH value). The cultures were incubated for a further 24h before removing the supernatant for measurements of the virus titre and detaching the cells by trypsin digestion for determination of cell numbers. Cells were not

cultured at the various pH levels for longer than 24h, due to the lack of pH control in monolayer cultures. The pH of the culture medium in each flask decreased by approximately 0.4 units after 24 hours incubation.

4.1.3 Culture of FLYRD18/LNC-hB7 Cells at Various Dissolved-Oxygen Concentrations

A 'Maestro' bioreactor (LSL Biolafitte, France) was used to control dissolved-oxygen concentration (dO_2) during the culture of FLYRD18/LNC-hB7 cells. In brief, two flat-bottomed glass vessels (connected to pH and oxygen probes) were inoculated with 8.5×10^6 cells in a small volume (20ml) of culture medium (DMEM:F-12 + 5% FCS) and the cells left to attach to the bottom of each vessel for 4 hours. After this time the number of cells remaining in suspension was counted to ensure over 95% of cells had attached before increasing the media volume to 200ml and leaving overnight at 37°C. The following day both vessels were connected to the controller, after replacing the medium in each for fresh. The pH control was set at pH 7.2, temperature at 37°C and dO_2 at 20% in one vessel and 50% in the other. The controlled cultures were maintained for 2 days before sampling the medium in each vessel for virus titre measurements and detaching the cells by trypsin digestion for determination of the final cell number cultured at each dissolved-oxygen concentration. The above experiment was then repeated using 50% and 80% dO_2. The use of 50% dO_2 in both runs allowed comparison between the two experiments by excluding inoculum conditions as a variable factor.

4.1.4 Culture of FLYRD18/LNC-hB7 Cells at Various Serum Concentrations

FLYRD18/LNC-hB7 cells were seeded into 6-well tissue culture plates at 1.6×10^5 cells per well in 3ml culture medium containing 2%, 4%, 6% or 8% FCS. After 24h incubation at 37°C, the culture medium in each well was changed for fresh (2-8% FCS) and after a further 24h the supernatant from each well was sampled for vector titre measurements (day 2). Cultures were then left for a further 3 days before sampling again for vector titre (day 5) and detaching the cells by trypsin digestion in order to determine the total cell number at each serum concentration.

In a further study, FLYRD18/LNC-hB7 cells were seeded into T-25 flasks at 2×10^4 cells/cm^2, as described in Chapter 2. In order to avoid any effect of the different serum concentrations on the percentage of cells which adhere, cells were seeded in medium containing 5% FCS. These cultures were left overnight before replacing the medium with fresh medium containing 1% or 5% FCS. The medium in each culture was replaced for fresh every day for up to 6 days. The culture supernatants were sampled on days 2 and 6 and the cells counted on day 6 as before. Replicate cultures were also maintained for up to 10 days, however no measurements from these cultures were taken, as a large proportion of the cells cultured in 1% FCS detached from the flasks and died during this prolonged culture period.

4.1.5 Effect of Serum on Stability of Vector Particle Activity

Vector Decay Rate in Culture Supernatants with or without Serum

The supernatant was collected from monolayer cultures of FLYRD18/LNC-hB7 cells (as described in Section 2), after overnight culture in medium with or without 5% FCS. The two supernatants were passed through a 0.45μm filter and divided into 1ml aliquots in cryovials.

Replicate vials were either stored immediately in liquid nitrogen (0h), or incubated at 32°C or 37°C for 3, 6, 9 and 24 hours before storing in liquid nitrogen. After all the samples were collected and stored in liquid nitrogen for at least 24h, they were thawed and used for vector titre measurements in order to determine the decay rate of the vectors at each temperature in the presence or absence of serum.

Effect of Heat-Inactivated Serum

The complement and antibody components of serum were denatured by incubation at 56°C for 30 min. FLYRD18/LNC-hB7 cells were seeded into 6-well plates at 2×10^5 cells per well in culture medium containing non-treated FCS (5%). After leaving the cells to attach overnight, the culture medium in each well was replaced for fresh medium containing heat-inactivated or non-treated FCS (5%). Each plate was cultured for 2 days and the supernatant in each well was sampled every day for vector titre measurements. On day 2 the cells in each well were counted to allow determination of the vector titre per cell.

4.1.6 Culture of FLYRD18/LNC-hB7 Cells in Serum-Free Medium

Aim-V™ and Episerf™ Serum-Free Medium

Monolayer cultures of FLYRD18/LNC-hB7 cells in T-25 flasks were established as described in Chapter 2. Cells in triplicate flasks were cultured in Aim-V™ serum-free medium (Life Technologies, Scotland), Episerf™ serum-free medium (Life Technologies, Scotland), or DMEM:F-12 + 5% FCS. The medium in each flask was changed for fresh daily for up to 6 days. The supernatant from each flask was sampled on Day 1, 4 and 7 for vector titre measurements and the cells from each flask were counted at the end of the culture period.

A further set of cultures was established in an attempt to adapt the FLYRD18/LNC-hB7 cell line to Episerf™ serum-free medium. Cells were seeded into T-25 flasks as before, in DMEM:F-12 + 5% FCS. After 24h the culture medium was changed for 50% Episerf™ in DMEM:F-12 +5% FCS or DMEM:F-12 +5% FCS alone (control). Cells were maintained in monolayer culture, as described in Section 2, and after 7 days the concentration of Episerf™ medium was increased from 50% to 75% (excluding the control flasks). Cells were maintained in 75% Episerf™ serum–free medium for up to 17 days. The cells were counted at each passage and the specific growth rates determined and compared to the control cultures. On Day 11 samples were taken for vector titres measurements.

Other Serum-Free Media

Six different serum-free media (courtesy of BioWhittaker U.K. Ltd.) were also tested for their ability to maintain the growth and vector production of FLYRD18/LNC-hB7 cells. These media are not commercially available, but are under development and referred to as: Hyb-F, Hyb-F/Version 2, Modified Hyb 2, LC-15, KF23 and Jab- 4F. Six-well plates were seeded with 2×10^5 cells per well in DMEM:F-12 +5% FCS and left overnight for cell attachment. The medium in each well was then replaced with one of the above serum-free media, or serum-containing medium (5%), using replicate wells for each. Each plate was cultured for up to 8 days and the medium sampled and changed for fresh daily. On day 8 the cells in each well were counted as before.

Further cultures in the serum-free media were maintained for up to 3 weeks, however no further cell growth was detected in any of the cultures and none of the monolayers reached a confluent state. Hence no data was collected from these cultures.

4.1.7 Effects of Sodium Butyrate and/or Dexamethasone on Vector Production

Sodium Butyrate Alone

Six-well plates were seeded with cells as described above and incubated until the monolayer of cells in each well was almost confluent (2 days). The medium in each well was then replaced for fresh medium containing 0, 1, 5, 10, 15 or 30mM sodium butyrate (Sigma, UK) and the plates incubated for a further 24h. The supernatant from each well was then sampled for vector titre measurements and the cells counted as before.

In a separate experiment, the above was repeated with the exception that the sodium butyrate was added at the *start* of the culture period. In this experiment the cells were exposed to sodium butyrate for up to 3 days.

Dexamethasone with/without Sodium Butyrate

Six-well plates, seeded with cells as described above, were incubated overnight before replacing the culture medium for fresh medium containing 0, 1 or 5μM Dexamethasone (Sigma, UK) alone or in the presence of sodium butyrate (5mM). After 24h, samples were taken for vector titre measurements and the cells in each well counted as before.

4.1.8 Effect of Fibroblast Growth Factor (FGF) on Vector Production

Tissue culture grade fibroblast growth factor (Sigma, UK) from bovine pituitary gland, was selected as a potent mitogen of various fibroblastic cell lines. A range of concentrations was assessed in order to determine whether cell proliferation and vector production could be further improved. Six-well plates were seeded with 2×10^5 cells per well, as described previously, and cultured in medium containing 0, 1, 10 or 100 ng/ml FGF for up to 3 days.

The medium in each well was sampled for vector titre measurements and changed for fresh daily. On day 3 the total number of cells per well was counted as before.

4.1.9 Effect of Peptone on Vector Titre

Six-well plates were seeded with FLYRD18/LNC-hB7 cells as described above and replicate wells cultured with 0, 1 or 2.5 mg/ml meat peptone (Sigma, UK) for up to 3 days. The medium in each well was sampled for vector titre measurements and changed for fresh daily. On day 3, the total number of cells per well was counted as before.

4.2 Results

4.2.1 Culture of FLYRD18/LNC-hB7 Cells at Various Temperatures

Daily retroviral vector titres in the culture supernatant of FLYRD18/LNC-hB7 cells cultured in monolayer at 32°C were compared to those from 37°C cultures (Figure 4.2.1.1). After day 2, virus titres were significantly higher in the 32°C cultures compared to the 37°C cultures, with a 10-fold increase by day 7. The mean cell concentration on day 8 was 6.3×10^5 cells/ml and 1.3×10^6 cells/ml at 32°C and 37°C, respectively (specific cell growth rate, μ, over 8 days was 7.6×10^{-3}/h at 32°C and 9.0×10^{-3}/h at 37°C). As a result of the higher virus titre and lower cell concentration in the 32°C cultures, the specific virus productivity (cfu/cell) was over 20-fold higher than 37°C cultures on day 8.

In contrast, the virus titres were negligible when this cell line was cultured at 27°C for 7 days (Figure 4.2.1.2). Cell proliferation was markedly inhibited at this lower temperature (the mean cell concentration on day 7 was 8.6×10^4 cells/ml and 1.7×10^6 cells/ml at 27°C and

37°C respectively). The specific virus productivity was 14-fold lower at 27°C compared to 37°C. The low virus production is likely to be due to the low cell proliferation at this culture temperature (as indicated by earlier studies in Chapter 3).

4.2.2 Culture of FLYRD18/LNC-hB7 Cells at Various pH Levels

The highest vector titre was measured in the supernatants from packaging cells cultured at pH 7.2. The titre at this pH was taken as 100% and all other titres calculated as a percentage of this (Figure 4.2.2). Since it is unlikely, under normal culture conditions, for the pH of the culture medium to increase, the effect of pH below 7.2 was of more concern than that above pH 7.2. Figure 4.2.2 illustrates the effect of such low pH on virus production and cell viability. While the cell viability remained above 90% at all pH levels tested except pH 6 (85% viability), the specific virus titre (cfu/cell) in cultures below pH 6.8 was significantly decreased compared to cultures at pH 7.2 (87%, 34% and 12.5% at pH 6.8, 6.4 and 6.0, respectively).

4.2.3 Culture of FLYRD18/LNC-hB7 Cells at Various Dissolved Oxygen Levels

No significant difference in cell numbers or vector titres was observed in cultures maintained for 2 days at 20%, 50% or 80% dO_2 (Figure 4.2.3). Hence the dissolved-oxygen concentration was not limiting to cell growth or virus production under the conditions employed in this study. Cell numbers increased almost 3-fold during the two days in culture.

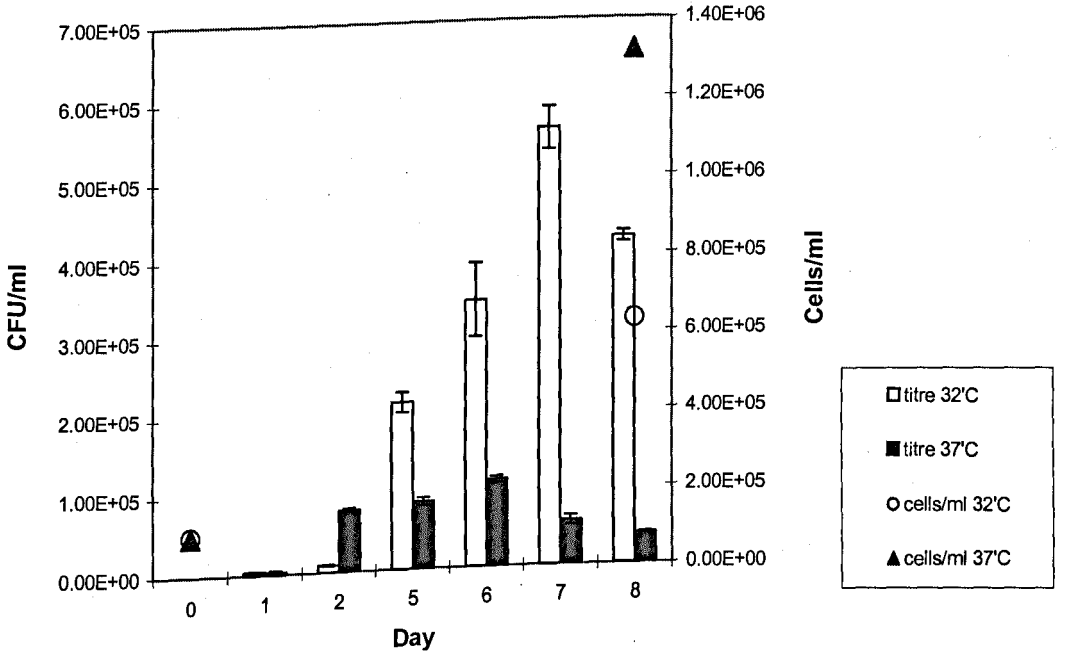


Figure 4.2.1.1

Virus titre and cell number during monolayer culture of FLYRD18/LNC-hB7 cells at 32°C or 37°C. Error bars represent observed range, n=2.

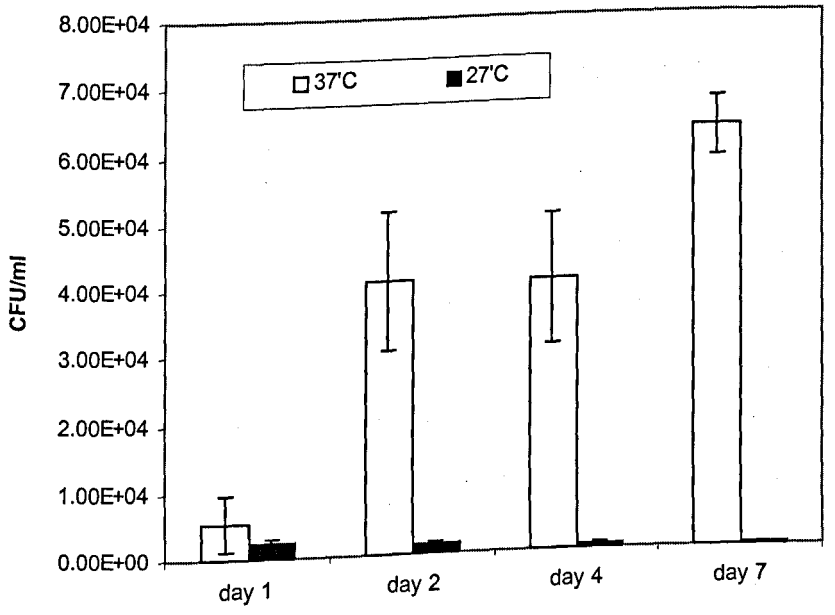


Figure 4.2.1.2

Daily vector titres produced by FLYRD18/LNC-hB7 cells during culture at 27°C compared to 37°C. Error bars represent standard deviation, n=6. Cell proliferation at 27°C was negligible.

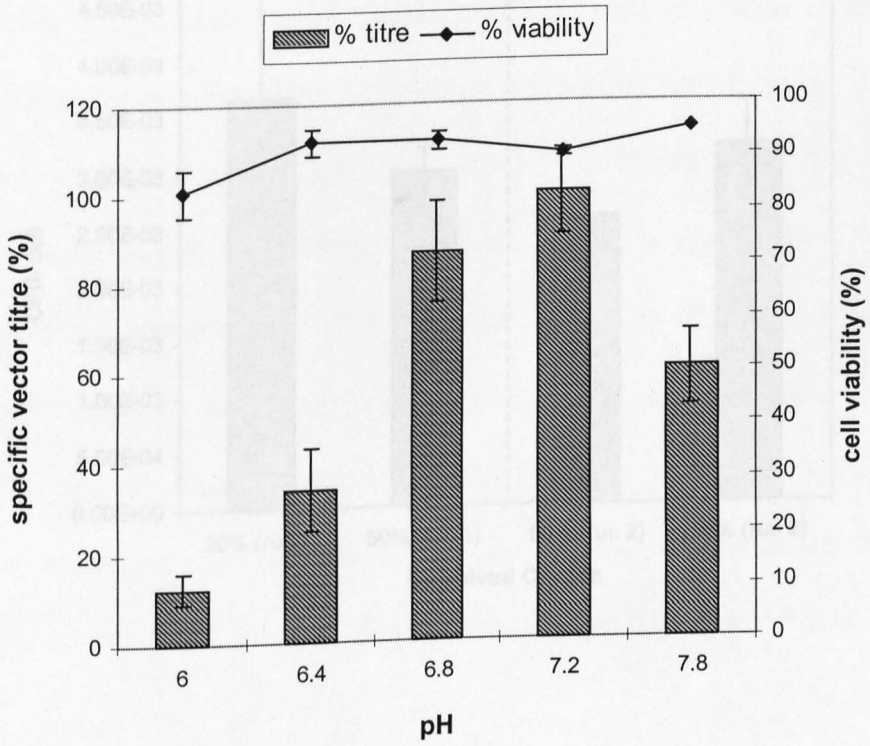


Figure 4.2.2

Specific virus titre (cfu/cell), as a *percentage* of that measured at pH 7.2. Samples measured after 24h culture at various pH levels. Error bars represent standard deviation, n=3.

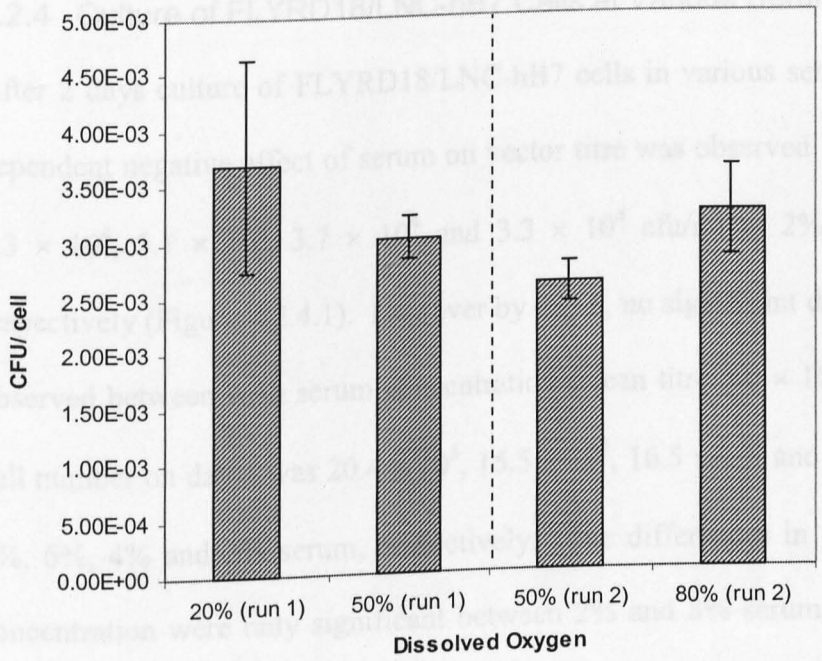


Figure 4.2.3

Specific virus titre (cfu per cell) after 2 days culture at various dissolved oxygen levels. Error bars represent observed range, n=2.

4.2.4 Culture of FLYRD18/LNC-hB7 Cells at Various Serum Concentrations

After 2 days culture of FLYRD18/LNC-hB7 cells in various serum concentrations, a dose-dependent negative effect of serum on vector titre was observed. The mean vector titre was 6.3×10^4, 5.1×10^4, 3.7×10^4 and 3.3×10^4 cfu/ml in 2%, 4%, 6% and 8% serum, respectively (Figure 4.2.4.1). However by day 5, no significant difference in vector titre was observed between each serum concentration (mean titre 3.0×10^5 cfu/ml). The mean total cell number on day 5 was 20.4×10^5, 15.5×10^5, 16.5×10^5 and 13.6×10^5 cells per well at 8%, 6%, 4% and 2% serum, respectively. The differences in cell number at each serum concentration were only significant between 2% and 8% serum, hence the vector titre *per cell* on day 5 was only significantly lower in the 2% serum cultures.

In a second experiment, cultures were maintained in 1% or 5% FCS for up to 6 days and the medium replaced for fresh every day. In contrast to the previous study, where the medium was left unchanged for 3 days, the cell specific vector titre (cfu/cell) was significantly higher in the 1% serum culture compared to the culture in 5% serum even after 6 days (Figure 4.2.4.2).

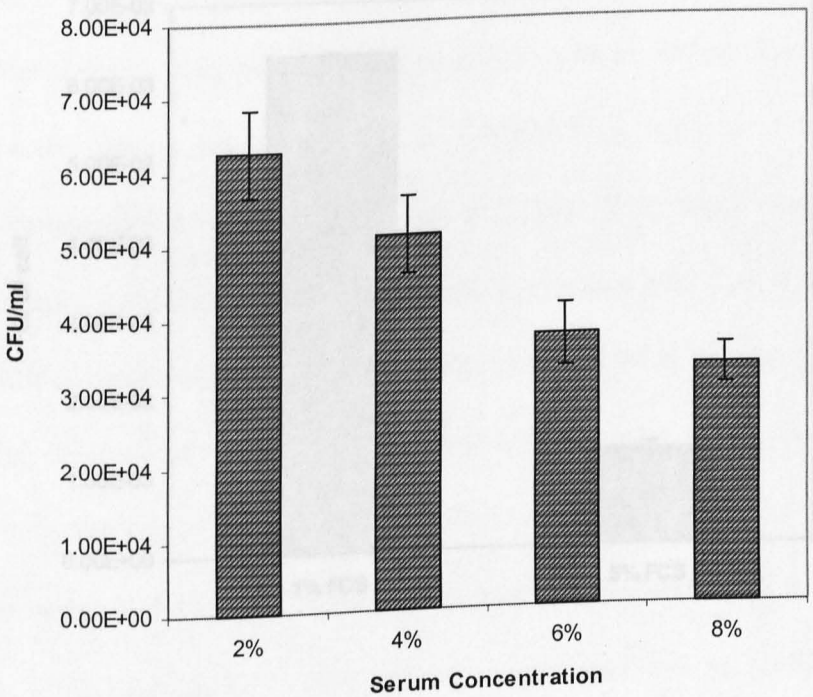


Figure 4.2.4.1

Virus titre after 2 days culture of FLYRD18/LNC-hB7 cells in various serum concentrations.

Error bars represent standard deviation, n=6).

