

**Injury in the transplanted lung: mechanisms,
clinical correlates, and the development of novel
therapeutic approaches.**

Bruce Foster Whitehead
Institute of Child Health
University College London

PhD Thesis

2001

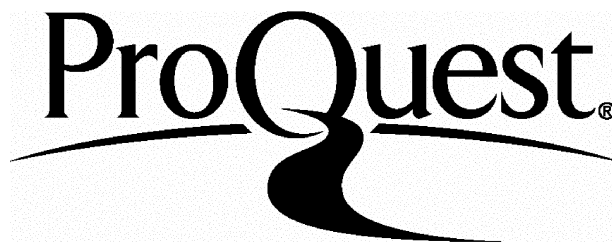
ProQuest Number: U642194

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest U642194

Published by ProQuest LLC(2015). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

This thesis reviews injurious events in the transplanted lung, describes the clinical correlates of these, and presents two novel therapeutic approaches. The mechanisms of injury in the transplanted lung have been detailed with special reference to allograft ischaemia, rejection, and infection. Clinical correlation of these injurious factors incorporated an extensive review of pulmonary function in a cohort of paediatric lung transplant recipients with cystic fibrosis. This study demonstrated an improvement in all dynamic lung function parameters at 12 months post transplant compared with pre transplant values ($p < 0.001$). Subsequently, there was a persistent decline in these values denoting the development of obliterative bronchiolitis. Measuring area under the curve of FEV₁% graphs (FEV₁% AUC) represented a unique method for assessing pulmonary function. This correlated negatively with graft ischaemic times (cold: $p < 0.05$, and total: $p < 0.01$) to 36 months post transplant, although not with the number of rejection or infection episodes in the first 12 months post transplant.

Novel therapeutic approaches included the evaluation of a non-viral, synthetic peptide gene vector system. This incorporated the administration, into the airway, of a ligand-polylysine (polylysine-molossin) vector in an *in vivo* rat lung model. The vector showed widespread distribution throughout the lung parenchyma, although limited attachment to the airway epithelium. However, gene transfer was not demonstrated despite the use of two reporter genes and additional methods to improve endocytic release. Antisense oligodeoxynucleotide (ODN) therapy for adenovirus infection constituted the other novel therapeutic approach. This study comprised the use of an *in vitro* biological assay to assess the efficacy of ODNs in modulating adenovirus infection. There was a small, but consistent reduction ($p < 0.005$) in adenovirus cytopathic effect associated with an antisense ODN directed to the E1A gene of adenovirus 5, compared with the nonsense control ODN. This result suggests a potential therapeutic role for ODNs in adenoviral infection.

Acknowledgements

First and foremost, I thank Professor John Fabre for his guidance, encouragement and patience from the initial experimental studies through to completion of the thesis. Other members of the transplant biology unit, initially at the Institute of Child Health, and later at King's College, London deserve special mention for their invaluable help throughout the process. They include: Greta Sawyer, Lorna Shewring, Rebecca Schofield, Helena Zhang, Sheng Yun, and Louise Collins. Kenth Gustaffson and Helena Kempinski at the Institute of Child Health, London also provided tremendous assistance for selected studies.

The cardiothoracic transplant team at the Great Ormond Street Hospital for Children, London deserves recognition for their support during the clinical studies. The team included: Pauline Whitmore, Paul Aurora, Marc de Leval, Martin Elliott, and Philip Rees. In addition, the Hearts for Kids Charity remained a generous supporter throughout.

I'm indebted to Rod Lane, John Rae, Aiden Lavery, and Gail Slade of the Respiratory Function Laboratory at the Great Ormond Street Hospital for Children, London for their help with the lung function component of this work.

During writing up at the John Hunter Children's Hospital, Newcastle, Australia, Kerry Rogers was of invaluable assistance with data analysis, statistical input and the reviewing of drafts. I also thank Jodi Hilton for her patience and support with the clinical workload that enabled me to complete the thesis.

Finally, I dedicate this work to my wife Vivienne and our children Callum, Mirren, Freya and Rowena, all of whom carried indirectly some of the burden of thesis writing. The thesis is also in memory of Dad, who was always a source of inspiration.

**Injury in the transplanted lung: mechanisms,
clinical correlates, and the development of novel
therapeutic approaches.**

Table of contents

Abstract	2
Acknowledgements	3
Table of contents	4
Figures and tables	7
Introduction to thesis	15
Section A Literature review	18
Chapter 1 Mechanisms of injury in the transplanted lung	19
1.1 Introduction	21
1.2 General overview of thoracic transplantation	22
1.3 Rejection of the lung allograft	27
1.4 Infection in the lung allograft	35
1.5 Other factors	43
1.6 Obliterative bronchiolitis	44

Chapter 2	Clinical correlates of injury in the transplanted lung	50
2.1	Introduction	51
2.2	Rejection and infection	52
2.3	Obliterative bronchiolitis	54
2.4	Pulmonary function post lung transplantation	56
2.5	Survival post lung transplantation	64
Chapter 3	The development of novel therapeutic approaches	69
3.1	Introduction	70
3.2	Gene transfer in the lung	72
3.3	Antiviral therapy with antisense oligodeoxynucleotides	84
Section B	Experimental results	90
Chapter 4	Correlation of pulmonary function with injurious events following lung transplantation	91
4.1	Introduction	92
4.2	Patients and methods	93
4.3	Results	98
4.4	Discussion	129

Chapter 5	<i>In vivo</i> gene transfer in the rat lung	133
5.1	Introduction	134
5.2	Methods	135
5.3	Results	151
5.4	Discussion	166
Chapter 6	<i>In vitro</i> modulation of adenovirus infection by antisense oligodeoxynucleotides	170
6.1	Introduction	172
6.2	Methods	173
6.3	Results	191
6.4	Summary of results	221
6.5	Discussion	223
Chapter 7	Nuclear localisation of antisense oligodeoxynucleotides	228
7.1	Introduction	229
7.2	Methods	231
7.3	Results	235
7.4	Discussion	241
Chapter 8	Concluding remarks	243
References		247
Appendix		289

Figures and tables

Chapter 1

Table 1.1	Bacterial organisms isolated from infected lung transplant recipients	39
Table 1.2	Types of virus infection in the pulmonary allograft	40
Table 1.3	Bronchiolitis obliterans syndrome staging scheme	45

Chapter 2

Table 2.1	Glossary of pulmonary function terms	57
Figure 2.1	Actuarial survival following adult heart-lung transplantation	64
Figure 2.2	Total lung transplant actuarial survival by procedure	65
Figure 2.3	Actuarial survival following paediatric heart-lung transplantation (1982-1999)	67
Figure 2.4	Actuarial survival following paediatric lung transplantation (1982-1998)	68

Chapter 4

Figure 4.1	Method for calculating area under the curve (AUC)	96
Table 4.1	Demographic data of lung transplant recipients and their respective donors, post transplant survival and outcome	99
Table 4.2	Graft ischaemic times and number of rejection and pulmonary infection episodes in paediatric lung transplant recipients	100

Table 4.3	Forced vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (FVC%)	103
Table 4.4	Forced expiratory volume in one second in paediatric lung transplant recipients expressed as percentage of predicted normal (FEV₁%)	104
Table 4.5	Percentage ratio of forced expiratory volume in one second to forced vital capacity (FEV₁/FVC) in paediatric lung transplant recipients	105
Table 4.6	Peak expiratory flow rate in paediatric lung transplant recipients expressed as percentage of predicted normal (PEFR%)	106
Table 4.7	Maximum expiratory flow at 25% of remaining vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (MEF₂₅%)	107
Table 4.8	Total lung capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (TLC%)	108
Table 4.9	Residual volume in paediatric lung transplant recipients expressed as percentage of predicted normal (RV%)	109
Table 4.10	Standardised lung diffusing capacity of carbon monoxide in paediatric lung transplant recipients expressed as percentage of predicted normal (KCO%)	110
Figure 4.2	Graph of median and range of forced vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (FVC%)	111
Figure 4.3	Graph of median and range of forced expiratory volume in one second in paediatric lung transplant recipients expressed as percentage of predicted normal (FEV₁%)	112
Figure 4.4	Graph of median and range of percentage ratio of forced expiratory volume in one second to forced vital capacity (FEV₁/FVC) in paediatric lung transplant recipients	113