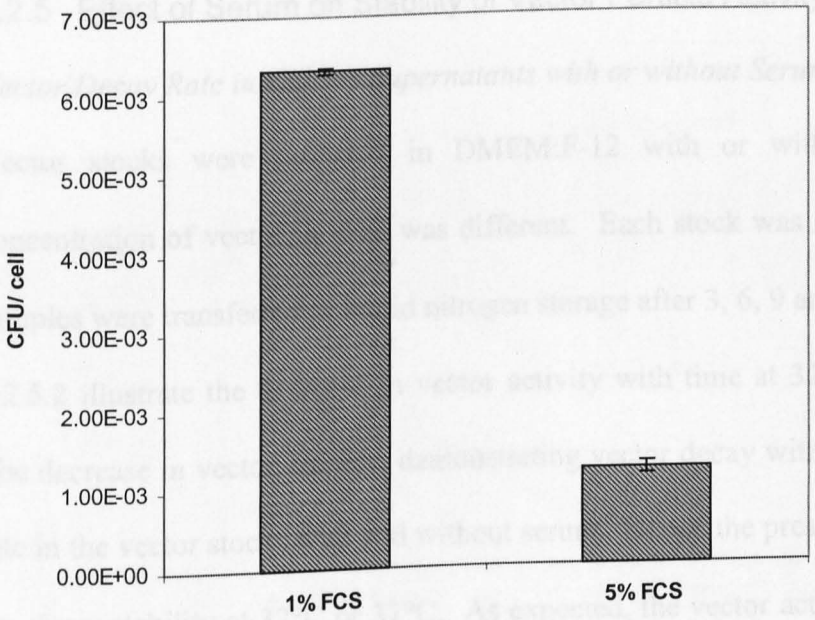


Figure 4.2.4.2

Cell specific vector titre after 6 days culture of FLYRD18/LNC-hB7 cells in 1% or 5% foetal calf serum (FCS). Error bars represent observed range, n=2.

4.2.5 Effect of Serum on Stability of Vector Particle Activity

Vector Decay Rate in Culture Supernatants with or without Serum

Vector stocks were prepared in DMEM:F-12 with or without 5% FCS, hence the concentration of vector in each was different. Each stock was stored at 32°C or 37°C and samples were transferred to liquid nitrogen storage after 3, 6, 9 and 24 h. Figures 4.2.5.1 and 4.2.5.2 illustrate the decrease in vector activity with time at 32°C and 37°C, respectively. The decrease in vector activity, demonstrating vector decay with time, occurred at the same rate in the vector stocks with and without serum. Hence the presence of serum had no effect on vector stability at 32°C or 37°C. As expected, the vector activity decay rate was greater at 37°C than at 32°C (this reduced vector half-life at higher storage temperatures has previously been demonstrated in Section 3).

Effect of Heat-Inactivated Serum

FLYRD18/LNC-hB7 cells were cultured in 5% heat-inactivated or non-treated serum for 2 days and the medium changed for fresh in each sample daily. The mean viable cell numbers were the same after 2 days culture in each type of serum (mean 4.4×10^5 cells per well). Similarly, there was no significant difference between the vector titres on Day 1 and 2 in the presence of heat-inactivated serum, compared to non-treated serum (Figure 4.2.5.3).

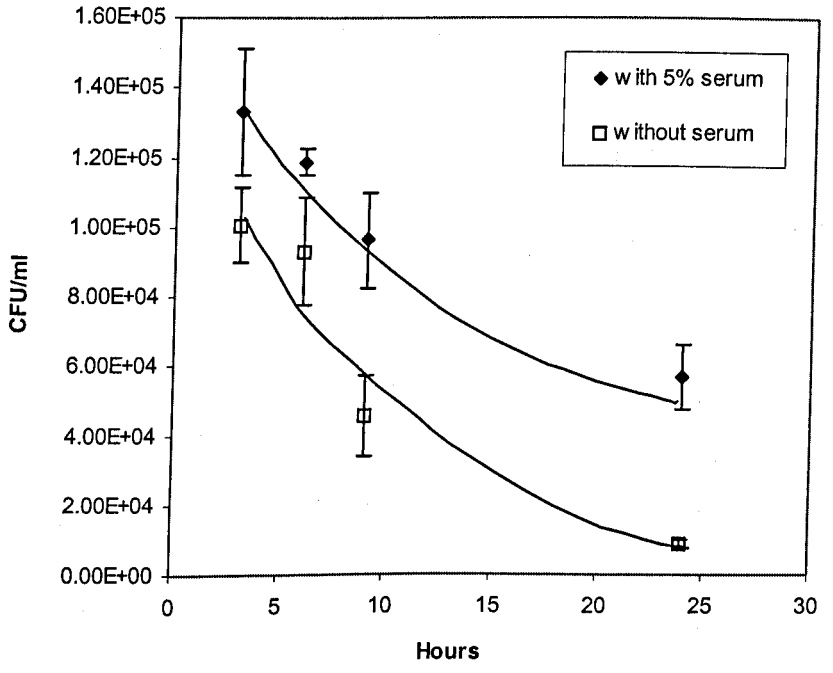


Figure 4.2.5.1

Vector activity decay during storage at **32°C**, in the presence or absence of 5% FCS. Error bars represent observed range, n=2.

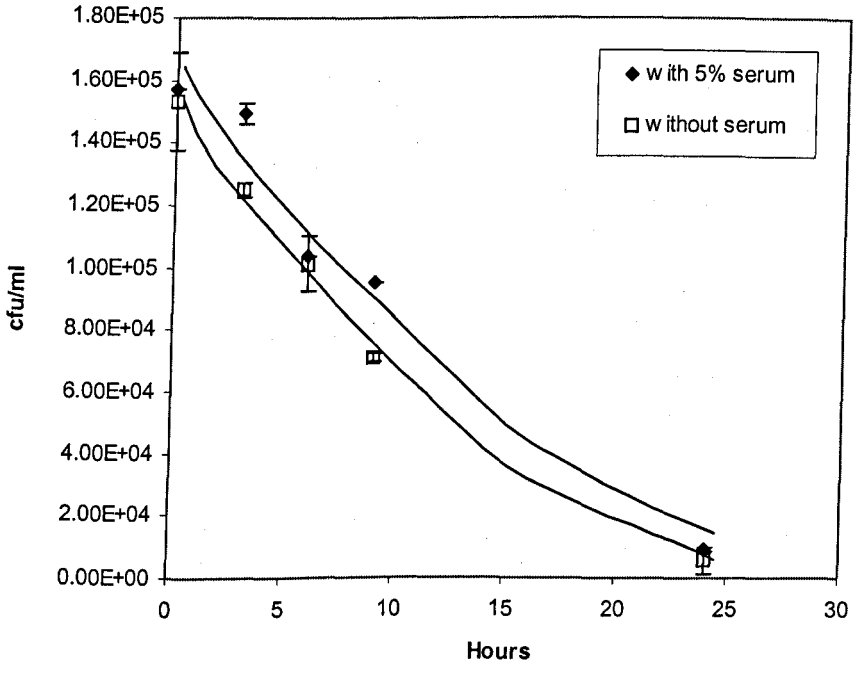


Figure 4.2.5.2

Vector activity decay during storage at **37°C**, in the presence or absence of 5% FCS. Error bars represent observed range, n=2.

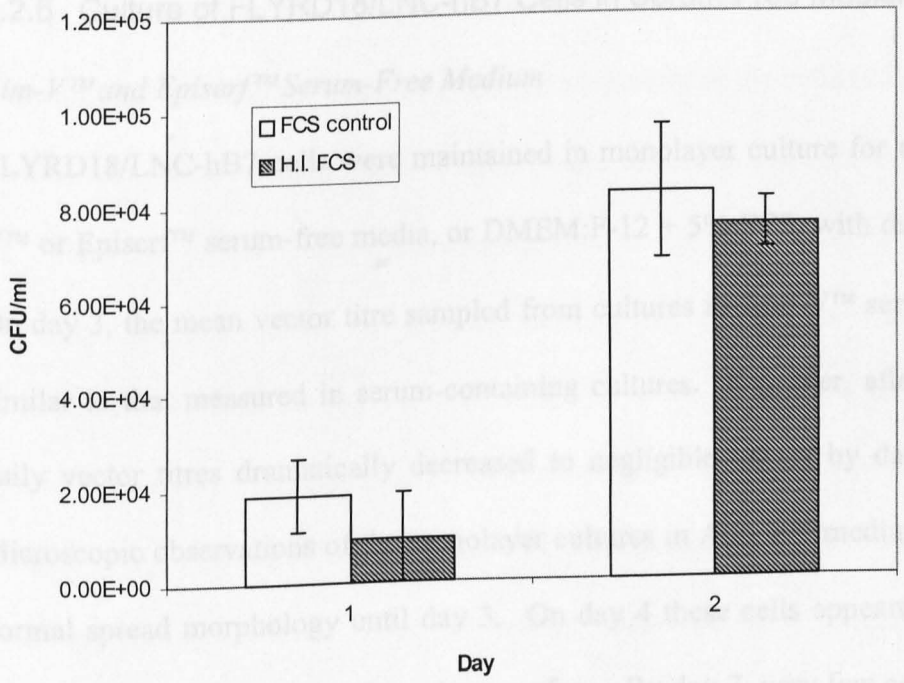


Figure 4.2.5.3

Daily vector titre in FLYRD18/LNC-hB7 cell cultures in 5% heat-inactivated foetal calf serum (H.I. FCS) or non-treated foetal calf serum (FCS control). Error bars represent standard deviation, n=4.

4.2.6 Culture of FLYRD18/LNC-hB7 Cells in Serum-Free Medium

Aim-V™ and Episerf™ Serum-Free Medium

FLYRD18/LNC-hB7 cells were maintained in monolayer culture for up to 7 days in Aim-V™ or Episerf™ serum-free media, or DMEM:F-12 + 5% FCS, with daily medium changes. On day 3, the mean vector titre sampled from cultures in Aim-V™ serum-free medium was similar to that measured in serum-containing cultures. However, after this time point the daily vector titres dramatically decreased to negligible values by day 7 (Figure 4.2.6.1). Microscopic observations of the monolayer cultures in Aim-V™ medium revealed cells with normal spread morphology until day 3. On day 4 these cells appeared more rounded and were starting to detach from the culture surface. By day 7, very few cells were left attached in the Aim-V™ cultures.

Vector titres sampled from cultures in Episerf™ serum-free medium also reached similar levels to those measured in serum-containing medium by day 3. These levels continued to increase and correlate well with the titres in 5% FCS until Day 4 (Figure 4.2.6.1). After this time point the vector titres in the Episerf™ cultures also dramatically decreased. On day 7 the mean vector titre in Episerf™ cultures was only 30% of that in the serum-supplemented cultures. Microscopic observations of the monolayer cultures in Episerf™ medium revealed the cells to be growing in clusters with a 'stellate' morphology from the start of culture. By day 4, the cells began to 'pile-up' in clusters instead of spreading across the culture surface. In comparison, the cells cultured in 5% FCS had a normal spread morphology and had formed a confluent monolayer by day 4. On day 7 the mean total number of cells per flask in Episerf™ medium was 1.8×10^6, compared to 1.23×10^7 cells in serum-containing cultures.

Due to the inability of the FLYRD18/LNC-hB7 cells to survive in either Aim-V™ or Episerf ™ serum-free medium for more than 4 days, adaptation of the cells to Episerf™ medium over a period of weeks was attempted. Cultures were established in 50% Episerf™ (in DMEM:F-12 + 5% FCS). After 11 days, the concentration of Episerf™ medium was increased to 75% and the cells maintained for another week. No vector titre was measurable in the supernatants sampled on day 11, indicating no vector production in the presence of 50% Episerf™ medium. Furthermore, as the percentage of Episerf™ medium was increased, the specific cell growth rate decreased and was consistently lower than that of cells cultured in serum-containing medium. The cell viability also decreased during culture in Episerf™ medium. The mean specific cell growth rates and percentage viability, compared to those measured in DMEM:F-12 +5% FCS alone, are given in Table 4.2.6. After 17 days, the cells were transferred to culture in 100% Episerf™ medium and once again the cells were observed to detach from the culture surface and die within a few days. Hence no adaptation of the cells to Episerf™ serum-free medium was achieved.

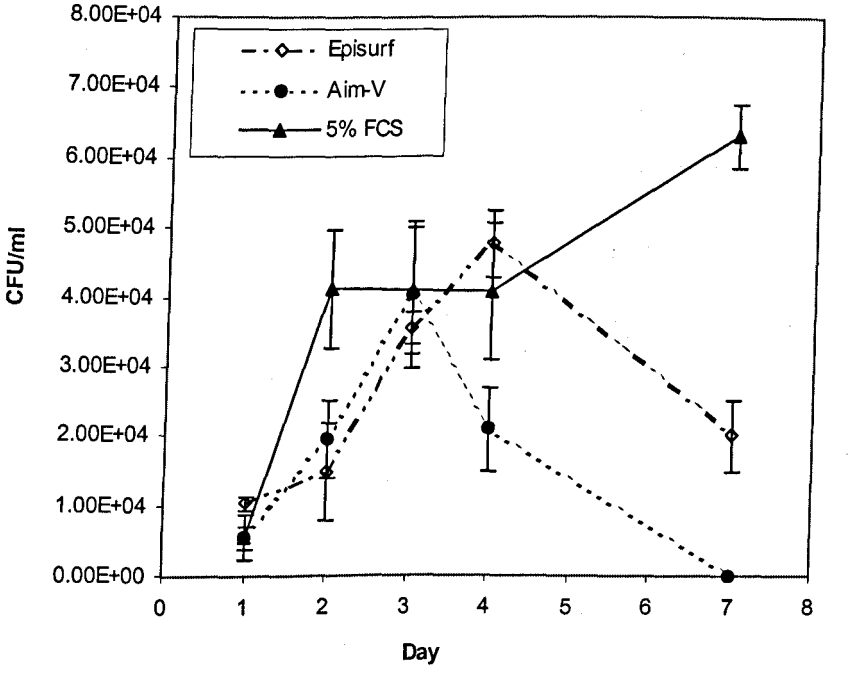


Figure 4.2.6.1

Daily vector titre measured in FLYRD18/LNC-hB7 cell cultures using two commercially available serum-free media compared to 5% serum. Error bars represent standard deviation, n=6. Decreased vector titres after Day 4 were due to cell death in the serum-free media.

Other Serum-Free Media

Six different serum-free media, under development by BioWhittaker Ltd., were tested for their ability to maintain cell growth and vector production by FLYRD18/LNC-hB7 cells. Figure 4.2.6.2 illustrates the mean vector titre and total cell numbers after 8 days culture in each media, compared to control cultures in medium containing 5% FCS. The cell numbers and vector titres in all six serum-free media were significantly lower than those obtained in 5% FCS. While cell numbers were similar between the different serum-free media, the vector titres measured on Day 8 varied markedly. No vector activity was detected in the culture supernatants of Hyb-F or Jab4F serum-free medium. The highest mean vector titre was in the LC-15 cultures, however this titre was less than 30% of that measured in the control cultures with 5% FCS. Microscopic observations of the cultures revealed different cell morphologies in the different media. Cells became rounded and withdrew into clusters in Hyb-F, Hyb-F/version 2 and LC-15 media. Cells maintained a spread appearance (similar to that of the control cultures) in KF23 medium, however, after reaching confluence on day 4, a number of 'holes' appeared in the cell monolayers. Cells in the Modified Hyb-2 medium showed mixed morphologies, some being rounded and some elongated. After 5 days, a number of cells started to detach from the culture surface in all serum-free media except Jab4F. The percentage cell viability remained above 80% in all media except KF23 and Modified Hyb-F which were only 50% and 77% viable, respectively. Surprisingly, the serum-free medium that yielded the highest cell number and greatest percent viability, namely Jab4F, showed no evidence of active vector production.

Day	Media Type	Specific Growth Rate, μ (h^{-1})	Cell Viability (%)
2	50% Episerf	2.1×10^{-2}	95
	DMEM:F-12 + 5% FCS	2.8×10^{-2}	96
4	50% Episerf	1.4×10^{-2}	93
	DMEM:F-12 + 5% FCS	2.6×10^{-2}	99
7	50% Episerf	1.3×10^{-2}	83
	DMEM:F-12 + 5% FCS	2.1×10^{-2}	96
11	75% Episerf	0.5×10^{-2}	72
	DMEM:F-12 + 5% FCS	1.5×10^{-2}	94

Table 4.2.6

Specific cell growth rates and percentage viability of FLYRD18/LNC-hB7 cells in various concentrations of Episerf™ serum-free medium, compared to cultures in 5% foetal calf serum.

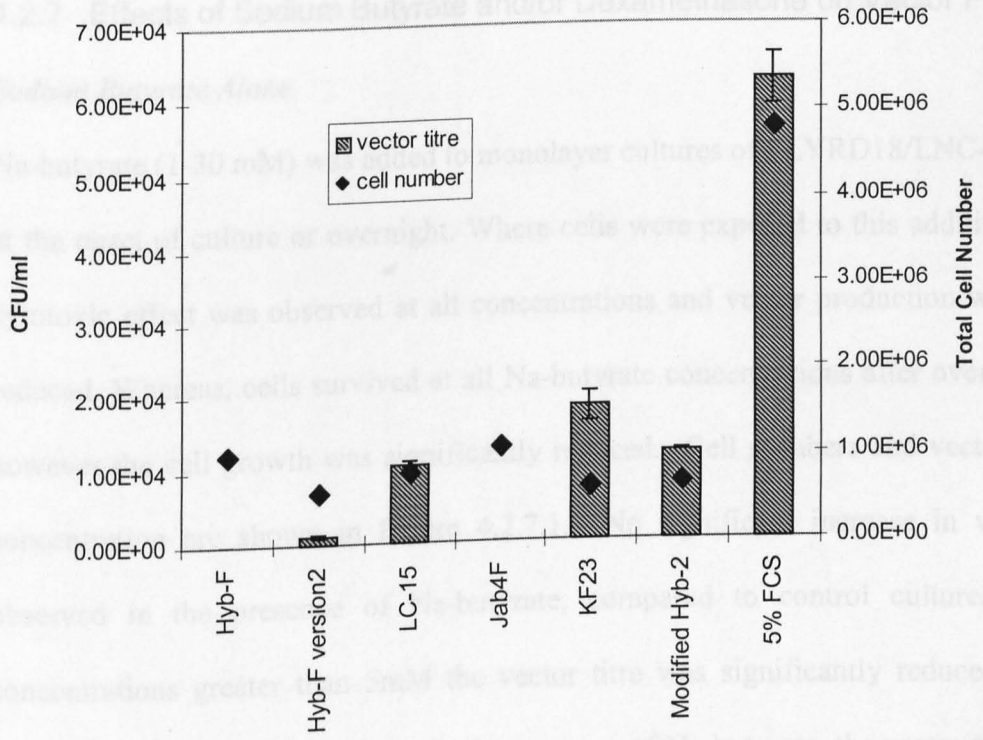


Figure 4.2.6.2

Vector titre and total cell number after 8 days culture of FLYRD18/LNC-hB7 cells in various serum-free media, compared to medium containing 5% FCS. Error bars represent observe range, n=2.

4.2.7 Effects of Sodium Butyrate and/or Dexamethasone on Vector Production

Sodium Butyrate Alone

Na-butyrate (1-30 mM) was added to monolayer cultures of FLYRD18/LNC-hB7 cells either at the onset of culture or overnight. Where cells were exposed to this additive for 3 days, a cytotoxic effect was observed at all concentrations and vector production was dramatically reduced. Whereas, cells survived at all Na-butyrate concentrations after overnight exposure, however the cell growth was significantly reduced. Cell numbers and vector titres at each concentration are shown in Figure 4.2.7.1. No significant increase in vector titre was observed in the presence of Na-butyrate, compared to control cultures. In fact, in concentrations greater than 5mM the vector titre was significantly reduced. Taking into account the reduced cell numbers in the presence of Na-butyrate, the vector titre *per cell* was higher in the presence of 1mM, but again significantly reduced in 5mM and above.

Dexamethasone with/without Sodium Butyrate

Dexamethasone (1-50 μM) was added alone or with Na-butyrate (5 mM), to replicate cultures of FLYRD18/LNC-hB7 cells. Where Na-butyrate was included, cell numbers at the end of the culture period were only 60% of those in cultures with dexamethasone alone, again due to the apparent cytotoxic effects of Na-butyrate. Likewise, vector titres were significantly reduced.

Cell numbers in the cultures with dexamethasone alone, up to 10 μM, were similar to those in control cultures with no additives. However, in 50 μM dexamethasone nearly all the cells died. Figure 4.2.7.2 illustrates the mean vector titre *per cell* 24h after exposure of the cells to dexamethasone alone. A slight increase in vector titre was observed with increasing

dexamethasone concentration up to 5 μM, however above this concentration vector titres were significantly reduced.

4.2.8 Effect of Fibroblast Growth Factor (FGF) Addition on Vector Titre

After 3 days culture of FLYRD18/LNC-hB7 cells in a range of FGF concentrations (in DMEM:F-12 + 5% FCS), the cell numbers were not significantly increased, compared to cultures without FGF addition. Likewise the daily vector titres over 3 days were not significantly different (Figure 4.2.8).

4.2.9 Effect of Peptone Addition on Vector Titre

The addition of meat peptone to FLYRD18/LNC-hB7 cell cultures (in DMEM:F-12 + 5% FCS) had no effect on cell number after 3 days. The vector titre on day 2 was slightly higher in the presence of 2.5mg/ml peptone, however by day 3 there was no significant difference in the vector titres in cultures with or without peptone (Figure 4.2.9).

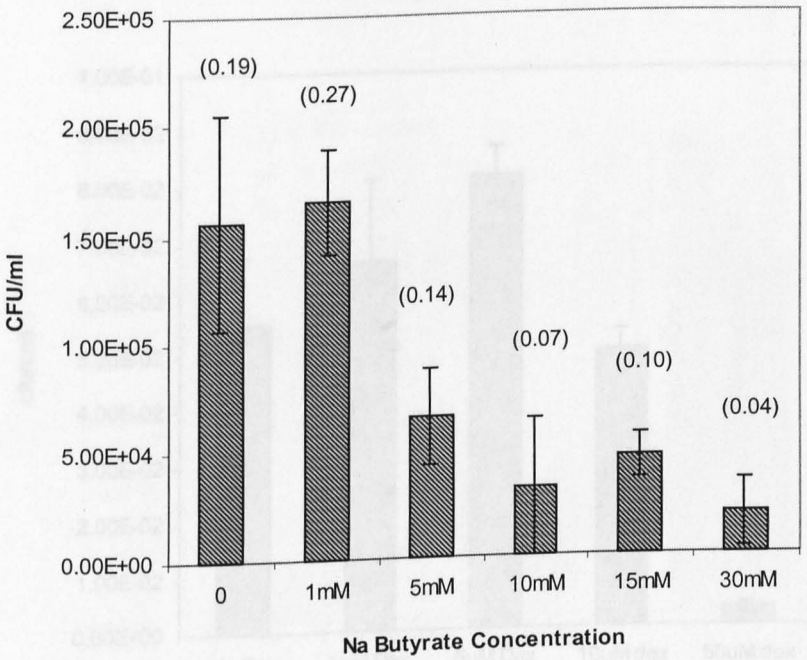


Figure 4.2.7.1

Vector titre in FLYRD18/LNC-hB7 cell supernatants after overnight addition of Na-butyrate (1-30mM), compared to control cultures without Na-butyrate. Figures in brackets represent mean vector titre *per cell*. Error bars represent standard deviation, n=4.