Figure 4.5	Graph of median and range of peak expiratory flow rate in paediatric lung transplant recipients expressed as percentage of predicted normal (PEFR%)	114
Figure 4.6	Graph of median and range of maximum expiratory flow at 25% of remaining vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (MEF₂₅%)	115
Table 4.11	Significance values for differences in pulmonary function measurements at 6 – 60 months post transplant, compared with pre transplant values	116
Table 4.12	Area under the curve of FEV₁% measurements (FEV₁% AUC) for each subject from 3 months to 12, 18, 24, 30, and 36 months post transplant and for the second and third post transplant years	119
Figure 4.7	(a) Graph of FEV₁% for two subjects (4 and 18) during the first 36 months post transplant. Despite similar FEV₁% values over the first 12 months, FEV₁% AUC values from 3 to 36 months were markedly different for each subject (b) and (c)	120
Figure 4.8	(a) Graph of FEV₁% for two subjects (5 and 19) during the first 36 months post transplant. Despite similar FEV₁% values at 36 months, FEV₁% AUC values from 3 to 36 months were markedly different for each subject (b) and (c)	121
Figure 4.9	Relationship between post transplant survival (from 3 years) and FEV₁% AUC from 3 to 36 months post transplant	122
Table 4.13	Significance values for correlations between FEV₁% AUC and other parameters	123
Figure 4.10	Relationship between graft cold ischaemic time (GIT-C) and FEV₁% AUC from 3 to 36 months post transplantation with regression line (p<0.05)	124
Figure 4.11	Relationship between graft total ischaemic time (GIT-T) and FEV₁% AUC from 3 to 36 months post transplantation with regression line (p<0.01)	125

Figure 4.12	Relationship between graft cold ischaemic time and FEV₁% AUC in the second year post transplantation with regression line (p<0.05)	126
Figure 4.13	Relationship between graft total ischaemic time and FEV₁% AUC in the second year post transplantation with regression line (p<0.05)	127
Figure 4.14	Relationship between recipient age at time of transplantation and FEV₁% AUC in the second year post transplantation with regression line (p<0.05)	128
 Chapter 5		
Figure 5.1	Polylysine-molossin (Poly-Mol) gene vector	136
Table 5.1	Components of tracheal instillate for <i>in vivo</i> gene transfer - Phase 1	140
Table 5.2	Components of tracheal instillate for <i>in vivo</i> gene transfer – Phase 2	141
Table 5.3	Preservation and staining methods used for rat lungs	145
Figure 5.2	X-GAL staining at varying pH of cubes of rat lung instilled with normal saline, and rat intestine	153
Figure 5.3	X-GAL staining at varying pH levels of cubes of rat lung instilled with naked DNA and DNA with Poly-Mol vector	154
Figure 5.4	X-GAL staining at varying pH levels of cubes of rat lung instilled with DNA, Poly-Mol vector and chloroquine (P8)	155
Figure 5.5	X-GAL staining at varying pH levels of cubes of rat lung instilled with DNA, Poly-Mol vector and fusogenic peptide (P10)	156
Figure 5.6	Micrographs (63x) of cryosections (10µm) of negative control rat lung (P26), (a) without, and (b) with, H₂O₂ quenching	158

Figure 5.7	Micrographs (63x) of immunoperoxidase stained cryosections from rat lung instilled with Thy-1 reporter gene (100µg/mL), Poly-Mol vector, fusogenic peptide and Lipofectamine	160
Figure 5.8	Micrographs as in Fig 5.7 at higher magnification (126x)	161
Figure 5.9	Poly-Mol vector distribution study (63x)	164
Figure 5.10	Poly-Mol vector distribution study (126x)	165
 Chapter 6		
Figure 6.1	Predicted secondary RNA structure of E1A	183
Figure 6.2	Predicted secondary RNA structure of PIX	184
Figure 6.3	Cartoon depicting method of viral plaque assay system with antisense oligodeoxynucleotides	186
Figure 6.4	Cartoon describing method of serial titring assay with antisense oligodeoxynucleotides	189
Figure 6.5	(a) Cell culture dish of A549 cells with no added virus (negative control) (b) Positive control cell culture dish of A549 cells with wild type adenovirus 5 (c) Plaques at higher magnification (x40)	192
Table 6.1	Plaque counts in A549 cells using various ODNs, following wild type adenovirus 5 application	195
Figure 6.6	Plaque counts at day 14 in A549 cells treated with ODNs (15µM concentration) following wild type adenovirus 5 application (as per Table 6.1)	195
Table 6.2	Plaque counts in A549 cells using E1AAS ODN at 1µM and 15µM concentrations, following wild type adenovirus 5 application	197

Figure 6.7	Plaque counts at day 14 in A549 cells using E1AAS ODNs at 1μM and 15μM concentrations, following wild type adenovirus 5 application (as per Table 6.2)	198
Table 6.3	Plaque counts in A549 cells using E1AAS and PIXAS ODNs at 1μM concentration with Lipofectamine (10μg/mL), following wild type adenovirus 5 application	200
Table 6.4	Plaque counts in A549 cells using E1AAS ODNs at 1μM concentration in the absence and presence of Lipofectamine (10μg/mL, without FCS or antibiotics), following wild type adenovirus 5 application	202
Figure 6.8	Plaque counts at day 14 in A549 cells using E1AAS ODNs at 1μM concentration in the presence or absence of Lipofectamine (10μg/mL, without FCS or antibiotics), following wild type adenovirus 5 application (as per Table 6.4)	203
Table 6.5	Plaque counts in A549 cells using end-protected and fully-protected E1AAS ODNs at 1μM concentration, following wild type adenovirus 5 application	205
Table 6.6	Plaque counts in A549 cells using end-protected and fully-protected E1AAS ODNs at 1μM concentration, following wild type adenovirus 5 application	206
Figure 6.9	Plaque counts at day 14 in A549 cells using end-protected and fully-protected E1AAS ODNs at 1μM concentration, following wild type adenovirus 5 application (as per Table 6.6)	207
Table 6.7	Plaque counts in A549 cells with E1AAS, E1AAS2, E1AAS3, PIXAS, PIXAS2, PIXAS3 ODNs at 1μM concentration, following wild type adenovirus 5 application	209
Figure 6.10	Plaque counts at day 14 in A549 cells with E1AAS, E1AAS2, E1AAS3 ODNs at 1μM concentration, following wild type adenovirus 5 application (as per Table 6.7)	210
Figure 6.11	Plaque counts at day 14 in A549 cells with PIXAS, PIXAS2, PIXAS3 ODNs at 1μM concentration, following wild type adenovirus 5 application (as per Table 6.7)	211

Table 6.8	Plaque counts in A549 cells with E1AAS, E1AAS4, E1AAS5, PIXAS4, PIXAS5 ODNs at 1μM concentration, following wild type adenovirus 5 application	212
Figure 6.12	Plaque counts at day 14 in A549 cells with E1AAS, E1AAS4, E1AAS5 ODNs at 1μM concentration, following wild type adenovirus 5 application (as per Table 6.8)	213
Figure 6.13	Plaque counts at day 14 in A549 cells with PIXAS4 and PIXAS5 ODNs at 1μM concentration, following wild type adenovirus 5 application (as per Table 6.8)	214
Table 6.9	Meta-analysis of E1AAS inhibition of viral plaque formation	215
Table 6.10	Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS, E1ANS, PIXAS and PIXNS ODNs at 1μM concentration	217
Table 6.11	Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS and E1ANS ODNs at 1μM concentration	218
Figure 6.14	Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS and E1ANS ODNs at 1μM concentration. Plaque counts from day 14 (as per Table 6.11)	219
 Chapter 7		
Table 7.1	Components of test medium for initial nuclear localisation experiments	232
Table 7.2	Conditions for second phase nuclear localisation experiments	234
Figure 7.1	Fluoroscopic photomicrographs of control slides of A549 cells after nuclear staining with DAPI at: (a) 20x, and (b) 100x magnification	236
Figure 7.2	Fluoroscopic photomicrograph (20x magnification) of A549 cells treated with fluoresceinated ODN	237

Figure 7.3	Fluoroscopic photomicrograph (100x magnification) of A549 cells treated with fluoresceinated ODN	238
Figure 7.4	Fluoroscopic photomicrograph (100x magnification) of A549 cells treated with fluoresceinated ODN in the presence of lipofectamine	239
Table 7.3.	Number of nuclei associated with fluorescent oligonucleotide per high power field (100x magnification) under various conditions	240

Introduction to thesis

This thesis is based on both clinical and non-clinical studies. The former were performed at the Great Ormond Street Hospital for Children NHS Trust, London. The latter were undertaken at two sites - the Institute of Child Health, University College London and the Institute of Liver Studies, King's College London. The work presented in this thesis has spanned over six years, with the author involved in cardiothoracic transplantation for 11 years.