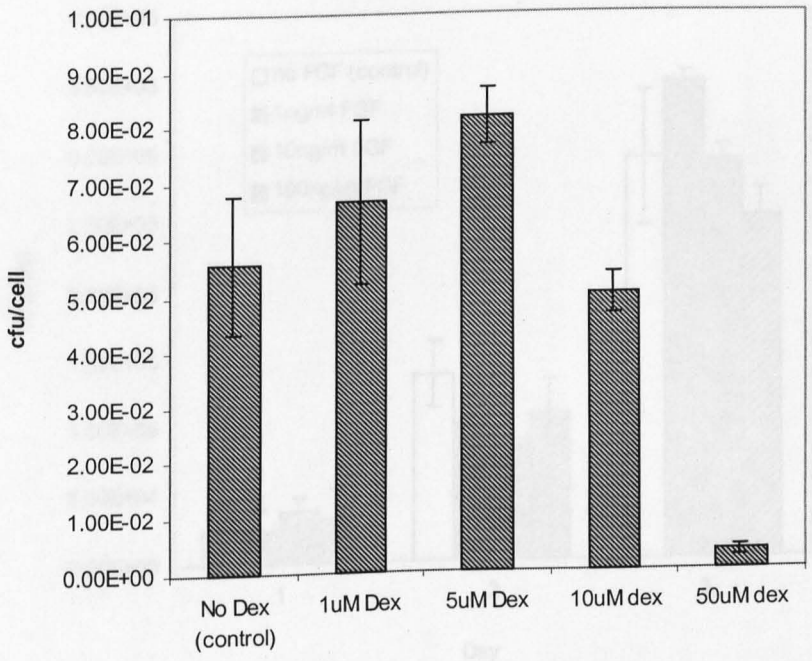


Figure 4.2.7.2

Mean vector titre per cell, after 24h exposure to increasing concentrations of dexamethasone, compared to control cultures with no dexamethasone addition. Error bars represent observed range, n=4.

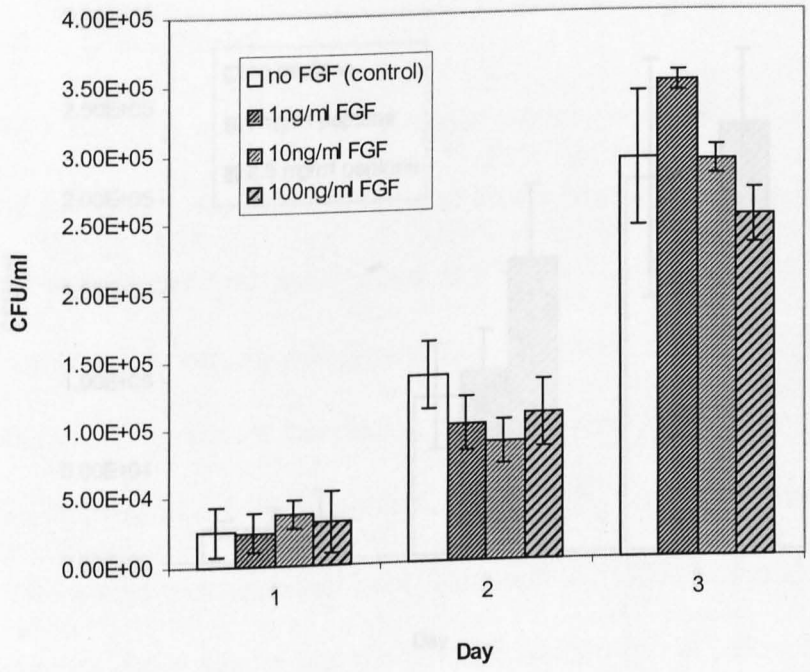


Figure 4.2.8

Daily vector titres in FLYRD18/LNC-hB7 cell cultures in a range of FGF concentrations, compared to control cultures without FGF addition. Error bars represent standard deviation, n=4.

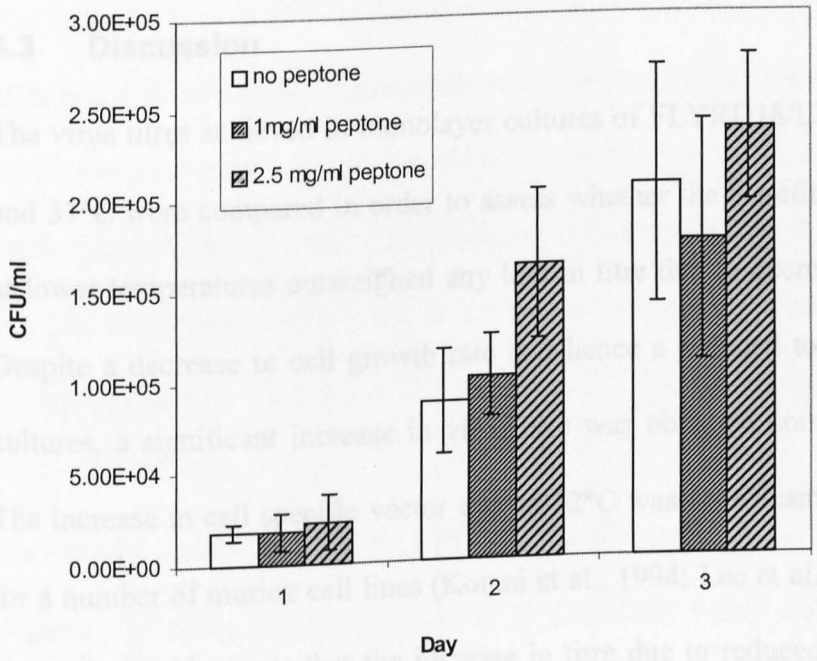


Figure 4.2.9

Daily vector titres sampled from FLYRD18/LNC-hB7 cell cultures in the presence of 1 or 2.5 mg/ml peptone, compared to cultures without peptone addition. Error bars represent standard deviation, n=4.

4.3 Discussion

The virus titres achieved in monolayer cultures of FLYRD18/LNC-hB7 cells at 27°C, 32°C and 37°C were compared in order to assess whether the benefits of increased virus stability at lower temperatures outweighed any loss in titre due to decreased cell metabolic activity. Despite a decrease in cell growth rate and hence a reduced total cell number in the 32°C cultures, a significant increase in virus titre was obtained compared to the 37°C cultures. The increase in cell specific vector titre at 32°C was significantly greater than that reported for a number of murine cell lines (Kotani et al., 1994; Lee et al. 1996; Kaptein et al., 1997). Hence it would appear that the increase in titre due to reduced culture temperatures varies between different cell lines. This is possibly due to different half-life values of the vectors produced by these cells. Cruz et al., (2000a) reported no increase in vector titre at 32°C, compared to 37°C, in PA317 packaging cell cultures. This group suggested that this was a consequence of the faster decay rates of the vectors used in their work, compared to others. The reduced stability of retroviral vectors at higher temperatures (37°C) has been hypothesised to be due to the inherent instability of the RNA genome or loss of reverse transcriptase activity (Palsson and Andreadis, 1997). It has also been suggested that loss of labile heterodimeric envelope glycoproteins causes loss of vector activity (Forestell et al., 1995; Pagès et al., 1995).

In order to rule out the possibility of an increased activity of certain serum components at 37°C compared to 32°C as a cause of reduced titres at 37°C, the stability of vector stocks with and without serum at 37°C and 32°C was compared. It was found that the vector decay rate was not affected by the presence of serum at either temperature. Hence it is likely that

the increased titre at 32°C is due to reduced thermal decay of the vector particles as opposed to reduced extra-cellular inactivation of vector particles by serum components. Le Doux et al. (1999) have used a mathematical model to analyse the effects of temperature on vector production and decay rates. The findings by this group also strongly suggest that increased titres at 32°C are due to reduced vector decay rates, despite slight reductions in vector production rates.

Conversely, culture of the FLYRD18/LNC-hB7 cells at 27°C resulted in a significant reduction in vector titres, compared to 37°C. While vector stability is expected to increase even further at this lower temperature, the cell proliferation rate at 27°C was also markedly reduced. Earlier studies in Chapter 3 demonstrated a correlation between cell proliferation and vector production. Hence it is likely that at this lower culture temperature, the vector production is reduced to negligible levels due to very low cell proliferation rates.

A range of pH levels were assessed in order to determine the effects on cell growth and vector production by FLYRD18/LNC-hB7 cells. An optimum vector titre was obtained at pH 7.2 and a significant decrease resulted from higher or lower pH levels. This observation has implications where packaging cells are cultured under batch, fed-batch or semi-continuous culture conditions where the pH is not normally controlled. In high cell density cultures the pH of the culture medium can drop as low as pH 6.5 over a 24-hour period (Smiley et al., 1989), hence where daily medium changes are employed for vector harvesting, it is unlikely that the optimum titre will be achieved. The use of a culture system where the pH is continuously controlled is therefore recommended for optimised continuous production of retroviral vectors.

The effect of dissolved-oxygen concentration on the production of retroviral vectors has not previously been reported. Surprisingly, at dissolved-oxygen concentrations between 20% and 80% air saturation, no difference in vector titre was observed, under the conditions employed in this study. Lower dissolved-oxygen concentrations were not tested but indeed should be assessed if concentrations below 20% are expected in any culture system used for vector production, as any oxygen limitation or deprivation could reduce cell viability by inducing apoptosis (Mercille and Massie, 1994; Simpson et al., 1997).

Serum was found to have a dose-dependent negative effect on vector titre over short-term culture. Reduced serum levels were found to significantly increase cell specific vector titres, however, where the medium was left unchanged, the difference in titre between cultures in 1% or 5% serum was lost after a few days. When this study was repeated, with daily medium changes, the negative effect of higher serum concentrations was apparent throughout culture. It is possible that where the medium was unchanged, inhibitory factors within the serum were either depleted or decayed with time and hence no difference was observed after a few days (between cultures in 1% or 5% serum). In contrast, where the medium was replaced with fresh on a daily basis, lost inhibitory factors would have been continuously replaced. This could explain why the negative effect of serum was apparent throughout culture in the latter study.

While reduced serum (1%) was found to significantly increase vector titres, compared to 5% serum, this serum level was found not to be sufficient to sustain cell cultures for longer than one week. For this reason, a low serum concentration is not suitable for increasing vector production in long-term cultures. Rather, it is recommended that the serum levels be reduced

prior to vector harvest in short-term cultures (less than one week). In addition to a significant increase in vector titre, reduced serum levels may also aid down-stream processing of vector stocks where the removal of proteins of animal origin is desirable.

It has been reported that retroviral vectors can be inactivated by complement or antibody components within serum (Takeuchi et al., 1994; Mason et al., 1999). In order to confirm that the negative effect of serum observed in the above studies was not due to extra-cellular inactivation of the vector particles, the stability of vector stocks with and without serum was compared, as mentioned previously. The vector decay rate was not significantly different in the presence or absence of serum, indicating that the negative effect of serum is *not* due to extra-cellular inactivation of vector particles. Furthermore, vector titres were not significantly different in cultures using heat-inactivated serum, compared to non-treated serum. The components in serum that could potentially cause extra-cellular vector particle inactivation would themselves be denatured after heat-treatment, therefore an increase in vector titre would be expected in cultures containing heat-inactivated serum, compared to non-treated serum. Since this was found *not* to be the case, this finding again confirms that serum did not reduce vector titres through extra-cellular inactivation of vector particles. It is suggested that the negative effect of serum on vector titre is due to inhibition of vector production or maturation, however the mechanism of this remains unknown.

Although not statistically significant, there was a slight *decrease* in the vector titre during culture in heat-inactivated serum. If this is indeed a real effect, this could be due to loss of nutrient/growth factor activity of various serum components during heat-treatment.

A range of serum-free media (commercially available or under development by the manufacturers) were assessed for their ability to support cell growth and vector production in FLYRD18/LNC-hB7 cell cultures. It was hoped that the use of a defined serum-free medium would eliminate the negative effects of serum on vector titre, whilst supplying essential nutrients to allow survival of the cells in culture. However, none of the serum-free media tested proved to be able to sustain cell growth during long-term culture. During the first *few days* of culture, the *cell specific* vector titres in a number of the tested media were similar to that measured in serum-containing cultures. Hence, while not suitable for long-term production of retroviral vectors, these media could be used to replace serum-supplemented medium prior to harvest of vector stocks from batch cultures as a means of eliminating serum from the final product.

Vector titres were *not* dramatically increased after the addition of sodium butyrate to the culture medium of FLYRD18/LNC-hB7 cells. In fact, where cells were exposed to this additive for more than 24h a cytotoxic effect was observed. Sodium butyrate is known to disrupt cell cycle progression, (Pagès et al., 1995), thus explaining the cell death during long term exposure to this additive. Despite this apparent cytotoxicity, a number of groups have demonstrated markedly increased vector titres after short-term (overnight) exposure of packaging cells to sodium butyrate, (reviewed in Chapter 1). Pagés et al. (1995) suggested that sodium butyrate can increase vector titres through modulation of MoMLV LTR expression. Despite the selectable marker gene of the FLYRD18/LNC-hB7 cell line being under the control of the MoMLV LTR, we observed only slight increases in vector titre after overnight exposure to *low* concentrations of sodium butyrate (higher concentrations resulted in reduced vector titres due to cytotoxic effects). At these low concentrations, the cell

specific titres were calculated to be greater than that of control cultures. This apparent increased titre *per cell* is probably misleading, as the cell number in cultures with sodium butyrate were significantly reduced and hence cells that were later killed in the presence of this additive could have previously produced a number of the vector particles harvested. Cruz et al (2000a) demonstrated only a small increase in vector titre produced by PA317 packaging cells in the presence of sodium butyrate. This group also found a decrease in cell number (due to the inhibitory effects of this additive) hence the cell specific titres were calculated to be higher in the presence of sodium butyrate.

Pagès et al., (1995) claimed that the modulation of the MoMLV LTR is dependent on the site of integration. This could explain the differences in responsiveness of a number of cell lines to sodium butyrate addition. Indeed, the responsiveness of different cell *clones* to sodium butyrate addition can vary 100-fold (Olsen and Sechelski, 1995). The dramatic increase in vector titre after addition of sodium butyrate seen by other groups could also be due to the low titres reported without this additive. For example, Olsen and Sechelski (1995) reported a 1000-fold increase in vector titre after sodium butyrate addition in cultures that previously produced very low titres. Where titres are already relatively high without sodium butyrate addition (as for Cruz et al., 2000a), other factors may well be limiting any further increase in vector titre, hence sodium butyrate may not have such a dramatic effect, if any, on vector titre in these cultures.

The above argument could also fall true for dexamethasone addition. We observed only slight increases in vector titres in the presence of dexamethasone, whereas Pagès et al. (1995) reported 7-10 fold increases in the presence of dexamethasone and sodium butyrate.

Interestingly, this group reported no effect of dexamethasone alone. The synergistic effect of dexamethasone and sodium butyrate was not observed in FLYRD18/LNC-hB7 cell cultures, probably due to the sensitivity of this cell line to sodium butyrate induced cell death.

The addition of fibroblast growth factor (FGF) to cultures of FLYRD18/LNC-hB7 cells in serum-supplemented medium gave no improvement on cell growth or vector production. It is likely that sufficient concentrations of FGF exist in the serum added to these cultures such that this growth factor is not limiting under the conditions employed in this study. However this does not rule out any potential benefits of using FGF in, for example, serum-free cultures or higher cell density cultures where growth factors may be limiting. This is also likely to be true for peptone additions, which also showed no benefits for cell growth or vector production during culture in serum-supplemented medium.

Chapter 5:

Alternative Culture Systems for Retroviral Vector Production

5.0 Introduction

The aim of this work was to assess culture systems alternative to simple monolayer cultures, which may be more suitable for larger scale production of retroviral vectors. The anchorage-dependence of the FLYRD18/LNC-hB7 cell line prohibits the use of typical suspension cultures, more suited for large volume cultures. Furthermore, studies in Chapter 3 showed the susceptibility of the FLYRD18/LNC-hB7 cell line to agitation during microcarrier culture caused cell death. For this reason, culture of these cells on suspended microcarriers in a stirred tank fermenter is also unlikely to be a suitable method for large-scale production of retroviral vectors. We therefore investigated the use of a packed bed culture system. In addition, the use of an alternative culture substratum with an expanded surface area was investigated.

Packed Bed

Packed bed systems have been used for the large-scale culture of a number of anchorage dependent mammalian cells (Looby and Griffiths, 1990). These systems offer a number of advantages including low surface shear, high cell density and productivity, ease of separation of product from cells, prolonged production times and potential for radial scale-up. Disadvantages include the poor homogeneity of the systems (though this may have less impact on products harvested from the supernatant, such as retroviral vectors), channel blockage and little potential for linear scale-up. The use of a packed bed system where the cells are immobilised on porous carriers (ImmobaSil™) was investigated as a potential method for large-scale production of retroviral vectors.

The packed bed system used throughout this chapter has already been well characterised (Bratch, 2000). However, in these latter studies, Fibra-Cel™ carriers were used, whereas in most of the present studies ImmobaSil-G ™ carriers were used. The effect of using a different carrier type on the media flow and oxygen transfer characteristics within the packed bed system was therefore assessed.

Expanded Surface Area T-Flasks

The porous silicone material of ImmobaSil™ carriers offers the benefits of a large surface area. This material has been incorporated into a tissue culture flask (T-flask) such that the culture surface (base) is comprised of porous silicone. This dramatically increases the available surface area for cell growth, thereby providing a potential means for scale-up of vector production by cells grown in these flasks. The cell growth and virus production in these flasks (prototypes constructed by Ashby Scientific Ltd.) was compared to standard T-flasks in order to assess their suitability as an intermediate scale-up of retroviral vector production.

5.1 Materials and Methods

5.1.1 Packed Bed

The packed bed system consists of a cylindrical glass reactor with medium entry and exit ports at the bottom and top of the reactor respectively (Figure 5.1.1). Medium is continuously circulated through the reactor via a peristaltic pump. In addition, a sampling port for removal of carriers (to check viability of attached cells during culture) is located on the side. The centre of the reactor contains coiled silicone tubing for delivery of oxygen

along the whole length. Carriers (ImmobaSil™ or Fibra-Cel™) were packed into the reactor around this silicone tubing.

5.1.2 Packed Bed Characterisation

Fluid Flow

A stimulus-response technique was used to determine the Residence Time Distribution (RTD) of fluid elements passing through the reactor. The information given by the RTD in continuous flow reactors is described in the discussion (Section 5.3). The packed bed vessel was filled with approximately 5.5×10^4 ImmobaSil-G™ porous silicone carriers (Ashby Scientific Ltd., U.K.) before filling the reservoir with 200ml water and re-circulating at 6 ml/min. A tracer dye, 0.5% Phenol Red solution (1ml), was injected as rapidly as possible into the feed stream, immediately adjacent to the inlet. Samples were subsequently taken immediately adjacent to the outlet, at one-minute intervals. The absorbance of each sample was read at 470nm, using a spectrophotometer (Pharmacia LKB Ultrospec III). This data was used to plot the concentration of dye in the outlet stream as a function of time, in order to calculate the RTD of this dye bolus. This experiment was repeated using open perfusion as opposed to closed, at the same fluid flow rate.

Oxygen Transfer

This experiment was conducted at 37°C, in order to obtain the same dissolved oxygen levels at air saturation as would be expected under normal culture conditions. A polarographic dissolved oxygen probe (Ingold) connected to an amplifier (LSL Ltd., Luton) was used for dissolved oxygen measurements. This probe was calibrated using medium saturated with

nitrogen gas for 0% dissolved oxygen and medium saturated with 5% CO_2 in air for 100% dissolved oxygen, before placing within the medium reservoir. The packed bed was filled with approximately 5.5×10^4 ImmobaSil-G™ carriers and 500ml of medium re-circulated through the vessel at 6ml/min. The whole system was initially purged with nitrogen to remove the dissolved oxygen from the re-circulating medium. Once the dissolved oxygen levels reached zero, the nitrogen source was removed and 5% CO_2 in air passed through the silicone tubing at approximately 20ml/min. The increasing dissolved oxygen levels were recorded every minute until saturation was reached. This data was used to determine the oxygen transfer coefficient in the packed bed system under these conditions.

5.1.3 Retroviral Vector Production in Packed Bed Cultures

Each packed bed was filled with 85ml settled volume of ImmobaSil-G™ porous silicone carriers, equivalent to approximately 5.5×10^4 carriers (Ashby Scientific Ltd., UK), except one study where 6g Fibra-Cel™ carriers (polyester fibre discs) were used. After addition of the carriers, the packed beds were sterilised in an autoclave at 121°C for 15 minutes.

Prior to inoculation of the packed bed with FLYRD18/LNC-hB7 cells, 100ml culture medium (DMEM: F-12 + 5% FCS) was re-circulated through the packed bed (containing sterile carriers) at 37°C for 30 minutes. Meanwhile the cells were detached from the T-flasks by trypsin digestion. The rinse medium was removed from the packed bed and the cells, suspended in fresh culture medium, were added through the top port (typically 1.8×10^8 cells in total). The packed bed system was rotated during this inoculation period to aid uniform cell attachment to the carriers. The percentage cell attachment was estimated by determining the number of unattached cells remaining in the medium (using haemocytometer counts).

When over 80% of the cells were attached (typically 4 hours post inoculation), the packed bed was connected to a reservoir containing an appropriate volume of culture medium to give a final cell concentration 3.6-6.0 × 10^5 cells/ml. This medium was re-circulated at a flow rate of 6 ml/min. Medium samples were taken daily for virus titre and glucose measurements and the culture medium changed for fresh every 1 or 2 days. Each sample was assayed twice for virus titre.

An exception to the above method involves *open* perfusion of the culture medium. In one study, the medium was perfused at a flow rate of 21.6 ml/h into a separate harvest bottle kept on ice, outside the incubator (Figure 5.1.3.1). This flow rate was chosen in order to obtain the same volume of medium per cell, per day, as used in all previous packed bed cultures. The aim of this study was to store the virus-containing supernatant at a lower temperature in order to reduce virus decay before collection.

A further exception to the above method is the use of a controller ('Maestro', Biolafitte, France) which was connected to the medium reservoir of a packed bed system (Figure 5.1.3.2). In this study, the temperature, pH and dissolved oxygen level in the medium reservoir was maintained at 32°C, pH 7.2 and 50% dO_2 respectively. The temperature of the medium reservoir (outside the incubator) was maintained at 32°C through the use of a water jacket, whilst the packed bed containing the cell-seeded carriers was kept within an incubator at 37°C. The pH was maintained throughout the culture period by the addition of 1M sodium hydroxide or CO_2 gas (monitored with a pH probe and controlled through the Maestro control unit). The dissolved oxygen levels were likewise monitored and controlled by the addition of oxygen or nitrogen gas.

Cell numbers in each packed bed culture were estimated by taking carrier samples and performing an MTT assay as described in Section 2.

5.1.4 Cell Growth and Retroviral Vector Production in ImmobaSil Flasks

FLYRD18/LNC-hB7 cells were inoculated into T-25 tissue culture flasks (surface area of base: 25cm^2), either with a standard tissue culture grade polystyrene culture surface, or with a porous silicone (ImmobaSil) culture surface. Cells were inoculated at 1×10^5 cells/ml and cultured at 37°C for 13 days. Vector titre and cell number in each culture were compared.