Despite many recent advances in the clinical management of the lung transplant recipient, injury to the pulmonary allograft remains a prominent feature. Various mechanisms play a role in producing this lung injury, both before and after the transplant procedure. Some are related to direct physical effects on the lung such as with ischaemia and surgery, while injury may occur as a consequence of other factors. Two of the major factors include rejection of, and infection in, the pulmonary allograft. The end result of these injurious events often comprises the development of obliterative bronchiolitis or chronic graft dysfunction. Once established, obliterative bronchiolitis is resistant to medical therapy and is a major cause of mortality.

There is a predictable emphasis on paediatric cardiothoracic transplantation in the clinical section of the thesis. Children undergoing lung transplantation present major challenges. Firstly, they appear to develop complications more readily than their adult counterparts, showing a greater incidence of rejection in the early post transplant period and an increased incidence of obliterative bronchiolitis. One of the reasons for these observed discrepancies may relate to the difficulty in monitoring children following lung transplantation. Non-invasive pulmonary function monitoring has not been well documented, while invasive monitoring by bronchoscopy and transbronchial biopsy has greater associated risks in the younger age group.

Accordingly, the mechanisms of injury in the lung allograft, obliterative bronchiolitis and the associated clinical aspects are reviewed in the first two chapters. Chapter 4 presents a detailed study of pulmonary function in a cohort of paediatric lung transplant recipients. Furthermore, the effects of identified injurious elements on post transplant pulmonary function are assessed.

Gene therapy represents an exciting and innovative method of treating diseases of the lung, especially as the lung is directly accessible for topical gene application. The main emphasis over recent times has centred on the correction of the genetic defect in the lungs of cystic fibrosis patients. There have been few reports on the role of gene therapy in the setting of lung transplantation, although studies assessing gene transfer techniques in other solid organ transplant models are beginning to emerge in the literature.

One of the major issues with gene therapy relates to the method of gene transfer and type of vector used. Viral gene vectors have been associated with untoward side effects including local inflammatory reactions. As a consequence, they induce a significant immunological response, which may reduce the efficacy of gene transfer. Non-viral gene vectors may represent a more effective and less hazardous method of gene transfer, and therefore may be an attractive alternative to viral vectors. These issues are addressed in Chapter 3, while the evaluation of a specific non-viral gene vector used in an *in vivo* rat lung model is described in Chapter 5.

Viral infections in the pulmonary allograft, although more rare than bacterial infections, are associated with a high incidence of morbidity, related directly to the degree of lung injury incurred. The pneumonitis induced by adenovirus may be fatal in the acute setting and may predispose to the early onset of obliterative bronchiolitis. Paediatric lung transplant recipients seem to be particularly prone to contracting adenovirus infection, presumably related to the lack of previous exposure. These primary infections, which may be transferred with the donor lung, appear to be the most clinically devastating. There is currently no specific treatment available for adenovirus infection.

A novel approach to the treatment of viral infections entails the use of antisense oligodeoxynucleotides (ODNs). These have been used with some success in *in vitro* models for the treatment of HIV, papillomavirus and other viral infections. Once again, the lung lends itself to this approach due to the direct accessibility of the airways. Therefore, a possible treatment option for adenoviral pneumonitis might involve administering antisense ODNs topically to the lung. Antisense methodology, oligodeoxynucleotide techniques and their application to the treatment of viral infections are reviewed in Chapter 3, while the development and assessment of specific antisense oligodeoxynucleotides for the treatment of adenovirus infection in an *in vitro* assay are described in Chapters 6 and 7.