Another study was performed in order to determine whether the ImmobaSil flasks could be re-used. One flask from the first experiment was rinsed in sterile PBS (after removal of the cells by trypsin digestion) and then soaked for 30 min in 70% (v/v) ethanol. The ethanol was removed and the flask was left to dry (under aseptic conditions). This flask was used, as above, in a 4-day culture. The cell number and vector titre from this culture was compared to that from a 'fresh' ImmobaSil flask. Cells were released from the flasks by trypsin digestion and counted using a haemocytometer. Two successive digestions were carried out to ensure all cells were removed. The second digestion released only 4% of the total cell yield.

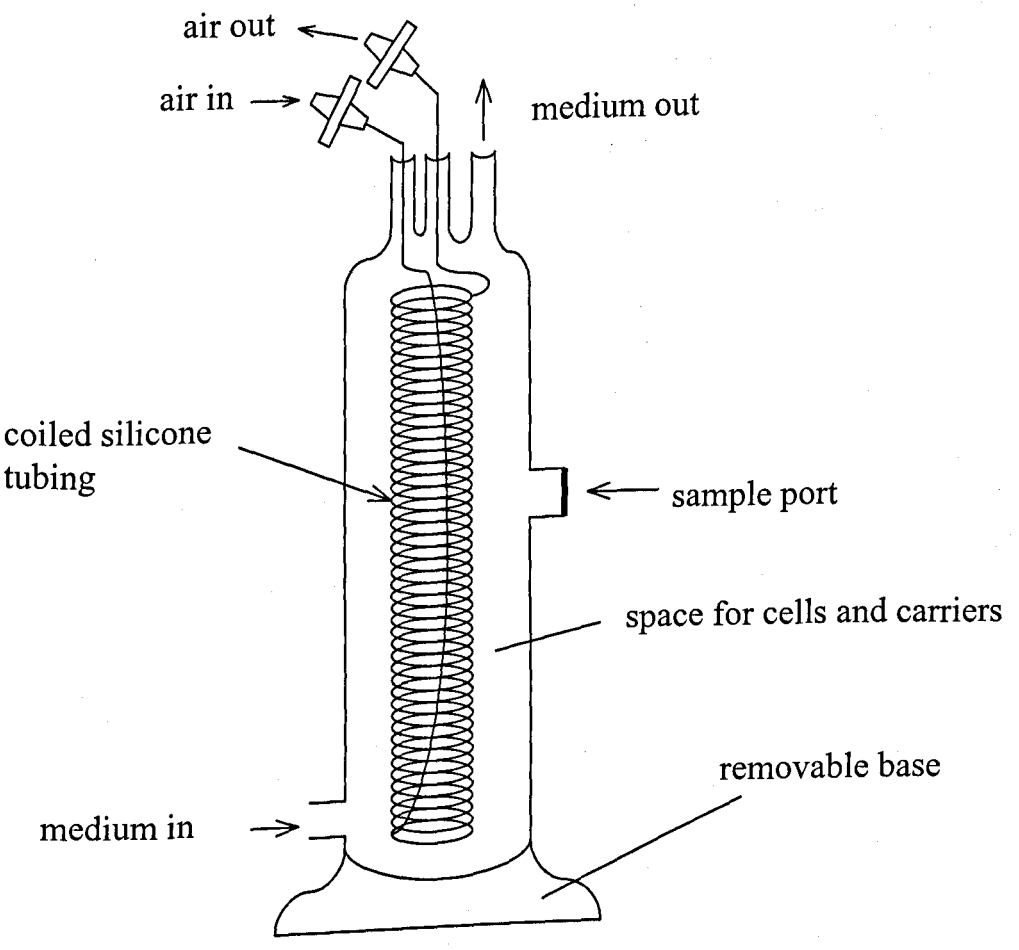


Figure 5.1.1

Schematic of packed bed bioreactor. Air is passed through silicone tubing via filters (0.2μm pore size).

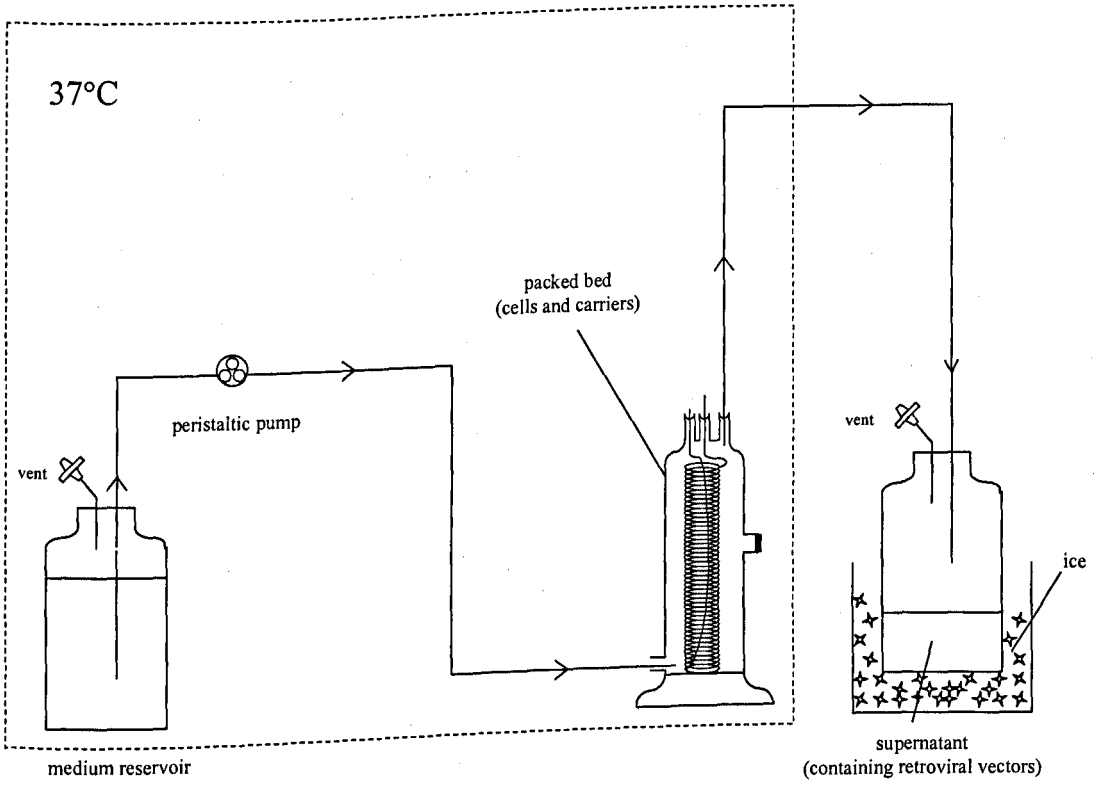


Figure 5.1.3.1

Schematic diagram of packed bed system with open perfusion of culture medium onto ice. Feed medium and immobilised cells in packed bed are maintained at 37°C, while the culture supernatant is collected and stored at 0°C until harvested.

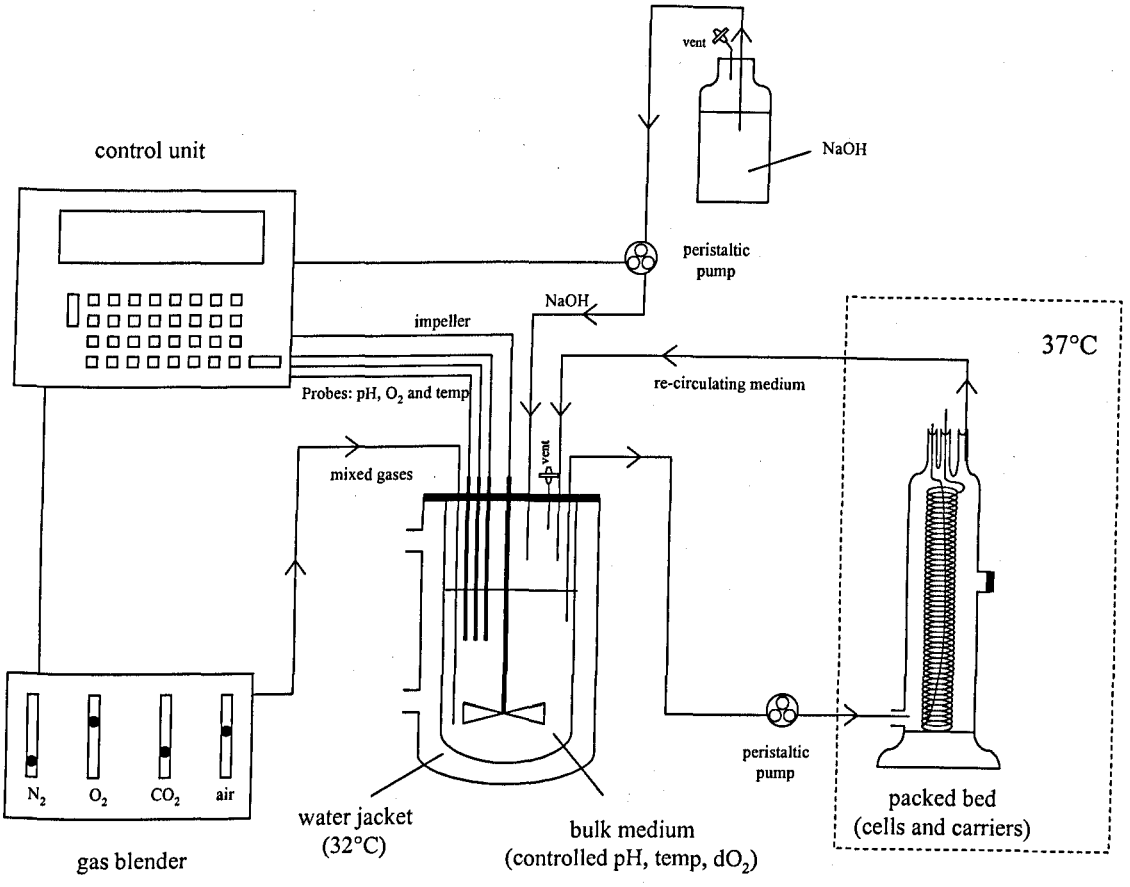


Figure 5.1.3.2

Schematic diagram of 'controlled' packed bed system. Medium is perfused through packed bed (closed circulation) via a medium reservoir. Dissolved oxygen concentration, pH and temperature of medium in the reservoir is monitored by relevant probes and the parameters maintained at set levels by a control unit (through the addition of O_2 or N_2, CO_2 or NaOH and a water jacket, respectively).

5.2 Results

5.2.1 Packed Bed Characterisation

Fluid Flow

A bolus of Phenol Red (1ml) was injected into the inward stream of a packed bed, during perfusion, and the dye concentration (absorbance at 470nm) in the effluent stream measured as a function of time. Figures 5.2.1.1 and 5.2.1.3 show the absorbance of samples from the effluent stream versus time during closed and open perfusion in the packed bed, respectively. These plots, representing the concentration of tracer exiting the system as a function of time, are referred to as C curves. The data from these curves can be normalised as follows:

$$\int_0^\infty C(t)\, d(t) = 1$$

Since the area under the C curve represents the total amount of tracer added, the data can be normalised by dividing the tracer concentration at time t, C(t), by the area under the curve. These values represent the age distribution function, E(t), of fluid elements leaving the packed bed (Fogler, 1992; Levenspiel, 1972). Hence:

$$E(t) = C(t) / \int_0^\infty C(t)\, d(t)$$

Where C(t) is the tracer concentration at time t and the integral in the denominator is the area under the C curve. The area under the curve in Figures 5.2.1.1 and 5.2.1.3 was determined using Simpson's graphical rule, which can be used for curves which fit a zero, first, second or third order polynomial function (Fogler, 1992). Figures 5.2.1.2 and 5.2.1.4 show E(t) versus time in the packed bed during closed and open perfusion respectively. The information given by these residence time distribution curves (E curves) is described in the discussion (Section 5.3.1).

It should be noted that during *closed* perfusion, the absorbance levels will not fall back to those measured before injection of the tracer because the 1ml bolus of Phenol Red has become mixed with the bulk of re-circulating fluid in the reservoir. This is illustrated in Figure 5.2.1.1.

Oxygen Transfer

The packed bed system was purged with nitrogen gas to remove all dissolved oxygen from the re-circulating medium. When the dissolved oxygen level reached zero, 5% CO_2 in air was passed through the silicone tubing at 20 ml/min. Figure 5.2.1.5 illustrates the increase in dissolved oxygen levels in the reservoir bottle, as a function of time. This data was used to construct Figure 5.2.1.6, where the natural log of the maximum dissolved oxygen level (95%) minus the percentage dissolved oxygen at time t, was plotted as a function of time. The slope of this plot was used to calculate the oxygen transfer coefficient (K_T) in the packed bed system under these conditions, using the following equation (Boraston et al., 1984; Hu et al., 1986; Bratch, 2000):

$$\text{Slope of } \ln(D100\%-Dot) \text{ vs. time} = -aK_T$$

$$\text{Hence, } \mathbf{K_T = slope / -a}$$

$$(a = A/V_T)$$

Where A is the total surface area of the silicone tubing (149.9cm^2) and V_T is the total volume of re-circulating medium (500ml). Hence 'a' equals 0.2998cm^2/ml. The slope from Figure 5.2.1.6 is -1.6×10^{-4} sec^{-1}, hence K_T was calculated as

5.3×10^{-4}cm/s.

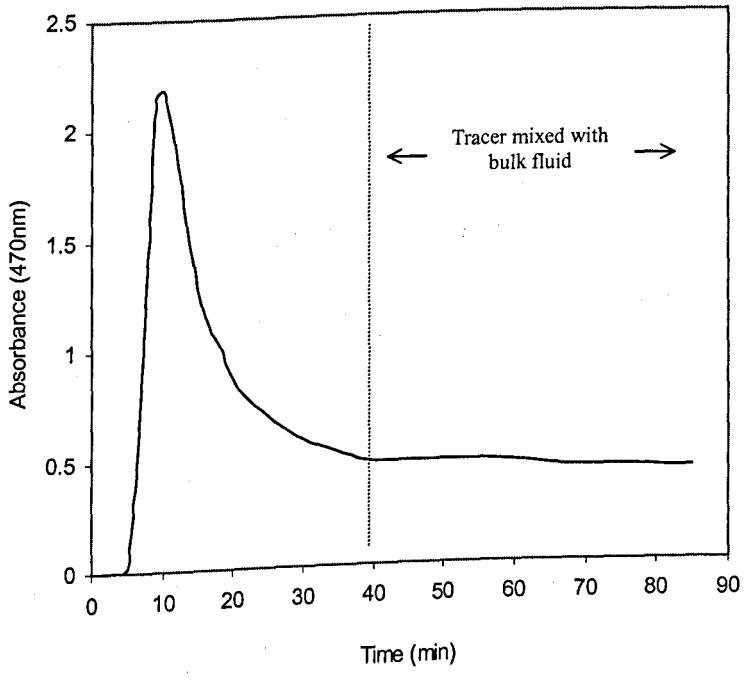


Figure 5.2.1.1

C curve: absorbance versus time for samples taken from the effluent stream in a packed bed after injection of a 1ml bolus of Phenol Red, during ***closed*** perfusion. Fluid flow rate of 6 ml/min and total fluid volume of 500ml. Packed bed filled with ImmobaSil-G™ carriers, void volume of 40ml.

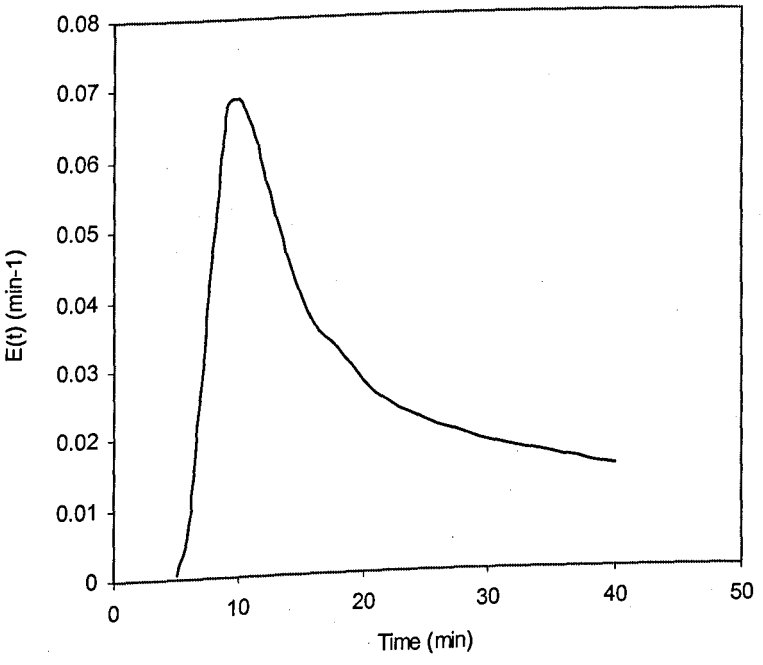


Figure 5.2.1.2

E curve: Residence Time Distribution in packed bed system during ***closed*** perfusion. Fluid flow rate of 6 ml/min and total fluid volume of 500ml. Packed bed filled with ImmobaSil-G™ carriers, void volume of 40ml.

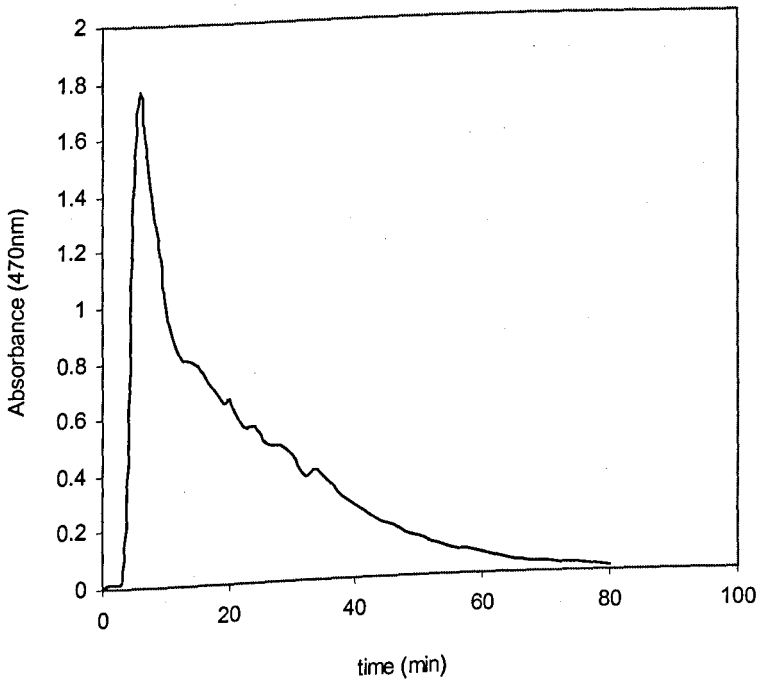


Figure 5.2.1.3

C curve: absorbance versus time for samples taken from the effluent stream in the packed bed after injection of a 1ml bolus of Phenol Red, during ***open*** perfusion. Fluid flow rate of 6 ml/min and total fluid volume of 500ml. Packed bed filled with ImmobaSil-G™ carriers, void volume of 40ml.

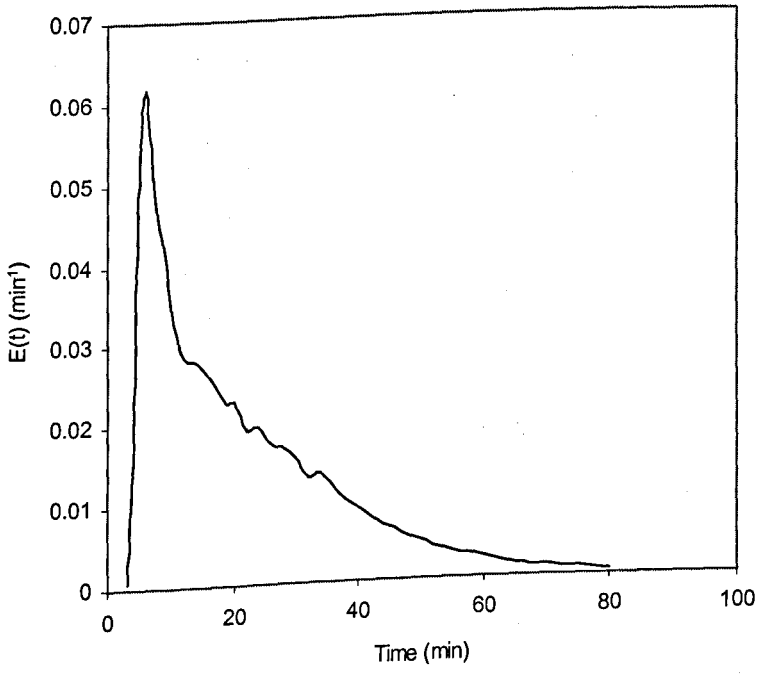


Figure 5.2.1.4

E curve: Residence Time Distribution in packed bed system during ***open*** perfusion. Fluid flow rate of 6 ml/min and total fluid volume of 500ml. Packed bed filled with ImmobaSil-G carriers, void volume of 40ml.

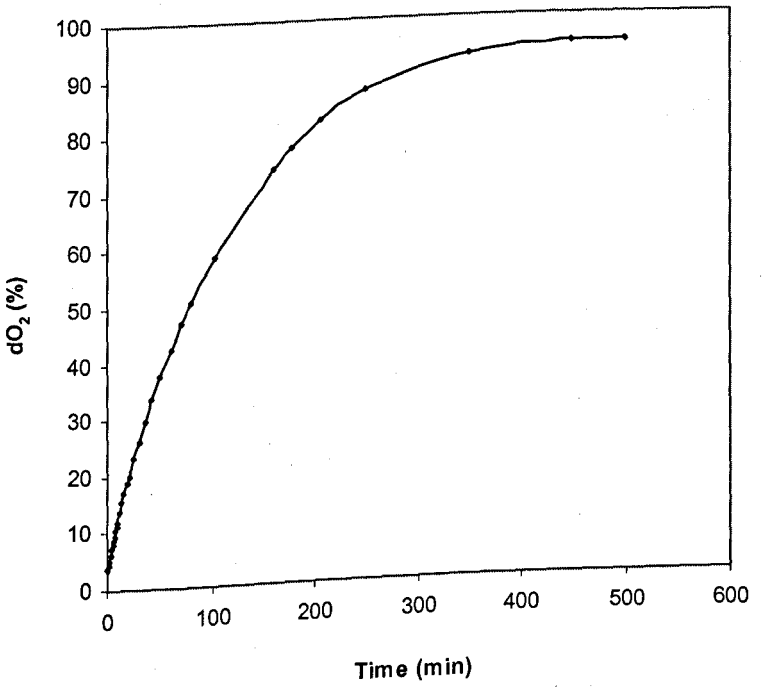


Figure 5.2.1.5

Dissolved oxygen levels in packed bed as a function of time, during perfusion of 5% CO_2 in air through silicone tubing at 20 ml/min (system initially purged with N_2). Packed bed was filled with ImmobaSil-G™ carriers and 500ml medium re-circulated through the system at 6 ml/min. Experiment conducted at 37°C.

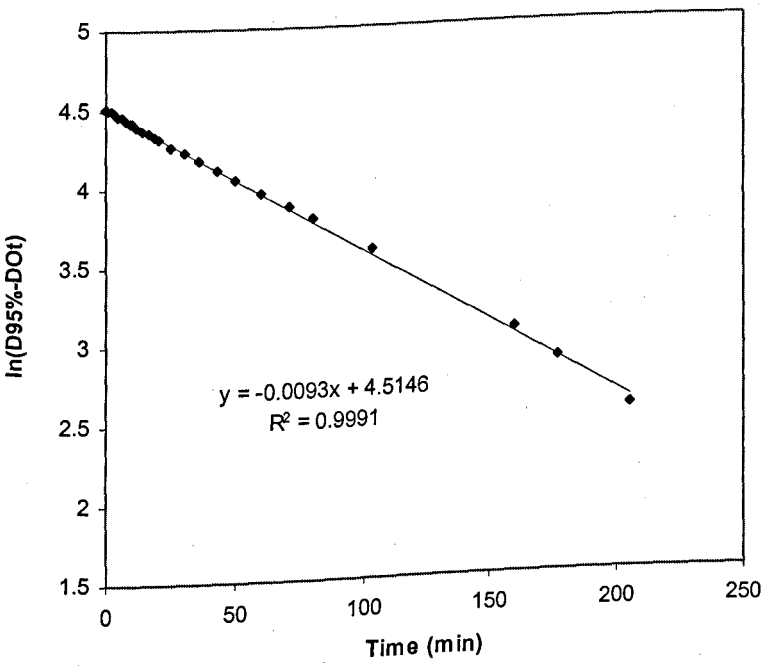


Figure 5.2.1.6

Natural log of maximum dissolved oxygen (95%) minus percentage dissolved oxygen at time t, plotted as a function of time. Data derived from Figure 5.2.5. The slope of this plot was used to determine the oxygen transfer coefficient in the packed bed system under these conditions.

5.2.2 Retroviral Vector Production in Packed Bed Cultures

Closed Perfusion at 37°C

The first packed bed culture was conducted at 37°C, where 6×10^5 cells/ml were cultured on ImmobaSil-G™ carriers during closed perfusion of the culture medium. The daily glucose levels and vector titres in the packed bed culture were compared to that of monolayer cultures from the same batch of cells, seeded at the same concentration. The glucose levels of the culture medium between daily changes, in both the packed bed and the monolayer cultures, were similar (Figure 5.2.2.1). Capiaumont et al., (1993) demonstrated a good correlation between cell growth and glucose depletion in culture medium. However, Zielke et al., (1984) claimed that in cultures with identical growth rates, the glucose utilisation was significantly lower when the initial glucose concentration was high (5mM) compared to cultures with low initial glucose concentrations (80μM). The use of glucose concentrations to monitor cell growth should therefore be used with caution. However, since the glucose levels did not drop below 7 mM, the data in Figure 5.2.2.1 indicates similar cell growth rates in the packed bed and monolayer cultures.

Scanning electron microscopy revealed the presence of attached cells throughout the pores of carriers removed from the packed bed on day 11. Some vacant areas were still present on the surface of some carriers (Figure 5.2.2.2), whereas some carriers were densely populated with attached cells (Figure 5.2.2.3).

On day 4, the daily vector titres in the packed bed culture were similar to those achieved in monolayer (packed bed titres were initially lower on day 2, but higher on day 3). From day 7 onwards, the titre appeared to decrease in the packed bed, whereas titres were maintained in

the monolayer cultures (Figure 5.2.2.4). An MTT assay of sample carriers (approximately 10 carriers per sample) on days 7 and 11 revealed no increase in cell number during this culture period (mean absorbance per carrier was 5.3×10^{-3}).

While vector concentrations were not greater in the packed bed, the total amount of active vectors collected from the packed bed system was obviously much greater than that collected from the monolayer cultures, due to the significantly larger volumes of culture medium harvested each day from the packed bed. Hence the volumetric productivity of the packed bed was 15-fold greater than that of the smaller scale T-flasks.

Open Perfusion onto Ice

A similar packed bed culture was conducted, using the same cell number as above, with *open* medium perfusion. The perfused medium was collected in a harvest bottle stored on ice outside the incubator (whereas the rest of the packed bed system was incubated at 37°C). A medium flow rate of 21 ml/h was selected in order to feed the cells with the same volume of medium over a 24 h period as was used in the above study (where the total volume was changed every 24 h). Figure 5.2.2.5 illustrates the daily vector titre in the packed bed culture, compared to monolayer cultures at 37°C using the same cell density. Initial daily vector titres in the packed bed culture were lower than those measured in the monolayer cultures. However, after day 3, titres in the packed bed reached or exceeded those in the monolayers. The maximum vector titre in this packed bed was achieved on day 3, and was almost 3-fold higher than the maximum titre of the earlier packed bed during closed perfusion at 37°C. Figure 5.2.2.6 shows the *relative* titres measured in both these packed bed cultures. Titres from each culture were calculated as a percentage of the maximum titre

achieved in the open perfusion system (measured on day 3). The increase in titre due to storage of the supernatant on ice, was not as high as would be expected from the increased vector stability at this lower temperature.

Closed Perfusion at 37°C - Fibra-Cel™ Carriers

In order to compare the performance of the ImmobaSil-G™ carriers to alternative commercially available carriers, a further packed bed, filled with 6g Fibra-Cel™ carriers (Bibby Sterilin, U.K.) was cultured at 37°C during closed perfusion. Slightly lower cell numbers were used in this study (3.9×10^5 cells/ml), approximately 65% of the cell concentration used in the previous studies. The maximum vector titre in this packed bed culture was approximately 60% of that measured in the previous packed bed culture using ImmobaSil-G™ carriers (Figure 5.2.2.7). Taking into the account the different cell densities inoculated into each system, the vector production per cell on Fibra-Cel™ and ImmobaSil-G™ carriers appears to be similar.

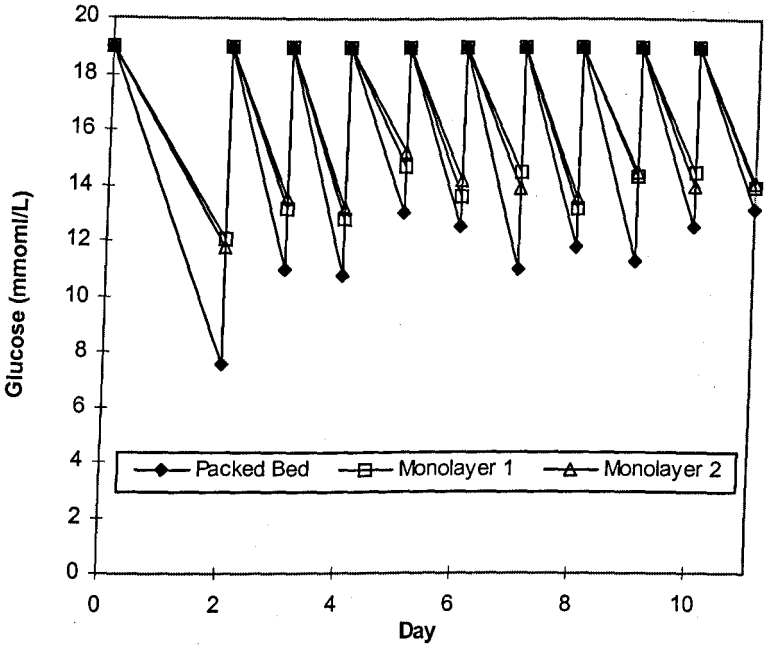


Figure 5.2.2.1

Glucose levels measured in the culture medium (changed daily) of packed bed and monolayer cultures of FLYRD18/LNC-hB7 cells. Packed bed culture conditions: 37°C, closed perfusion, ImmobaSil-G™ carriers.

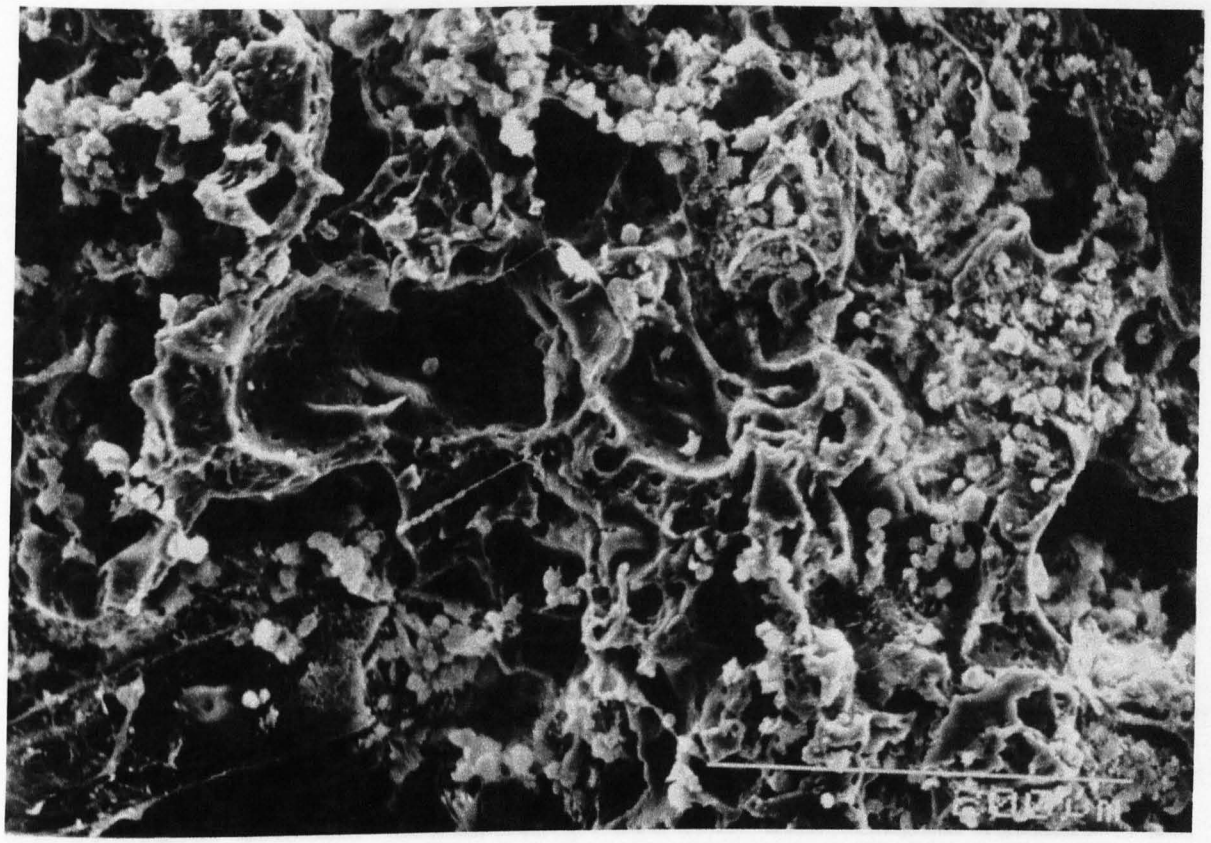


Figure 5.2.2.2

Scanning electron micrograph of an ImmobaSil™ carrier seeded with FLYRD18/LNC-hB7 cells and cultured in packed bed at 37°C (day 11). Bar represents 200μm.

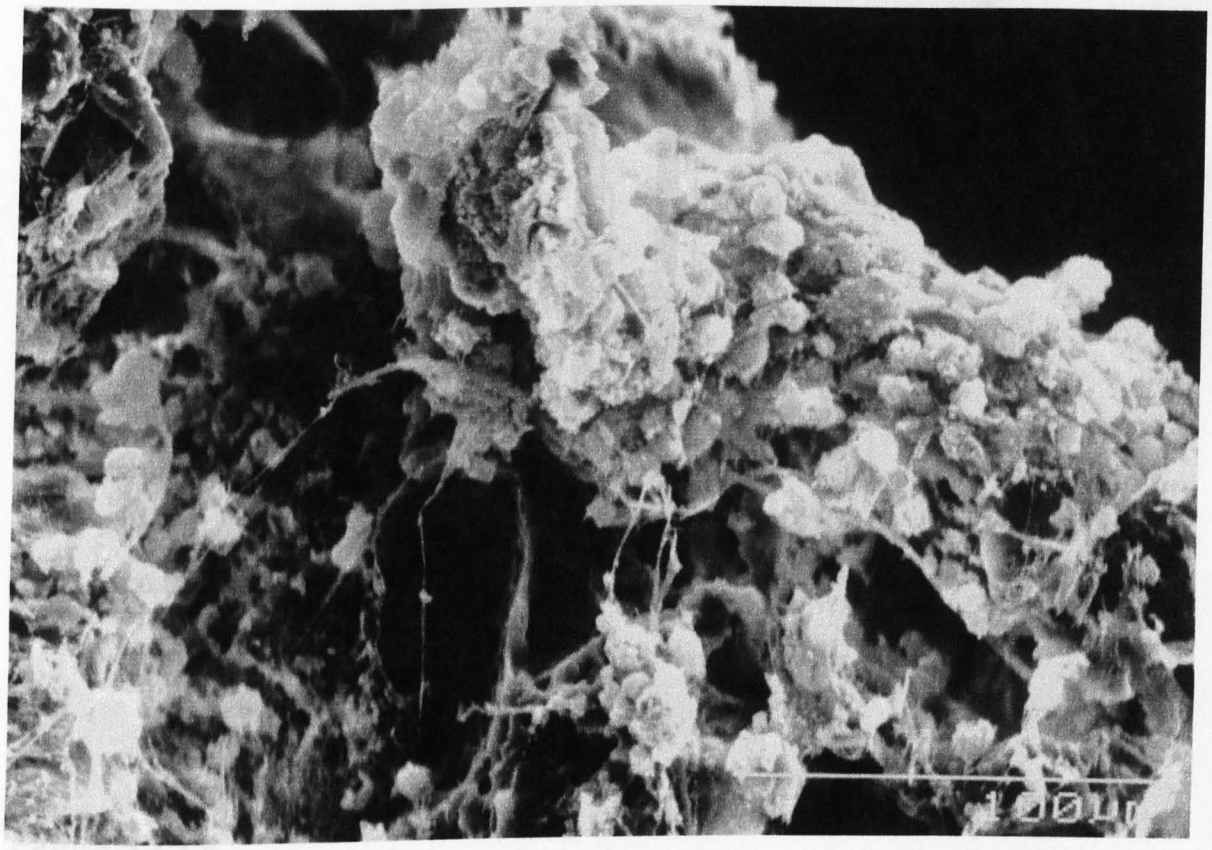


Figure 5.2.2.3

Scanning electron micrograph of an ImmobaSil™ carrier seeded with FLYRD18/LNC-hB7 cells and cultured in packed bed at 37°C (day 11). Bar represents 100μm.

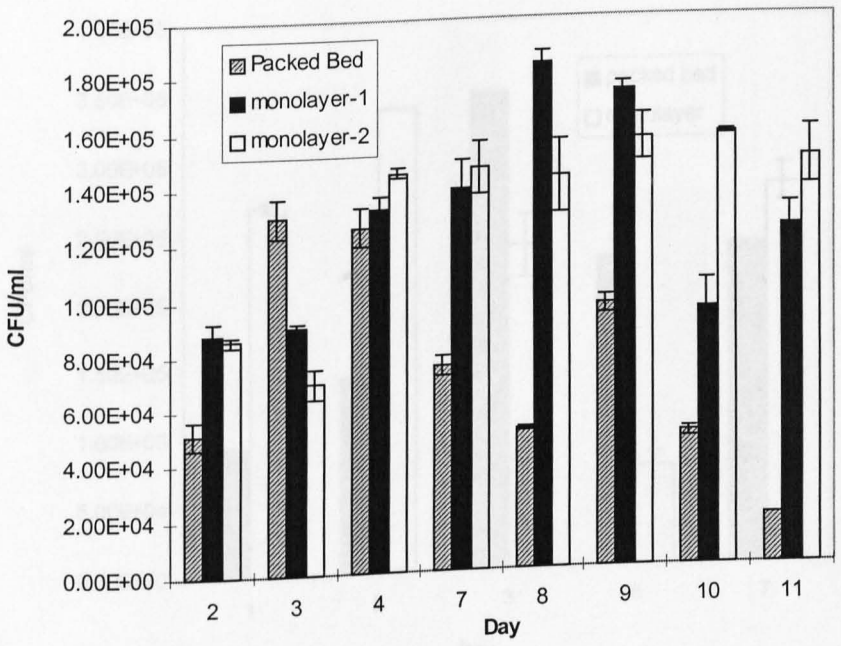


Figure 5.2.2.4

Daily vector titres in packed bed and monolayer cultures of FLYRD18/LNC-hB7 cells. Error bars represent observed range, n=2.

Packed bed culture conditions: 37°C, closed perfusion, ImmobaSil-G™ carriers.

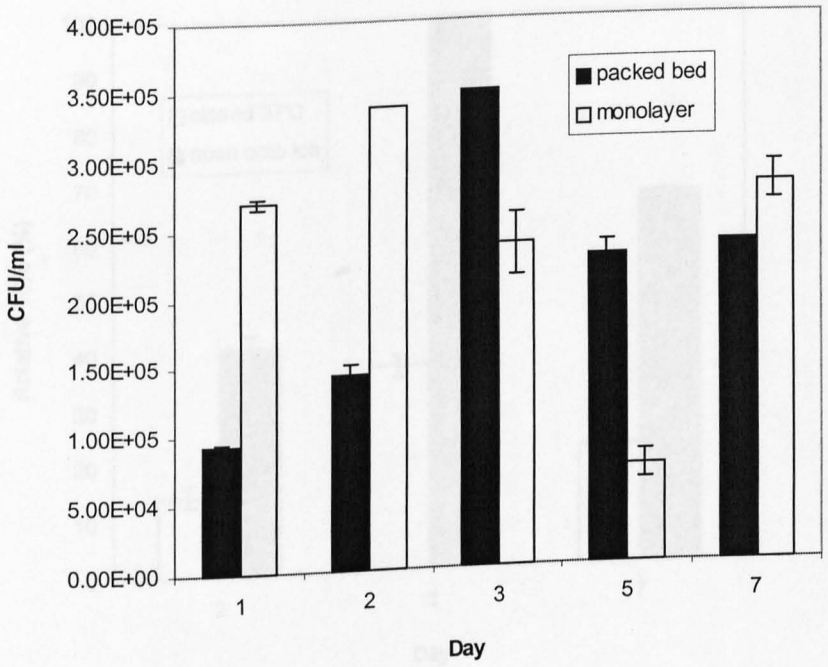


Figure 5.2.2.5

Daily vector titres in packed bed and monolayer cultures of FLYRD18/LNC-hB7 cells. Error bars represent observed range, n=2.

Packed bed culture conditions: 37°C, **open perfusion, supernatant harvested onto ice,** ImmobaSil-G™ carriers

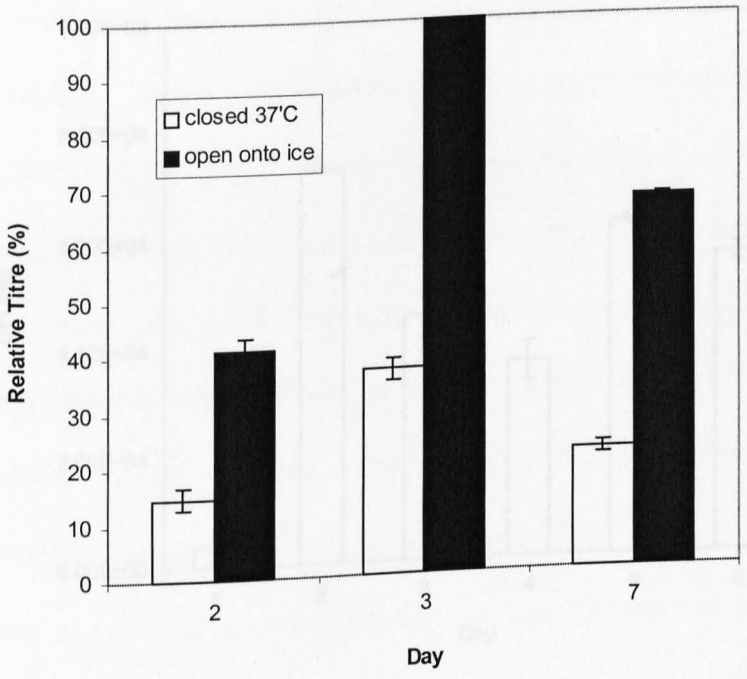


Figure 5.2.2.6

Comparison between virus titres in packed bed cultures during closed perfusion at 37°C or open perfusion with supernatant harvested onto ice. Relative virus titres were calculated as a percentage of the titre in the supernatant collected onto ice on day 3 (maximum titre measured). Error bars represent observed range, n=2.

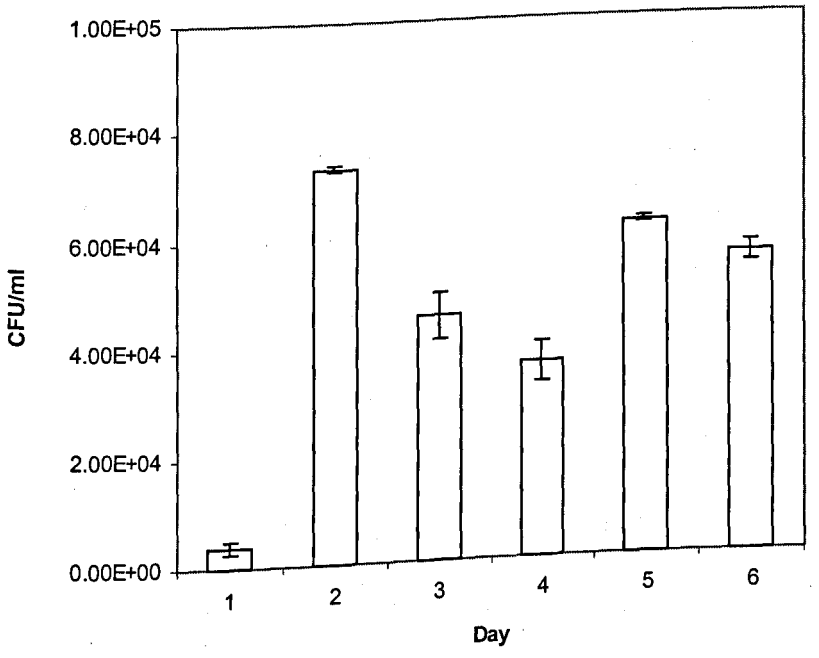


Figure 5.2.2.7

Daily vector titres in packed bed culture of FLYRD18/LNC-hB7 cells on **Fibra-Cel™** carriers (37°C; closed perfusion). Error bars represent observed range, n=2.