Section A
Literature review

Chapter 1 Mechanisms of injury in the transplanted lung

1.1 Introduction

1.2 General overview of thoracic transplantation

1.2.1 Historical aspects of cardiac transplantation

1.2.2 Cyclosporine

1.2.3 Historical aspects of lung transplantation

1.2.4 Paediatric thoracic transplantation

1.3 Rejection of the lung allograft

1.3.1 Mechanisms of rejection

1.3.1.1 Major histocompatibility complex (MHC)

1.3.1.2 Rejection effector cells

1.3.1.3 Allorecognition and alloresponsiveness

1.3.1.4 Humoral responses

1.3.1.5 Hyperacute, acute and chronic rejection

1.3.2 Immunosuppression agents

1.4 Infection in the lung allograft

1.4.1 Predisposing factors

1.4.2 Infectious agents

1.4.2.1 Bacteria

1.4.2.2 Viruses

1.4.2.3 Fungi

1.4.2.4 Other agents

1.5 Other factors

1.6 Obliterative bronchiolitis

1.6.1 Pathophysiology

1.6.2 Incidence

1.6.3 Aetiology

1.6.4 Research aspects

1.1 Introduction

Of all the solid organs currently transplanted, the lung presents as one of the most challenging. It is a relatively fragile organ as regards surgical handling and does not cope with ischaemia well. Due to its communication with the environment, it is constantly exposed to infective and other toxic elements. Presumably as a response to this exposure, the lung has become an immunologically active organ, with highly developed immune responses. As a consequence, the lung appears to be more prone to rejection processes than other transplanted organs.

Many of these factors, together with other mechanisms, may result in significant injury to the transplanted lung. Much of this injury is reversible with appropriate therapy, although repeated insults may eventually lead to the development of chronic, irreversible lung damage. In the setting of lung transplantation, this chronic damage is known as obliterative bronchiolitis, also termed bronchiolitis obliterans. This disease remains the greatest impediment to the long-term success of lung transplantation.

This chapter, therefore, gives a brief overview of lung transplantation in both adult and paediatric recipients and explores the various mechanisms of injury in the lung allograft. The two most important elements include rejection of, and infection in, the pulmonary allograft and these two topics are discussed in detail. Other factors involved in producing injury in the transplanted lung are detailed and finally, a review of obliterative bronchiolitis is presented.

1.2 General overview of thoracic transplantation

1.2.1 Historical aspects of cardiac transplantation

John Hunter was the first to suggest transplantation of solid organs in 1778. However, it was not until the 20th century that major advances in the field allowed successful clinical transplantation to become a reality. Within the discipline of cardiothoracic transplantation, Carrel and Guthrie described the first experimental cardiac transplantation in a dog (Carrel 1905). They implanted the heart into the neck of the animal (heterotopically) to establish the techniques of vascular anastomoses. Later, in 1933, Mann *et al*, performing similar procedures in dogs, demonstrated lymphocytic infiltrations in the transplanted organs and postulated that “some biological factor” produced failure rather than problems with the surgical technique (Mann 1933). This of course was untreated, acute graft rejection.

Numerous cardiothoracic surgical advances were made during the 1950s and included the use of hypothermia (Neptune 1953) and preservation methods for donor hearts (Webb 1957). Most of the experimental models in use at the time incorporated the heterotopic approach (ie: an auxiliary heart in a different anatomical position), which could not support the circulation. It was Lower and Shumway in 1960 who initially reported a successful method of orthotopic transplantation in canines in which the native heart was removed and a donor heart implanted into the same site (Lower 1960). The implanted hearts not only supported the circulation but also enabled survival of the dogs for up to 21 days. Immunosuppression was not administered to these animals and once again histological changes of acute graft rejection were observed. However, it was clear that the surgical techniques employed were practicable and successful.

The recognition and acceptance of brain stem death criteria paved the way for the use of cadaveric human donor organs and in 1967, Christian Barnard performed the first successful cardiac transplant in man using a human donor heart (Barnard 1967). The immunosuppression regimen was primitive and aggressive with the result that

the recipient succumbed after 18 days from pneumonia caused by *Pseudomonas spp.* In the same year, Adrian Kantrowitz performed an orthotopic cardiac transplant in a neonate with severe congenital heart disease. Sadly, the recipient only survived for six hours (Kantrowitz 1968).

Following these two reports, a worldwide wave of enthusiasm for cardiothoracic transplantation arose over the ensuing two to three years. This excitement soon dwindled due to the poor results obtained, mainly as a consequence of the inadequate immunosuppression protocols available at the time. A few centres, including Stanford University in California, persevered and during the 1970s made major advances in donor organ retrieval methods (Watson 1979) as well as immunosuppression strategies. The latter included the development of rabbit anti-thymocyte globulin for induction therapy and for the treatment of graft rejection (Griepp 1972).

1.2.2 Cyclosporine

Without question, the most significant event during this period was the discovery of the immunosuppressive qualities of cyclosporine A (CsA) by Jean François Borel and colleagues in Basle, Switzerland (Borel 1991). This agent, a neutral, lipophilic cyclic peptide, was derived from the fungus *Tolypocladium inflatum* Gams. Its inhibitory action on lymphocytes is mediated via an immunophilin, which downregulates the transcription and hence the production of interleukin 2. CsA has been shown to be an effective agent in abrogating allograft rejection.

The introduction of CsA into clinical practice revolutionised the approach to immunosuppression in solid organ transplantation during the late 1970s and early 1980s (Calne 1978, Cohen 1984). No longer was it necessary to utilise the relatively unsophisticated combinations of "broad spectrum" agents such as high dose corticosteroids and azathioprine. Instead, a more specific approach to combat allograft rejection with immunosuppression therapy was possible. The initial

experience in renal transplantation demonstrated that CsA could be used in combination with lower doses of corticosteroids and azathioprine with superior results and fewer side effects than had been obtained previously.

This encouraging experience was reproduced in the cardiac transplant arena (Reitz 1982). There was an impressive increase in post-transplant survival and a concomitant reduction in immunosuppression related side effects, particularly infections. However, CsA was not devoid of deleterious side effects with nephrotoxicity one of the more serious (Copeland 1986). Many of these effects appeared to be dose related and therefore assays were developed to monitor CsA levels and correlate these with clinical status. Subsequently, therapeutic ranges were derived, although these were often empiric in nature (Rodighiero 1989). Nevertheless, CsA had made a huge impact upon cardiac transplantation and had paved the way for the resurgence of lung transplantation as a therapeutic modality.

1.2.3 Historical aspects of lung transplantation

Demikhov, a Russian surgeon, reported the first experimental heart-lung transplants performed in dogs during the 1940s (Demikhov 1960). The longest post-operative survival was six days. Dogs, as well as cats, require innervation of the lungs to maintain ventilation (ie: an intact Hering-Breuer reflex) and without this, long term survival is not possible (Nakae 1967). However, Castaneda and co-workers showed that this reflex was not required to maintain ventilation in baboons (Castaneda 1972) and postulated that long-term survival following transplantation of the lungs could be expected in other primates including man.

Another aspect of lung transplantation to be considered was the airway anastomosis. The poor healing of the airway anastomosis complicated initial attempts at clinical lung transplantation. This was presumably related to the high dose corticosteroid therapy used to prevent the rejection response. At Stanford University, the cardiothoracic transplant team had developed an improved surgical technique for the

tracheal anastomosis in their model of combined heart-lung transplantation (Reitz 1980). Together with the introduction of CsA and its associated corticosteroid-sparing effects, they were able to perform the first successful heart-lung transplant in a woman with primary pulmonary hypertension in 1981 (Reitz 1982a). This marked a turning point in clinical lung transplantation.

There had been some previous (unsuccessful) attempts at single lung transplantation during the 1960s (Hardy 1963, Wildevuur 1970) and it was not until well after the initial Stanford experience with heart-lung transplantation that isolated lung transplantation became a viable technique. Single lung, double lung and bilateral sequential single lung transplantation were developed after the initial problems with bronchial airway healing were overcome (Toronto Lung Transplant Group 1986, Cooper 1989). The procedure of choice depends very much upon the original diagnosis of the patient and debate still exists as to the place of heart-lung transplantation and subsequent "domino" transplantation of the heart from the heart-lung recipient (Cavarocchi 1989).

1.2.4 Paediatric thoracic transplantation

Thoracic transplantation in the paediatric age group was considered a more viable proposition following the introduction of CsA and the encouraging results obtained with lung transplantation in adults. Concerns including the long term outcome of lung transplantation, the risks associated with chronic immunosuppression and its effects on growth, and the socio-ethical implications were still a major consideration and produced a delay in the application of this therapy in children.