Closed Perfusion at 32°C

Studies in Chapter 4 demonstrate the ability to increase vector titres in monolayer cultures of FLYRD18/LNC-hB7 cells by reducing the culture temperature from 37°C to 32°C. However, it has been reported that the optimal temperature for retroviral vector production in monolayer may not be the same in bioreactor cultures (Andreadis et al., 1999). This surprising phenomenon may be due to the production of different levels of inhibitors in different culture systems. Therefore a packed bed culture was conducted at 32°C to determine whether similar increases in vector titre, to those observed in monolayer cultures, could in fact be achieved. A packed bed was cultured with 6×10^5 cells/ml (as for previous 37°C packed bed culture) with closed perfusion at 32°C. Figure 5.2.2.8 illustrates the daily glucose consumption in this packed bed system. As expected, less glucose was consumed by the cells cultured at 32°C, compared to 37°C. The daily vector titres increased throughout the culture period of 10 days (Figure 5.2.2.9). This was not observed in the previous packed bed cultures conducted at 37°C. The increase in titre was accompanied by an increase in absorbance per carrier, measured in an MTT assay conducted on sample carriers taken on day 3, 6 and 10 (Figure 5.2.2.10), indicating an increase in cell number throughout the culture period. Interestingly, such an increase in cell number was not observed in packed bed cultures at 37°C. The mean absorbance per carrier at 32°C was initially lower than that measured in carriers sampled from the 37°C culture (up to day 6). This is to be expected, as the cell growth rate of FLYRD18/LNC-hB7 cells is reduced at 32°C, compared to 37°C (see Chapter 4). However, by day 10 the mean absorbance per carrier was higher in the 32°C packed bed compared to day 11 in the 37°C packed bed (10×10^{-3} and 5.2×10^{-3} respectively). It is not expected that cell numbers at 32°C would be greater than those

reached at 37°C, hence this result is surprising. It is possible that cell numbers in the packed bed at 37°C reached a maximum between days 7 and 11, when carriers were not sampled for MTT assay. Cell numbers may have decreased by day 11 due to sub-optimal culture conditions. In contrast, the cell numbers in the packed bed at 32°C may reach a maximum at a later time point (due to the reduced cell growth rate at this temperature), hence a decrease in cell number was not measured before day 10.

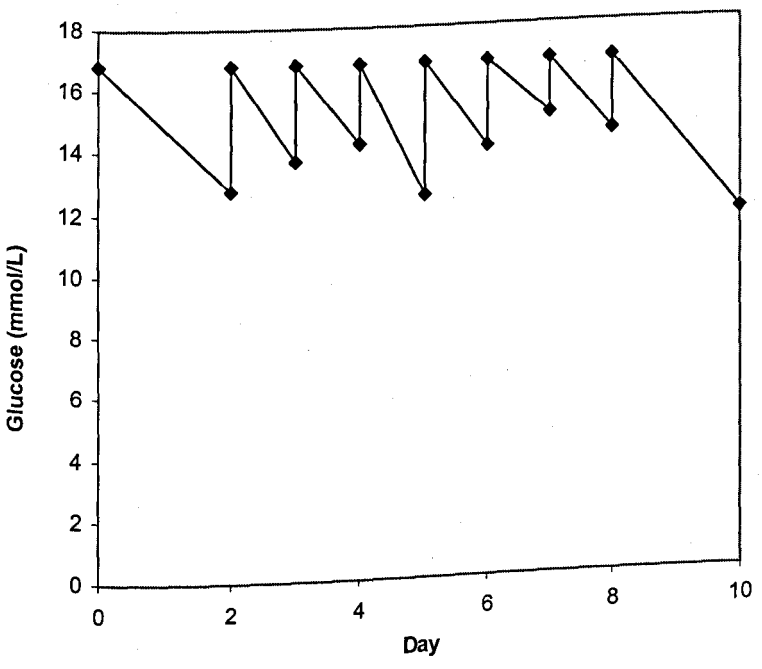


Figure 5.2.2.8

Glucose levels measured in the culture medium (changed daily) of a packed bed culture of FLYRD18/LNC-hB7 cells. Packed bed culture conditions: **32°C**, closed perfusion, ImmobaSil-G™ carriers.

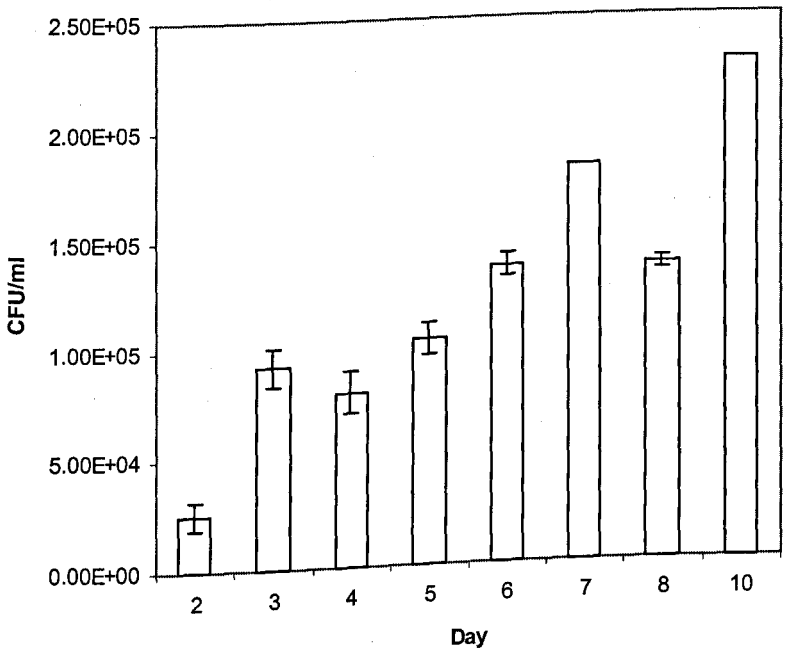


Figure 5.2.2.9

Daily vector titres in packed bed culture of FLYRD18/LNC-hB7 cells. Error bars represent observed range, n=2.

Packed bed culture conditions: **32°C**, closed perfusion, ImmobaSil-G™ carriers.

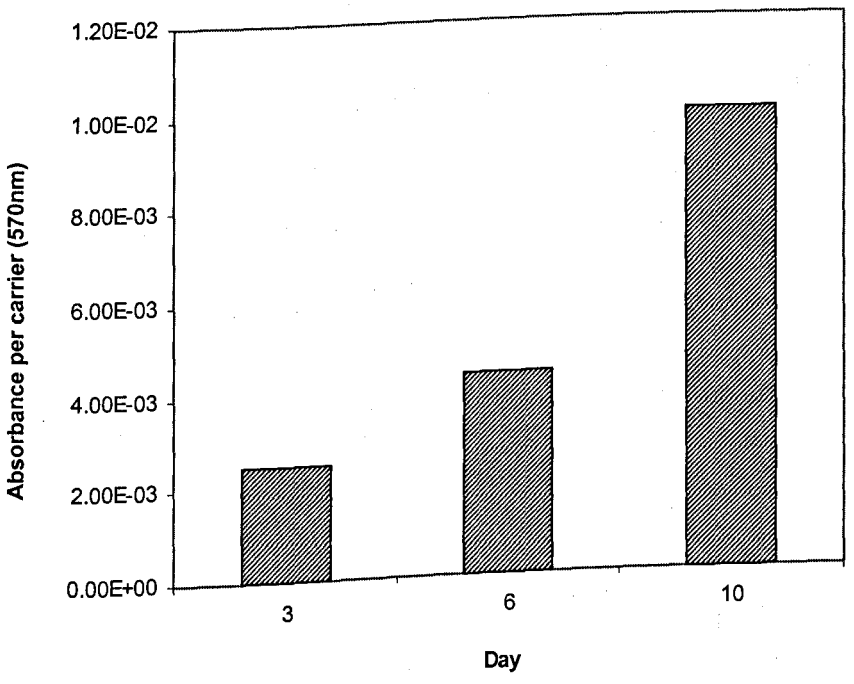


Figure 5.2.2.10

MTT Assay: Absorbance per carrier (mean of 10 carriers), as a function of time. Carriers sampled from packed bed culture of FLYRD18/LNC-hB7 cells: closed perfusion, 32°C, ImmobaSil-G™ carriers.

Long Term Packed Bed Culture

In order to determine whether the culture of FLYRD18/LNC-hB7 cells on ImmobaSil-G™ carriers within a packed bed system is suitable for the long term production of retroviral vectors, a similar closed perfusion culture was conducted for 35 days (inoculated with 3.6×10^5 cells/ml). The culture temperature was maintained at 37°C for the initial four weeks, then reduced to 32°C for the final week. As for previous packed bed cultures, the medium was changed for fresh daily. Vector titres were maintained between 1×10^4 and 2×10^5 cfu/ml throughout the 35-day culture period and again significantly increased after the culture temperature was reduced to 32°C (Figure 5.2.2.11). The mean daily vector titre increased from 3×10^4 cfu/ml at 37°C to 2×10^5 cfu/ml at 32°C respectively. The maximum vector titre was measured on day 35 at 32°C (2.5×10^5 cfu/ml).

A sample was also taken to determine the transduction *efficiency* of the vector preparation on Day 5. As described in Chapter 2, successfully transduced cells were detected by labelling vector-exposed target cells with an FITC-conjugated anti-human B7 antibody. A high proportion of the target cell culture was successfully transduced by this vector preparation (as indicated by an increase in fluorescence intensity with respect to control cultures not exposed to vector preparations). This demonstrates the ability of the packed bed system to allow the production of good 'quality' vector supernatants with similar transduction efficiencies to that produced in monolayer cultures (see Chapter 3). The distribution of fluorescence intensity per cell, compared to target cells not exposed to the vector preparation, is shown in Figure 5.2.2.12.

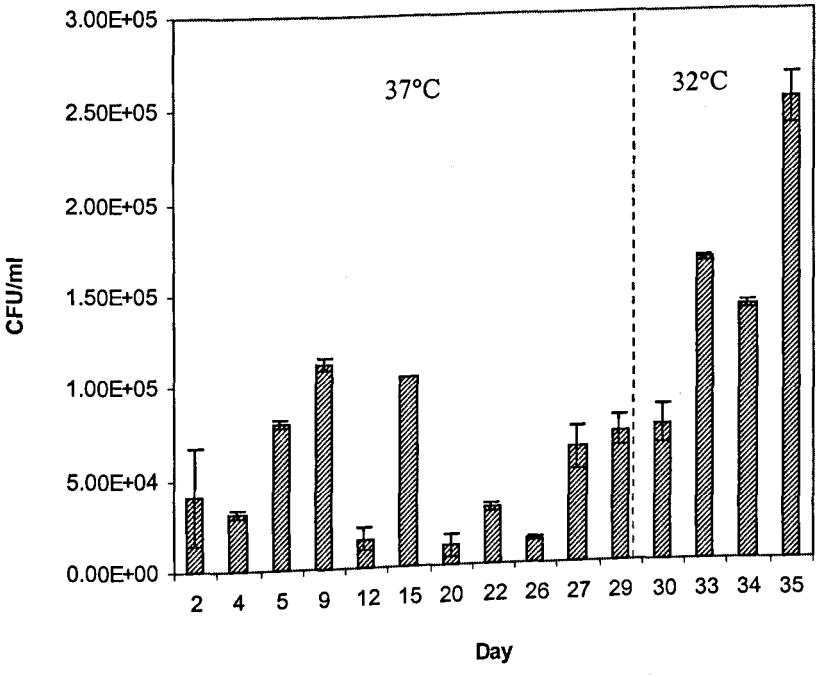


Figure 5.2.2.11

Daily vector titres during **long term** packed bed culture of FLYRD18/LNC-hB7 cells. Error bars represent observed range, n=2.

Packed bed culture conditions: closed perfusion, ImmobaSil-G™ carriers, 37°C for four weeks, followed by 32°C for one week.

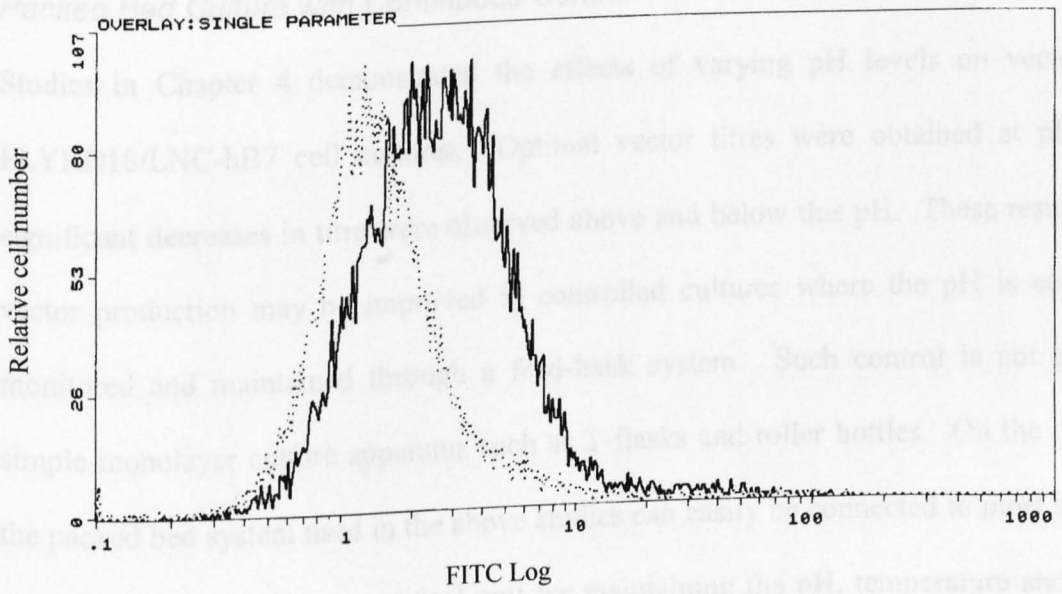


Figure 5.2.2.12

Distribution of fluorescence intensity of A2780cp cells labelled with FITC-conjugated anti-human B7 antibody (measured by flow cytometry) 48h after exposure to vector preparation, compared to control cultures not exposed to vector.

Vector preparation: day 5 of long term packed bed culture of FLYRD18/LNC-hB7 cells at 37°C.

Dotted line represents cells not exposed to vector preparation (control), solid line represents cells exposed to vector preparation for 8h.

Packed Bed Culture with Continuous Control

Studies in Chapter 4 demonstrated the effects of varying pH levels on vector titre in FLYRD18/LNC-hB7 cell cultures. Optimal vector titres were obtained at pH 7.2 and significant decreases in titre were observed above and below this pH. These results suggest vector production may be improved in controlled cultures where the pH is continuously monitored and maintained through a feed-back system. Such control is not possible in simple monolayer culture apparatus such as T-flasks and roller bottles. On the other hand, the packed bed system used in the above studies can easily be connected to most stirred tank fermenters which possess a control unit for maintaining the pH, temperature and dissolved oxygen levels in the medium. The culture vessel of such stirred tank reactors can be used as a medium reservoir for the perfusion of medium to and from a packed bed containing immobilised cells. The medium in this reservoir can be monitored by pH, temperature and dissolved oxygen probes and the conditions maintained at set levels via a control unit.

Such a packed bed culture (filled with ImmobaSil-G™ carriers) was inoculated with 3.6 x 10^5 cells/ml and the medium in the reservoir maintained at pH 7.2, 50% dissolved oxygen and 32°C. The packed bed vessel itself was placed within an incubator at 37°C. Hence the cells were maintained at a temperature optimal for cell growth, whereas the bulk of vector-containing supernatant was maintained at 32°C, in an attempt to reduce the vector decay rate. The system was initially tested with water alone in order to confirm that the medium leaving the reservoir and entering the incubator could reach 37°C before entering the packed bed vessel. This occurred at a flow rate of 4 ml/min. The bulk of medium in the reservoir was changed daily for fresh. Figure 5.2.2.13 shows the daily vector titre in this packed bed culture. Daily levels were less variable compared to previous packed bed cultures. The

mean vector titre was higher than that achieved in non-controlled packed bed cultures conducted at 32°C or 37°C (except where the supernatant was harvested onto ice).

At the end of the 11-day culture period, the medium in the packed bed system was replaced with MTT solution (5 mg/ml) which was re-circulated through the vessel at 37°C for 3 hours. Photographs of the MTT stained system were taken after 1 and 3 hours in order to assess the distribution of attached, metabolically active, cells throughout the vessel (Figures 5.2.2.14 and 5.2.2.15a). The intensity of purple formazan staining of each carrier was seen to vary throughout the packed bed, indicating a non-uniform distribution of viable cells within the culture. Such variation in staining was even more noticeable when a number of carriers were removed from the packed bed and laid adjacent to one another (Figure 5.2.2.15b). This variation in cell number per carrier is to be expected in such a 'non-ideal' reactor, as explained in Section 5.3.1.

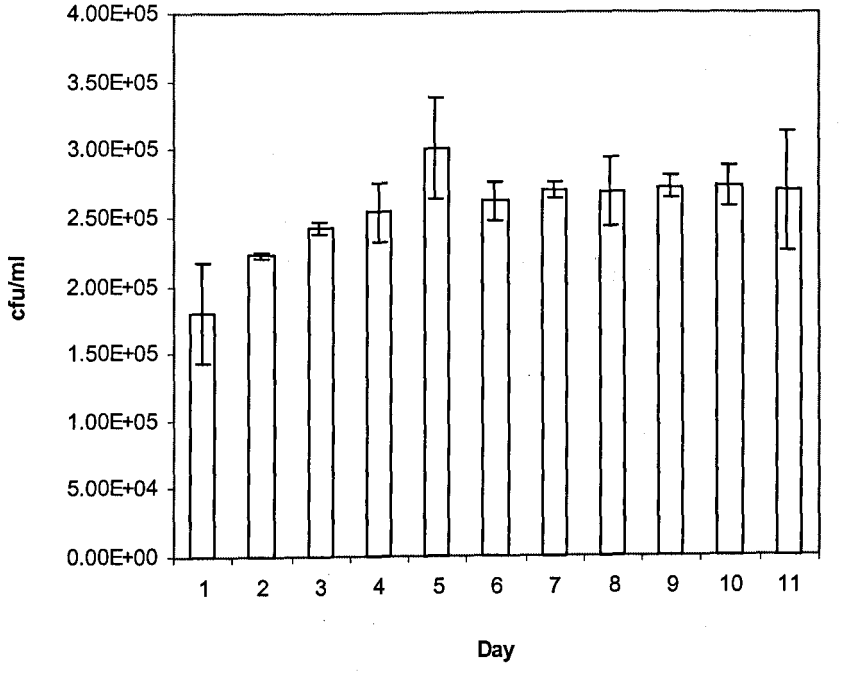


Figure 5.2.2.13

Daily vector titres during **controlled** packed bed culture of FLYRD18/LNC-hB7 cells. Error bars represent observed range, n=2.

Packed bed culture conditions: closed perfusion, ImmobaSil-G™ carriers, pH 7.2, 50% dissolved oxygen, cells at 37°C, bulk medium at 32°C.

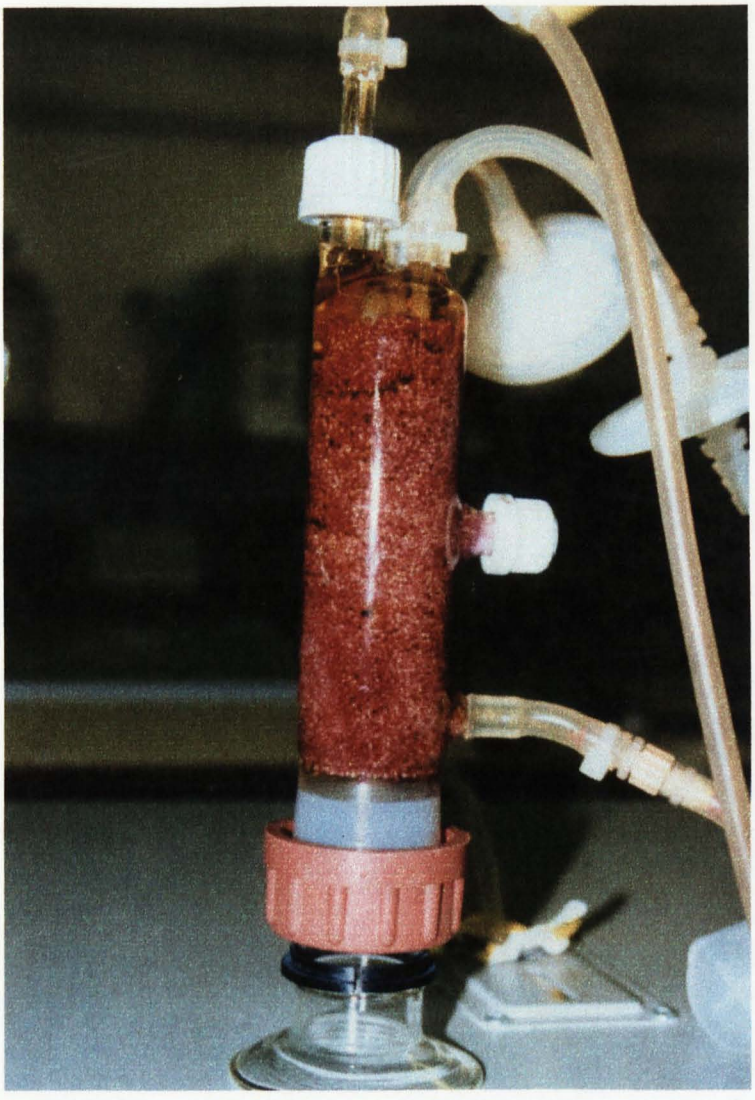


Figure 5.2.2.14

Immobilised cells in packed bed stained with MTT (5 mg/ml) on day 11. **One hour** at 37°C.

Note non-uniform distribution of purple formazan on carriers throughout system.

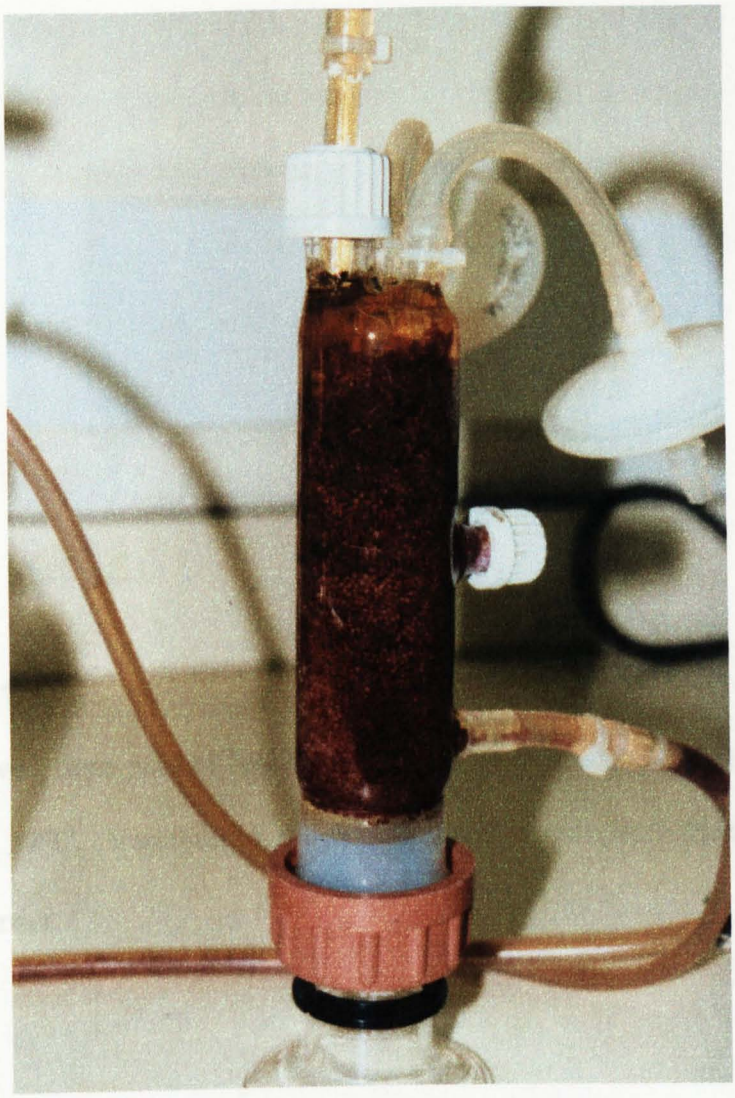


Figure 5.2.2.15a

Immobilised cells in packed bed stained with MTT (5 mg/ml) on day 11. **Three hours** at 37°C. Note non-uniform distribution of purple formazan on carriers throughout system.

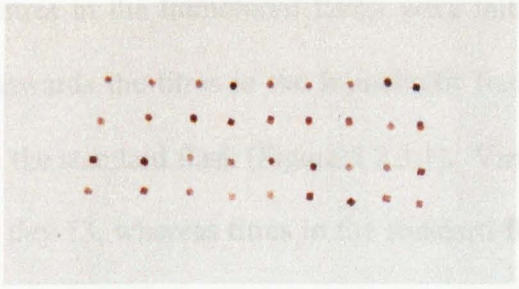


Figure 5.2.2.15b

Carriers removed from packed bed (Figure 5.3.20a) after exposure to MTT (5 mg/ml) for three hours at 37°C. Variation in staining intensity is evident, indicating variation in cell number per carrier.

5.2.3 Cell Growth and Retroviral Vector Production in ImmobaSil Flasks

Cell growth and vector production in ImmobaSil flasks was compared to that in standard T-flasks. While virus titres in the ImmobaSil flasks were initially lower than in the standard flasks, from day 6 onwards the titres in the ImmobaSil flask were equal to or greater than those harvested from the standard flask (Figure 5.2.3.1). Virus titres in the ImmobaSil flasks increased through to day 13, whereas titres in the standard flasks were reduced after 5 days. Cell numbers harvested from the ImmobaSil flasks and standard T-flasks were 5×10^6 and 7×10^6, respectively.

In a second experiment, an attempt was made to re-use an ImmobaSil flask. Despite similar cell numbers after 4 days (compared to culture in a fresh ImmobaSil flask), the virus titres were significantly lower (Figure 5.2.3.2).

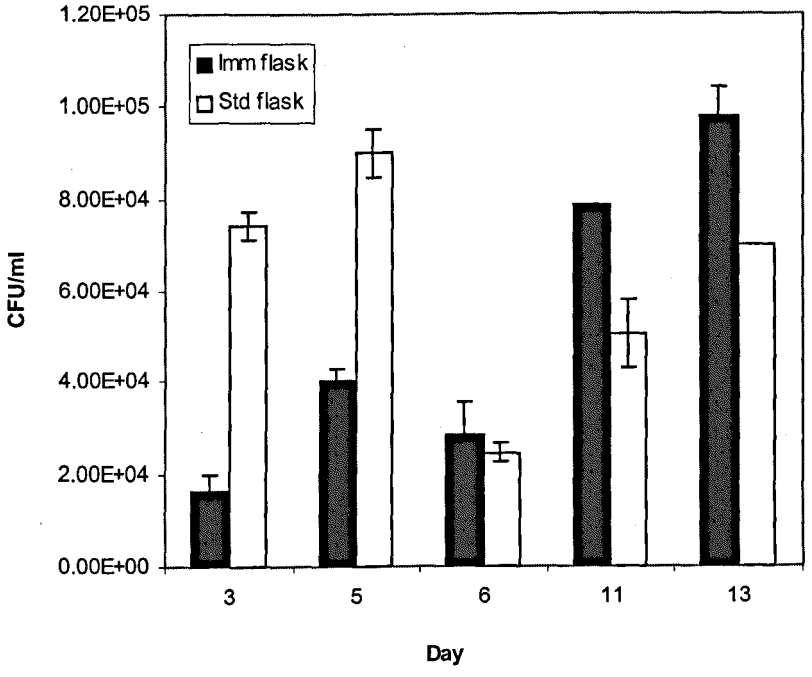


Figure 5.2.3.1

Daily vector titres (CFU/ml) in ImmobaSil flask cultures of FLYRD18/LNC-hB7 cells, compared to standard T-flask cultures. Error bars represent observed range, n=2.

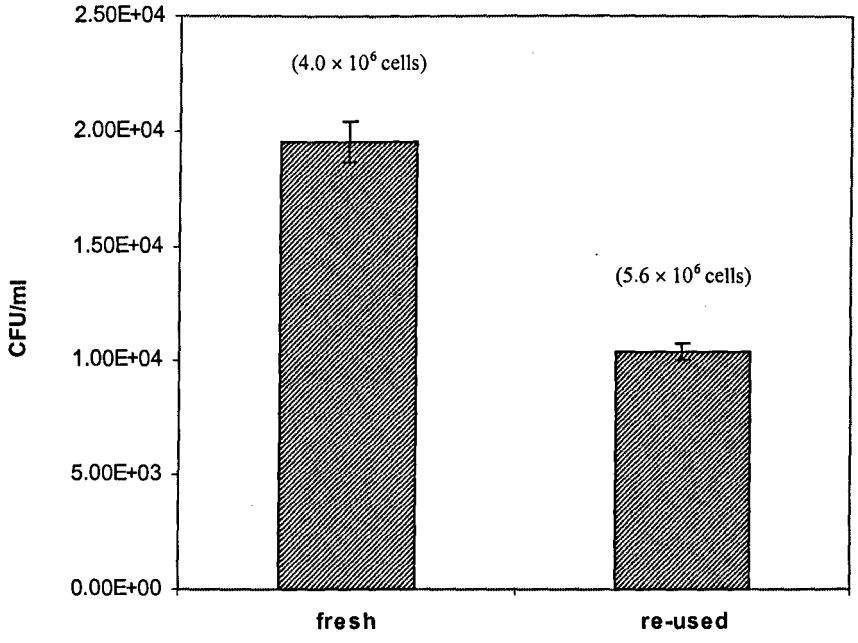


Figure 5.2.3.2

Vector titre (cfu/ml) after 4 days culture of FLYRD18/LNC-hB7 cells in fresh or re-used ImmobaSil flasks. Total cells harvested from each culture are indicated in brackets. Error bars represent observed range, n=2.

5.3 Discussion

5.3.1 Packed Bed Characterisation

Fluid Flow

In order to characterise the fluid flow within a continuously perfused reactor, such as the packed bed system used in this chapter, the distribution of residence times of fluid elements passing through the vessel should be determined. Since different elements of fluid will take different routes through the packed bed, they will take different lengths of time to pass through the vessel and enter the effluent stream. The distribution of these times is called the exit age distribution, E(t), or the Residence Time Distribution (RTD). Since the Residence Time Distribution reveals the length of time various fluid elements remain within a reactor during circulation, the RTD is characteristic of the mixing that occurs within each reactor (Fogler, 1992; Levenspiel 1972).

A stimulus-response technique was chosen for determination of the RTD due to the simplicity of this method. Various tracers can be used for this method, such as salts, dyes or radioactive species. Phenol Red was chosen as it has a similar viscosity and density to water and hence will not disturb the normal flow patterns through the reactor. Furthermore, the concentration of Phenol Red in the effluent stream can be readily measured using absorbance measurements, with minimal costs.

In an 'ideal' reactor, the tracer would pass through the vessel as a plug flow and exit as one undiluted bolus, giving a vertical line in the E(t) versus time plot (E curve). However, in reality, the tracer pulse is widened out to create a distribution curve. It obviously takes a finite time to inject the tracer, hence a small amount of diffusion will occur at the point of

entry. This results in a steep, but not vertical, peak in the concentration versus time curve (C curve), and hence in the E curve, as the tracer reaches the effluent stream (as seen in Figures 5.2.1.1 and 5.2.1.3). The curve is often further widened due to various mixing and channelling of fluid elements (as described below). Hence after an initial steep peak, the tracer concentration decreases more gradually producing a 'tail' region.

Closed perfusion was used for most of the packed bed cultures described in this chapter, however, where the culture supernatant was kept at a lower temperature to the cultured cells (supernatant perfused onto ice), the cultures were performed under open perfusion. Therefore E curves were constructed under open and closed perfusion (Figures 5.2.1.2 and 5.2.1.4 respectively) in order to characterise the fluid flow under both conditions. Comparison of these curves to those of Bratch (2000) using the same packed bed system under closed perfusion with Fibra-Cel™ carriers, allows an assessment of the effects of different carriers on fluid flow. Using the same fluid flow rates, similar curves were obtained. Both showed sharp peaks, with similar upward slopes. However, the downward slope in Figure 5.2.1.2 is more gentle than that of Bratch (2000), with a longer tail region, indicating a longer residence time of some fluid elements in the packed bed filled with ImmobaSil-G™ carriers. This could be attributed to the high porosity and different geometry of the ImmobaSil-G™ carriers compared to the Fibra-Cel™ carriers. The ImmobaSil-G™ carriers are cylindrical (length 1mm; diameter 0.8mm), whereas the Fibra-Cel™ carriers are flat discs (thickness < 1mm; diameter 5-6mm). Dye entering the pores of the ImmobaSil-G™ carriers may take longer to diffuse out of each carrier than that entering the Fibra-Cel™ carriers, hence the longer tail region in Figure 5.2.1.2. At the very end of the curve, the slow decline could be due to diffusional effects as opposed to fluid flow.

The E curves for closed and open perfusion in the packed bed, Figures 5.2.1.2 and 5.2.1.4 respectively, like those of Bratch (2000), resemble that of a tubular reactor with dead zone channelling (Fogler, 1992). Typically in a packed bed reactor, an uneven flow distribution is observed. This is due to the uneven packing of carriers/particles (as illustrated in Figure 5.3.1.1), causing 'channelling' where certain fluid elements will follow the route of least resistance to the exit stream whilst others flow around the carriers taking a longer route before exiting. Furthermore, wall effects come into play, where some fluid elements follow the shortest route along the wall of the vessel as opposed to passing through the packed carriers (see Figure 5.3.1.2). Both these effects contribute to the distribution of contact times of various fluid elements with the cell-seeded carriers.

While the Residence Time Distribution of fluid in the packed bed system is far from 'ideal', any modifications to the packed bed could only slightly improve the distribution but never reach that of ideal plug flow. It must therefore be borne in mind that factors such as wall effects in a packed bed system may mean that certain elements of medium will not reach cells attached to the carriers within this region. Also, stagnant regions are likely to exist within the reactor, where cells may be deprived of nutrients and die. This could explain the decrease in retroviral vector titre occasionally observed after 3-5 days culture in the packed bed system (see Section 5.2.2). However, most of the medium passing through the packed bed should be sufficient to maintain other cells attached to carriers outside these stagnant regions. Hence after an early decrease, titres remain fairly constant in most packed bed cultures.

A wide RTD in a packed bed system can lead to the generation of a non-uniform culture of cells. Since certain elements of the medium will be in contact with the attached cells for different lengths of time, some elements will, for example, undergo relatively little glucose depletion while others (in the extreme) may have a high glucose depletion. The same is true for the deposition of metabolic by-products into the culture medium. Hence the pH and nutrient levels of various elements of medium may vary throughout the reactor, leading to non-uniform cell growth and virus production. Since the virus particles released by the cells are all pooled in the reservoir bottle, their final concentration is a mean of that from the high and low producing cells in the packed bed. Hence the resultant virus titre may not be optimal, but variation in production throughout the packed bed is not detected.

A further consequence of a wide RTD is that virus particles released by the cells will reside in the vessel for different lengths of time. Where the culture vessel is maintained at a different temperature to that of the medium reservoir (for closed perfusion) or harvest reservoir (for open perfusion), the activity of particles in different parts/elements of the medium may differ (as loss of activity is temperature and time dependent). This is discussed in more detail in Section 5.3.2.

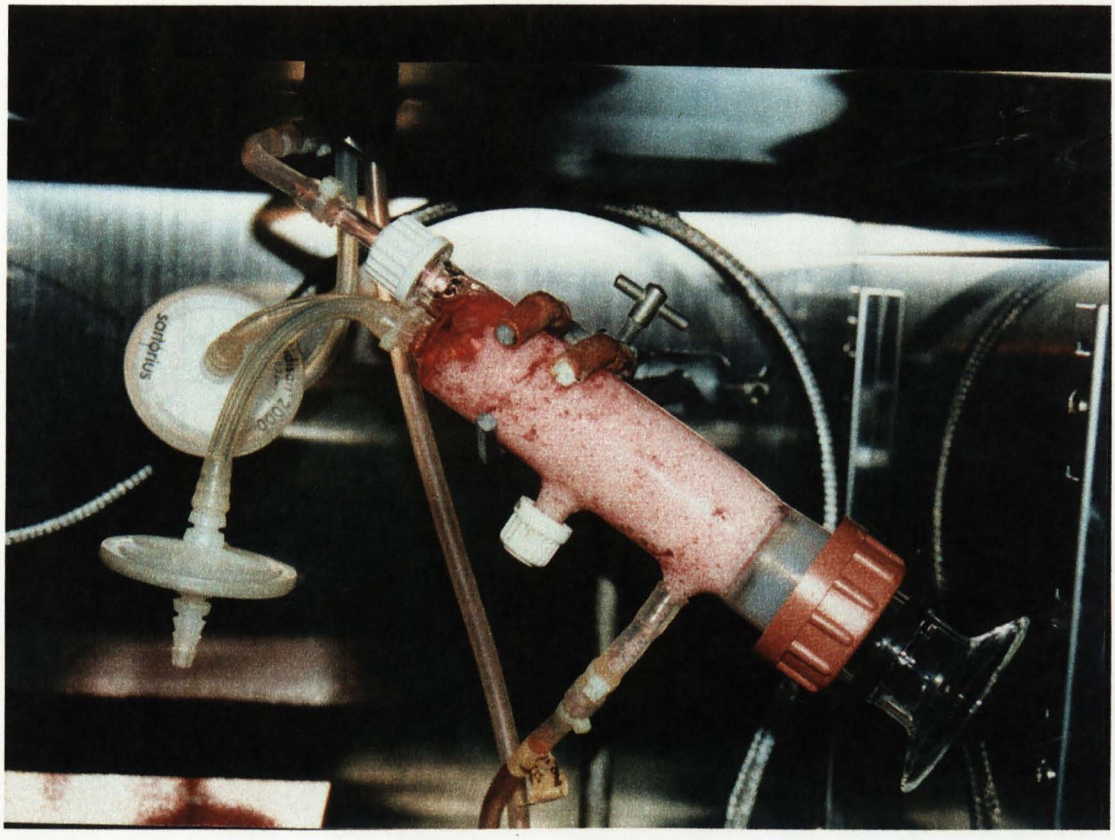


Figure 5.3.1.1

Packed bed vessel filled with ImmobaSil-G™ carriers and culture medium. This photograph illustrates non-uniform packing of the carriers, responsible for so-called channelling of fluid elements.

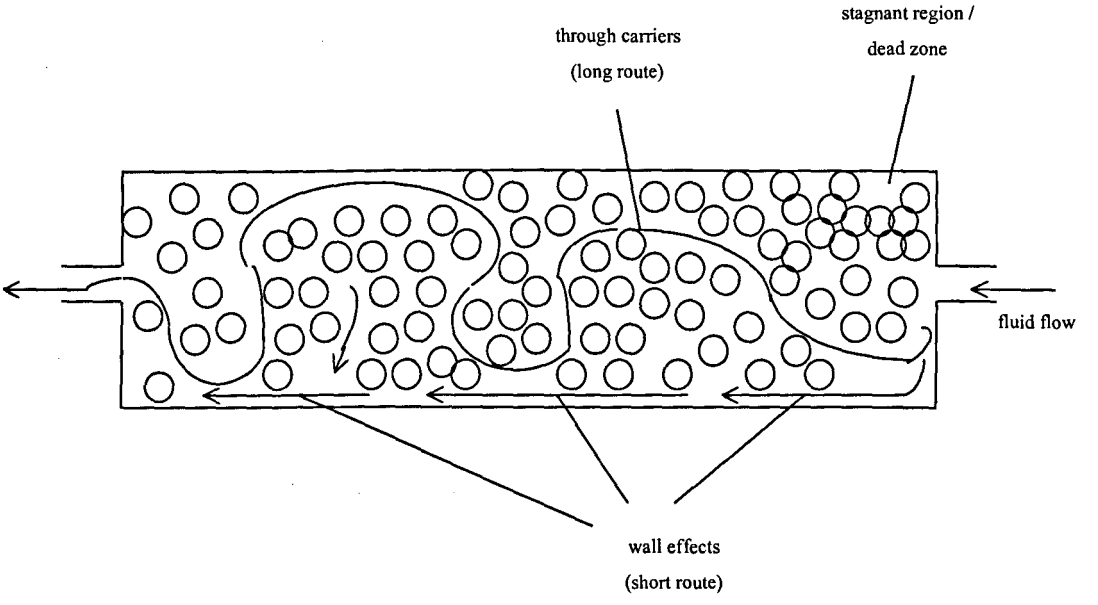


Figure 5.3.1.2

Diagram to show various fluid movements through a packed bed with non-uniform distribution of carriers.

Oxygen Transfer

Oxygen delivery is one of the key challenges in high density cell cultures. While the oxygen demand of such cultures is high, oxygen is only sparingly soluble in culture medium. In fact the oxygen concentration of culture medium saturated with air, at 37°C, is only approximately 0.2 mmol/L (Boraston et al., 1984; Henzler and Kauling, 1993). Oxygen levels within the culture medium can be maintained through sparging, however, problems of cell lysis due to bubble bursting have frequently been reported (Kioukia et al., 1992). A number of groups have demonstrated the transfer of oxygen from the gas phase to liquid culture medium via silicone tubing (Fleischaker and Sinskey, 1981; Mano et al, 1990). Emery et al. (1995) demonstrated equivalent oxygen transfer rates using immersed silicone tubing and pure oxygen, compared to sparging with air. This method of oxygen delivery avoids any agitation and bubble-bursting problems often associated with sparging. Pure oxygen should be used with care, as high oxygen levels within cultures has been shown to cause cell death through the production of superoxides and free radicals creating 'oxidant stress' (Ellis, 1991; Emery et al., 1995; Simpson et al., 1997). The medium within the packed bed system used throughout this chapter was aerated using 5% CO_2 in air passed through immersed silicone tubing. This allowed oxygenation of the medium as well as maintenance of the pH through the presence of 5% CO_2 (which buffers any breakdown of sodium bicarbonate within the medium).

In this packed bed system, oxygen delivered to the cells must cross a number of barriers. These include the transfer of oxygen across the wall of the silicone tubing, the transfer of the oxygen from the gas phase to the liquid phase (dissolving in culture medium) and the delivery of the oxygenated medium to the attached cells. All of these factors will affect the

oxygen transfer coefficient of the system. Priessman et al., (1997) demonstrated the penetration of oxygen only reached a maximum depth of 300μm in their cell-seeded carriers. This resistance to oxygen transfer through the carriers was reported to cause cell death in the centre of these carriers. The ImmobaSil-G™ carriers used in the packed bed studies within this chapter should not show such inhibited oxygen transfer, since the manufacturers claim that the silicone material of these carriers is oxygen permeable throughout.

The oxygen transfer coefficient, K_T, is a useful parameter for characterising the oxygen transport from the gas phase, within the silicone tubing, to the liquid phase, in the bulk of the culture medium. In a packed bed filled with ImmobaSil-G™ carriers, K_T was calculated as 5.3×10^{-4} cm/sec. This value is almost 4-fold lower than that calculated by Bratch (2000), using the same packed bed under the same conditions but filled with Fibra-Cel™ carriers. The lower K_T found in the packed bed filled with ImmobaSil-G™ carriers may be attributed to the smaller pore size of these carriers and/or their tight packing within the packed bed. The geometry of the Fibra-Cel™ carriers (flat discs) does not encourage uniform tight packing within the vessel. Furthermore, only 6g of Fibra-Cel™ discs were added to the packed bed in the studies by Bratch. This amount of Fibra-Cel™ carriers does not completely fill the packed bed vessel (85ml capacity), hence the carriers were not tightly packed in these experiments. In the present study, ImmobaSil-G™ carriers were tightly packed throughout the vessel, hence any oxygen passing across the wall of the silicone tubing and dissolving into the surrounding culture medium may diffuse into the pores of the surrounding carriers and hence take longer to diffuse out again. This would increase the time taken for oxygenated medium to reach the reservoir bottle, and hence increase the measured oxygen transfer coefficient of the system. The addition of packed carriers to the system is

also likely to affect the mixing within that vessel, which in turn could reduce the mass transfer rate. Indeed, Bratch (2000) demonstrated a significantly reduced oxygen transfer coefficient in the presence of carriers compared to that of a packed bed without carriers. More importantly, the ImmobaSil™ carriers, unlike Fibra-Cel™, are permeable to oxygen. This property may also affect the oxygen transfer within the system and may be the major factor causing reduced K_T.

More importantly, the ability of the packed bed system to deliver enough oxygen to the cells attached within the vessel should be assessed. It is essential that the oxygen uptake rate (OUR) of the attached cells does not exceed the oxygen transfer rate of the packed bed system. Furthermore, the dissolved oxygen level within the packed bed should not fall below the critical oxygen concentration (Stanbury et al., 1995). The OUR of the FLYRD18/LNC-hB7 cell line attached to ImmobaSil™ carriers was previously measured (see Chapter 3). This data, along with the oxygen transfer coefficient in the packed bed, can be used to determine the total number of cells that can be supported by the reactor ($X_{v,\,total}$).

In order to maintain cell growth and viability within the packed bed, the oxygen transfer rate should equal the oxygen uptake rate by the cells. Hence:

$$AK_T\,(C\text{-}CL) = QO_2\,X_{v,total}$$

Where A is the total surface area of the silicone tubing (149.9cm^2), QO_2 is the specific oxygen uptake rate of the cells, C is the concentration of oxygen in air-saturated medium (0.2125 mmol/L) and CL is the required oxygen level in the packed bed. Since 20% dissolved oxygen is the lowest level of oxygen shown not to effect the growth and vector production of FLYRD18/LNC-hB7 cells (see Chapter 4), the oxygen level within the system

should not fall below this level. Therefore CL is the concentration of oxygen at 20% air saturation (i.e. 0.20C). Hence C-CL is 0.80C (i.e. 0.170 mmol/L $\equiv 0.170 \times 10^{-3}$ mmol/cm^3). The oxygen uptake rate (OUR) of FLYRD18/LNC-hB7 cells attached to ImmobaSil™ carriers is 1.55×10^{-15} mol/cell/min ($\equiv 2.58 \times 10^{-14}$ mmol/cell/sec), see Chapter 3. Therefore the total number of cells that can be supported within the packed bed ($X_{v,total}$), under the conditions described in this chapter, can be calculated as follows:

$$X_{v,\,total} = AK_T(0.80)C \,/\, QO_2$$

$$\equiv \frac{149.9\ \mathbf{cm^2} \times 5.3 \times 10^{-4}\ \mathbf{cm/sec} \times 0.170 \times 10^{-3}\ \mathbf{mmol/cm^3}}{2.58 \times 10^{-14}\ \mathbf{mmol/cell/sec}}$$

$$= 5.27 \times 10^8 \text{ cells}$$

This value is higher than the number of cells actually inoculated in the packed bed cultures described in this chapter (typically 1.8×10^8). However, this value is not magnitudes higher than the number of inoculated cells and a 5-fold increase in cell number could well lead to oxygen limiting conditions within a closed batch culture. In the packed bed cultures described within this chapter, the medium in the reservoir bottle was changed for fresh every 24 h, in order to harvest the retroviral vectors. Hence medium with a high dissolved oxygen level was introduced to the system daily. As a consequence of this, the dissolved oxygen levels within the packed bed system were observed not to fall below 60% during a typical 10-day culture. However, it should be borne in mind that where the medium reservoir is *not* changed daily, the oxygen delivery to cells immobilised in the packed bed system could become a limiting factor for cell growth and virus production if the cell number increases. This can be overcome by continuous control of the dissolved oxygen concentration (as conducted in the last study of section 5.2).

In order to sustain greater cell numbers, the oxygen delivery within such a system can be increased by increasing the total surface area of the silicone tubing and/or using oxygen-enriched air. The use of oxygen-enriched air can achieve dissolved oxygen levels of greater than 100%, however supra-optimal oxygen levels which may cause cell death should be avoided, as discussed earlier.

5.3.2 Cell Growth and Retroviral Vector Production in Packed Bed Cultures

The studies within this chapter demonstrate the feasibility of using a packed bed system for the long-term production of large quantities of retroviral vectors from the FLYRD18/LNC-hB7 cell line. ImmobaSil-G™ carriers appeared to perform well, compared to Fibra-Cel™ carriers, with respect to the maintenance of cell viability and production of retroviral vectors. Other carrier types were not assessed.

Conditions within the packed bed can be optimised to increase vector titres. For example, decreasing the culture temperature from 37°C to 32°C lead to an increase in vector titre harvested from the packed bed (Figures 5.2.2.8 and 5.2.2.11). Combining this lower temperature with continuous control of the pH and oxygen levels further increased the vector titre as well as reducing the variation in daily vector titres (Figure 5.2.2.13). The reduced variation in vector titre was not expected as the vector containing stocks were replaced with fresh medium, in both the non-controlled and the controlled packed bed systems, on a daily basis. It is possible that the cells in the non-controlled cultures underwent periods of inhibited vector production before each medium change and their recovery may have varied each day. A further optimisation of the packed bed culture conditions involved the use of open perfusion of the culture medium, where the vector-containing supernatant could be

collected in a separate bottle stored on ice. This system allowed a reduction in the vector decay rate. This significantly increased the maximum vector titre, compared to closed perfusion at 37°C (Figure 5.2.2.6).

The above improvements in vector titre with each modification were not as great as expected. In monolayer cultures of FLYRD18/LNC-hB7 cells, a decrease in culture temperature from 37°C to 32°C resulted in a greater increase in vector titre (Chapter 4) compared to that seen in the packed bed cultures. Furthermore, previous studies demonstrated an almost 10-fold increase in vector half-life when stored on ice, compared to storage at 37°C (Chapter 3). Hence the increase in titre during open perfusion onto ice, is not as great as would be expected. This is possibly as a result of the wide RTD of fluid elements in the packed vessel, as reported earlier. Fluid elements which have a *short* residence time will exit the packed bed more rapidly and enter the harvest bottle stored on ice, hence vector particles within these fluid elements will have a reduced decay rate. However, since there is a wide RTD, some fluid elements have a much *longer* residence time and hence remain in the packed bed vessel at 37°C for longer periods. Vector particles in these fluid elements will not have such a reduced decay rate. The expected increase in titre with recovery of supernatants onto ice will therefore not be optimal.

Furthermore, the non-uniformity of the packed bed means that not all cells are grown under optimum conditions. Stagnant regions may explain the early decrease in titre, observed in some of the 37°C cultures (Figures 5.2.2.4 and 5.2.2.5), as discussed in Section 5.3.1. However, this early decrease in vector titre was not observed in all packed bed cultures at 37°C. This may be attributed to the fact that each vessel was filled with carriers by hand

before sterilising, hence there will be variability in the packing of these carriers between experiments. Hence in some experiments the uniformity of carrier packing may be better than in others, causing a decrease or increase in channelling and stagnant regions. Alternative geometry of the carriers may aid in more uniform packing of the vessel.

Interestingly, both packed bed cultures conducted at 32°C yielded a progressively increasing daily vector titre. This is in contrast to the daily vector titres in packed bed cultures at 37°C which only increased for the first 1-2 days, then remained fairly constant, though sometimes after an initial decrease. The daily increase in vector titre in the 32°C packed bed cultures was accompanied by an increase in MTT conversion per carrier, indicating that the increasing titres were a result of increasing cell numbers. It is possible that in the 37°C cultures, the higher metabolic activity of the cells caused growth-limiting conditions at an earlier time point. Glucose levels were *not* found to be limiting in the 37°C cultures however, high concentrations of metabolic by-products, produced by the high cell numbers over 24 h, may have caused inhibition of cell growth and/or vector production. Furthermore, labile medium components, such as glutamine, may have a faster decay rate at 37°C compared to 32°C. Limiting conditions are likely to be reached at a later time point in the 32°C packed bed cultures, where high cell numbers are reached later due to the reduced cell growth rates at this lower temperature.

It can be concluded from the above studies that the packed bed system is a suitable candidate for the large-scale production of retroviral vectors, though some optimisation is required. For example, a reduction in the non-uniformity of the packed bed culture may be achieved through the use of carriers with a different geometry (to reduce channelling and stagnant

regions). Such modifications may allow the immobilised cells to be cultured under optimal conditions throughout the packed bed. The continuous control of parameters such as pH, temperature and dissolved oxygen levels is recommended for the optimisation of vector production throughout the culture period and reduction of vector titre variability.

5.3.3 Cell Growth and Retroviral Vector Production in ImmobaSil Flasks

Vector titres in the ImmobaSil flasks were similar to those in standard T-flasks. Surprisingly, the number of cells counted in the ImmobaSil flasks was lower than that in the standard flasks, indicating increased vector productivity of the cells cultured on the ImmobaSil surface. However, it is possible that not all the cells were released after trypsin digestion in the ImmobaSil flasks. The ImmobaSil culture surface is highly porous and hence cells may migrate into the pores rendering them difficult to detach for counting. If all the cells were not removed by trypsin digestion the cell numbers in the ImmobaSil flasks will be higher than those counted.

The vector titre in the ImmobaSil flasks continually increased through to day 13, whereas titres in the standard flasks were reduced after 5 days. Hence it appears that maximum titres in the ImmobaSil flasks are reached at a later time point than in standard flasks. This could be due to reduced cell growth rates on this alternative culture substratum, compared to standard tissue culture grade polystyrene.

The progressive increase in vector titres in the ImmobaSil flasks suggests that cell numbers were increasing. The increased surface area in the ImmobaSil flasks may ultimately allow increased cell numbers per flask, however longer culture periods in these prototype flasks

would be necessary to determine whether this is indeed an advantage. If so, such flasks could be used for simple scale-up of retroviral vector production.

Re-use of the ImmobaSil flask resulted in lower vector titres. A possible explanation for this is the attachment of vector particles to proteins deposited onto the ImmobaSil surface during the previous culture (though this was not tested). Data from this experiment suggests that these flasks should only be used as single-use disposable culture vessels for vector production.

Chapter 6:

General Discussion and Conclusions

The production of retroviral vectors for gene therapy applications faces a number of challenges. Of primary concern is the low titre of vector stocks produced by packaging cells in culture. Furthermore, the inherent instability of retroviral activity presents problems in optimising production processes. Retroviral vector production should aim to increase both titre and 'quality' (transduction efficiency). Quality can be improved by increasing vector stability and decreasing the concentration of inhibitors to infection, such as inactive vector particles and free envelope proteins (Forestell et al., 1995) or proteoglycans released by the packaging cells (Le Doux et al., 1998). Vector titres can be improved at both the genetic level and the cell culture level. While a number of groups have achieved higher vector titres through the construction of new and improved packaging cell systems, few reports exist on optimisation of the culture processes for vector production.

In order to reduce production costs, the maximum titre achievable for each packaging cell system should be determined and maintained throughout production. In this thesis, an assessment of culture parameters that can influence vector titre was conducted. In addition, the use of an alternative production system, which is readily scalable, was investigated. These latter studies were conducted since the quantity of vector particles required for future clinical applications is likely to exceed those achievable using current production methods.

Culture Parameters

While a number of factors had no measurable effect on vector production (e.g. dissolved oxygen concentration or addition of FGF, peptone, dexamethasone and sodium butyrate), studies within this thesis show that a number of parameters can significantly affect the vector titre achieved in cultures of FLYRD18/LNC-hB7 cells. Cell proliferation rate was found to be closely linked to vector productivity (Chapter 3). For this reason, production cultures should be kept in a highly proliferative state in order to achieve maximum titres. Moreover, unlike a number of mammalian cells producing recombinant proteins, the arrest of FLYRD18/LNC-hB7 cells in any particular phase of the cell cycle affords no benefits in retroviral vector production. Cruz et al., (2000) recently reported a correlation between cell proliferation and vector production, in PA317 retrovirus packaging cells, hence this finding may be true for a number of retroviral packaging cell lines.

A reduction in culture temperature was demonstrated to increase vector titres through a reduction in vector decay rates. This was only true within a small temperature range, since reduction of the culture temperature also leads to a decrease in vector production (due to lower cell proliferation/metabolic rates). Hence the optimum culture temperature is that which allows accumulation of vector particles in the culture medium (due to lower decay rates) whilst maintaining vector production at a sufficient rate. This may vary between different packaging cell systems due to their different production rates and vector half-lifes.

Serum concentration was found to have a dose-dependent negative effect on vector titre. Low serum levels can therefore improve vector titres as well as reduce the protein concentration of vector preparations, though it would appear from the literature that the

effects of serum on vector production vary between different packaging systems. Since contaminating proteins such as those in serum should ideally be removed in the final product, the use of low serum concentrations during production can reduce the demands on down-stream processing.

The pH of the culture medium was also observed to be a critical parameter in vector production (Chapter 4). Titres were significantly decreased when pH levels were higher or lower than the optimum level (pH 7.2). Continuous control of the pH throughout culture is therefore necessary in order to maintain optimal vector production. Surprisingly, this parameter has received little attention in the literature on retroviral vector production (possibly because typical culture methods such as roller bottles do not allow continuous pH control). The simplest method for continuous pH control involves monitoring the culture medium with a pH probe and the addition of a base, such as NaOH, when the pH drops below a critical level, or the addition of CO_2 gas when the pH rises (using a control unit). However, it has been reported that the addition of NaOH during prolonged culture can lead to dilution of the product and increased osmolarity of the culture medium (Harbour et al., 1989). Increased osmolarities can adversely effect the productivity of a number of cell lines. This should therefore be assessed for each packaging cell system. Due to the short half-life of retroviral vectors, the culture supernatant of packaging cell cultures is usually harvested at frequent intervals (e.g. every 24h). The dilution of vector-containing supernatants due to NaOH addition may therefore not be significant over such a short period, but should be borne in mind.

Large-Scale Vector Production

Few reports exist on the development of culture methods for the scale-up of retroviral vector production. Recently, Pan and Whitley (1999) investigated the use of a closed hollow fibre bioreactor and showed promising results. In this system, cells and released vectors are retained in the extra-capillary space, while medium is perfused through the capillaries. Nutrients are delivered to the cells via diffusion across the capillary walls. Such a system allows the concentration of the vector-containing supernatant and titres were significantly increased compared to monolayer cultures. However, the total volume in the extra-capillary space of this system is relatively small, reducing the volumetric productivity. A further problem in such a hollow fibre system is the limited space for cell growth (anchorage-dependent cells attach to the capillary walls). At high cell concentrations, Pan and Whitley reported that two thirds of the extra-capillary space were filled with cells after prolonged culture, leaving even less volume for supernatant production. Large cell masses surrounding the capillaries can also reduce the ability of nutrients to diffuse across the capillary walls. Hence such a system may be limiting during intensive production from anchorage-dependent cells.

Studies in Chapter 5 demonstrate the feasibility of using a novel packed bed system for the production of retroviral vectors. The vector titres produced in this perfused culture system were similar to those achieved in monolayer cultures. Large volumes of culture supernatant can be harvested from the packed bed system at regular intervals (or continuously) and scale-up of the system is relatively easy. Furthermore, the ability to maintain vector production over prolonged culture periods can reduce production costs by reducing the 'down-time' of each system between batches. Further optimisation of the packed bed system is, however,

necessary to reduce the residence time distribution (RTD). A reduced RTD could lead to improved titres, as each component of the culture supernatant will be retained in the packed bed for the same length of time and hence be exposed to the same optimum conditions.

In Chapter 3, the dependence of vector half-life on temperature was clearly demonstrated. The increased vector half-life at low temperatures was exploited in a packed bed culture where *open* perfusion of the culture medium allowed the recovery of the vector-containing supernatants onto ice, thereby increasing the stability of harvested vectors (Chapter 5). The use of such open perfusion cultures not only allows a reduction in vector decay rates, but also provides the potential for integration of the production process with primary down-stream processing, such as vector isolation and purification (Braas et al., 1996; Lyddiatt and O'Sullivan, 1998; Cruz et al., 2000b). Continual harvesting of vector stocks may also be a more reliable method, since it has been recognised that during batch culture it is difficult to predict the optimal harvest time, when there is a 'peak' in titre (Shen et al., 1996).

In such open perfusion cultures, reduced flow rates of the culture medium can increase the product concentration released by immobilised cells. However, if perfusion rates are too low, nutrient levels may become limiting and the concentration of metabolic by-products may become toxic (Griffiths et al., 1992; Ozturk, 1996). A further consideration in perfused cell cultures for retroviral vector production is that a decrease in flow rate may lead to an increase in vector decay rate. This can occur where the vectors are retained in the culture vessel at a higher temperature for long periods before exiting into the recovery vessel (normally at a lower temperature). Low perfusion rates may therefore lead to a reduced active particle/total particle ratio, thereby reducing the quality of the vector preparation. The

quality of the vector stock may be further compromised by an increased concentration of inhibitors (released by the packaging cells) at lower flow rates. It is therefore necessary to adapt the perfusion rate of such culture systems according to the cell specific production rates and vector stability of each retroviral packaging cell system. In a recent paper by Cruz et al., (2000a) a mathematical model was developed to determine the best retrovirus harvest strategy in perfused cultures, in order to optimise the vector yield. It was claimed that this model could be used in any perfusion culture, provided the parameters of the cell system used are determined.

The suitability of packed bed reactors for retroviral vector production has also been demonstrated in a recent paper by Kang et al., (2000). This group compared vector production in monolayer, stirred microcarriers and packed bed cultures. Despite the rapid cell growth in the stirred microcarrier culture, the vector titre was 10-fold less than that obtained in monolayer cultures. Further studies on the effects of agitation indicated that this reduced titre was due to the effects of shear forces on the vector particles. This loss in vector particle activity due to agitation was also observed by Kenworthy (2000). The packed bed cultures conducted by Kang and co-workers yielded slightly lower vector titres than those from monolayer culture. However, a high cumulative titre and high volumetric productivity was demonstrated when using the packed bed system in continuous mode.

Kang et al. initially compared vector production in packed bed cultures using three different carrier types for cell immobilisation (Siran™, MIF-5 and Fibra-Cel™). Fibra-Cel™ gave the best results and was therefore chosen for use in further studies. Studies in Chapter 5 of this thesis employed the use of ImmobaSil-G™ carriers for packed bed culture of the packaging

cells. A study was conducted to compare these carriers to Fibra-Cel™ and similar vector tires per cell were obtained using each carrier type (Figure 5.2.2.7). Hence ImmobaSil™ carriers are likely to perform well against other carrier types.

The suitability of culture flasks with an ImmobaSil™ culture surface was also assessed in Chapter 5. These prototype flasks have an increased surface area for cell growth and hence may be suitable for the intermediate scale-up of vector production by anchorage-dependent cells (a scale between standard T-flasks and roller bottles/packed beds). Initial results were promising and demonstrated the use of these flasks as a simple disposable system for increased vector production. However, unlike the packed bed system, critical culture parameters such as pH cannot be continuously controlled in closed flasks.

Clinical Requirements

The studies in this thesis have demonstrated the importance of optimising culture conditions for improving vector titre. Obviously the titre requirements for clinical use will depend upon the application, where the number of cells which require transduction and the site of administration both vary. For example, a higher titre product may be necessary for successful systemic administration, where the vectors will become diluted and a high percentage may decay before reaching the target cells. On the other hand, site directed injections, such as those into solid tumours, may not require such high doses. *Ex vivo* gene delivery is unlikely to require such high titre vector stocks (in comparison with *in vivo* delivery), since successfully transduced cells can be 'selected' before transplantation back into the patient. However, production of vector stocks with the highest titre possible will inevitably reduce production costs.

Although outside the realms of this thesis, down-stream processing is also vital for clinical use of retroviral vector preparations. This includes concentration of the vector stock, purification (to remove contaminating proteins and inhibitors of transduction), preservation, storage and formulation. Common methods for concentration include ultrafiltration, and centrifugation. Alternative concentration methods under investigation include PEG (polyethylene glycol) precipitation, two-phase extraction (using Dextran, PEG or PVA), membrane filtration, liquid chromatography and adsorption chromatography (Andreadis et al., 1999; Lyddiatt and O'Sullivan, 1998; Braas et al., 1996). While membrane filtration is a promising candidate, the membranes are prone to fouling and high shear forces may act on the envelope proteins of vector particles, leading to a decrease in activity (Kenworthy, 2000; Cruz et al., 2000b). An underlying problem with most concentration methods is the co-concentration of inhibitors to transduction, such as proteoglycans (Le Doux et al., 1999). Hence additional steps such as enzyme digestion of inhibitors may be required to increase the transduction efficiency of concentrated vector stocks.

Long term storage is also a problem for the inherently unstable vectors. The effectiveness of liquid nitrogen storage was demonstrated in Chapter 3, however, in large-scale this method of long-term storage becomes costly and impractical. Furthermore, significant losses in activity are incurred with the first freeze-thaw process. Lyophilisation is an alternative option, though significant activity loss is again a problem. Recently, ambient temperature vacuum dehydration has shown promising results (Andreadis et al., 1999), but needs further work.

Clearly it is difficult to purify and store retroviral vector stocks without destroying some of their activity. Therefore, production processes should aim to produce vector stocks with high titres and minimal contaminating protein concentrations (to reduce the demands on down-stream processing).

The production of vectors for clinical use requires not only stable products with high purity and high titre, but also safety is a key issue. Manufacturers need to ensure the final product is free of mycoplasma, endotoxins and other adventitious agents such as replication competent retrovirus (RCR). The production of RCRs is of major concern in the administration of retroviral vectors in humans. Stringent testing of vector stocks for the presence of RCRs is therefore essential. The importance of this issue was made clear at a forum sponsored by the FDA and NIH in 1996, where the development and evaluation of Phase I gene therapy vector products was discussed. A major topic for discussion at this meeting was the testing for RCRs during vector production and patient follow-up. The current requirement by the FDA was the testing of 5% of supernatant volumes and 1% of packaging cell cultures before products could be used in the clinic. However, as the size of production lots increased (to hundreds of litres), this testing became more logistically burdensome (and costly). Furthermore, significant quantities of product must be used for such testing, thus reducing the output. Steps to revise these requirements were therefore suggested. Statistical models to validate the testing strategies were presented which took into account both the volume of test samples and the sensitivity of assays for the detection of RCRs. The FDA also proposed the provision of standardised stocks of RCRs to investigators, in order to allow 'calibration' of their assay sensitivity.

Whatever the methods employed for retroviral vector production, good control of the manufacturing processes are necessary to meet the current Good Manufacturing Practice (cGMP) requirements. cGMP requires safety (as outlined above), efficacy and potency of a product as well as consistency between batches. In typical monolayer culture methods it is difficult to control the culture parameters and the titre and quality of the vector stock can significantly vary from batch to batch. The packed bed system described in Chapter 5 may help to reach the goals of cGMP, as the culture parameters can be continuously controlled. Furthermore, such a system allows the implementation of a continuous perfusion culture, which is advantageous in the production of inherently unstable biologicals, as they can be rapidly removed from the bioreactor (Kadouri and Spier, 1997).

Summary

The studies within this thesis have demonstrated the importance of temperature, pH, and serum concentration during retroviral vector production. In addition the proliferation of packaging cells was shown to be vital for the production of high titre vector stocks. Finally a packed bed system was proven to be a good candidate for the production of large quantities of active vectors. These findings should be considered during the development of appropriate procedures for the large-scale production and handling of retroviral vector stocks for human gene therapy applications.

Chapter 7: Publications

Papers:

McTaggart S. and Al-Rubeai M., (2000). Relationship between Cell Proliferation, Cell Cycle Phase and Retroviral Vector Production in FLYRD18 Human Packaging Cells. Biotechnology and Bioengineering. Submitted.

McTaggart S. and Al-Rubeai M., (2000). Effects of Culture Parameters on the Production of Retroviral Vectors by a Human Packaging Cell Line. Biotechnology Progress, 16, 859-865.

McTaggart S., Gerin P., Al-Rubeai M., (1999). Optimisation of the Production of Retroviral Vectors for Human Gene therapy. Bioforum International, 3 (1), 13-17.

Presentations:

McTaggart S. and Al-Rubeai M. The role of cell proliferation and cell cycle in the production of retroviral vectors. 8th Meeting of the European Society of Gene Therapy (ESGT), October, 2000, Stockholm. Poster (abstract published in The Journal of Gene Medicine, supplement to Vol 2, p32).

McTaggart S. and Al-Rubeai M. Process Development of Retroviral Vector Production for Gene Therapy. 219th American Chemical Society National Meeting, March, 2000. San Fransisco, U.S.A. Oral presentation.

McTaggart S. and Al-Rubeai M. Effect of Cell Cycle and Environmental Factors on the Production of Retroviral Vectors by a Human Packaging Cell Line. ESACT-UK 10th Annual Meeting, January, 2000. Keele, U.K. Oral presentation.

McTaggart S. and Al-Rubeai. Developing an Optimal Culture Method for the Production of High Titre Retroviral Stocks. ESACT-UK 9th Annual Meeting, January, 1999, Oxford, U.K. Oral presentation.

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