The first reports of paediatric lung transplantation began appearing at the end of the 1980s and early 1990s (Smyth 1989, Starnes 1991). These suggested that there were other problems associated with the procedure in this younger age group. Firstly, obtaining appropriately sized donor organs limited its application. Furthermore, a greater incidence of graft related complications were reported - in particular

rejection and infection. These in turn appeared to predispose to the early onset of chronic graft dysfunction in the form of obliterative bronchiolitis. It was not surprising then that early results of paediatric lung transplantation were inferior to those obtained in adults (see below).

Nevertheless, lung transplantation offered to many seriously ill children an opportunity of prolonged quality survival. Consequently, many transplant centres commenced performing lung transplantation in children with terminal cardiopulmonary conditions, including cystic fibrosis and primary and secondary pulmonary vascular disease, with increasing success (Noyes 1994, Spray 1994, Whitehead 1995, Bridges 1996). However, it became readily apparent that to achieve real clinical success in both adult and paediatric lung transplantation, greater understanding of the mechanisms of injury to the lung allograft, particularly by rejection and infection, and their management, was essential.

1.3 Rejection of the lung allograft

Pulmonary allograft rejection is a common event and, together with infection, poses a major threat to graft (and patient) survival. It is an almost invariable occurrence early after lung transplantation, particularly in children. Rejection may produce an immediate deterioration in pulmonary function that is usually reversible with augmentation of immunosuppression. However, recurrent episodes of rejection may occur leading to more persistent lung damage and an accompanying reduction in respiratory capacity. This may eventually result in the condition known as “obliterative bronchiolitis” or “bronchiolitis obliterans”, about which more will be discussed in Section 1.6. Furthermore, increased immunosuppressive therapy used to treat rejection raises the risks of pulmonary infection intervening, thus contributing to the process of lung damage. The following discussion outlines the currently understood basic concepts of allograft rejection and how these apply in the setting of lung transplantation.

1.3.1 Mechanisms of rejection

1.3.1.1 Major histocompatibility complex (MHC)

Histocompatibility antigens are glycoproteins present on the cell membrane. In all vertebrate species, histocompatibility antigens can be divided into a single major histocompatibility system that is extremely complex (hence major histocompatibility complex or MHC), and numerous minor systems. It is incompatibility for antigens of the MHC between a donor and a recipient, which are the main determinants of rejection and destruction of the allograft.

In man, the MHC is known as the human leucocyte antigen (HLA), and can be further subdivided into Class I and Class II antigens, based on their structure, tissue distribution and function (Klein 1981). Class I antigens are expressed on most cells, whereas Class II antigens demonstrate a restricted distribution. The latter are

expressed on B lymphocytes, activated T lymphocytes, macrophages, dendritic cells and endothelium. In Class I antigens, polymorphism is mainly restricted to the α_1 and α_2 domains of the heavy chain, while the polymorphism in Class II antigens is confined mainly to the α_1 domain of the α chain and the β_1 domain of the β chain. The HLA-DR A gene, however, is essentially non-polymorphic. The difference between MHC alleles, either Class I or II, is due to relatively small differences in amino acid sequences in the polymorphic chains of the respective molecules. The classical HLA Class I and Class II genes are the most polymorphic yet discovered, with hundreds of alleles described so far (Parham 1996).

Incompatibility for either Class I or Class II antigens can give rise to an immune response resulting in graft rejection. However, it is possible that Class II antigen incompatibility may cause a more marked immune response to an allograft than Class I incompatibility due to some of the mechanisms which are discussed below.

1.3.1.2 Rejection effector cells

Neutrophils are usually the first inflammatory cells recruited in the host response to the transplanted organ. They may infiltrate the graft within hours after interaction with selectins upregulated on vessel walls after the nonspecific ischaemic insult. These cells may in turn release biochemical mediators that increase the expression of adhesion molecules and enhance vascular permeability, enhancing the infiltration of the graft by host lymphocytes and macrophages (Sedmark 1991).

Both CD4⁺ and CD8⁺ T lymphocytes are found in acutely rejecting allografts. CD4⁺ is a ligand for MHC class II molecules distributed on dendritic cells, circulating B lymphocytes, monocytes, and vascular endothelial cells (an important target for immune injury). CD4⁺ T lymphocytes can be further divided into Th1 and Th2 subpopulations: the former produce the proinflammatory lymphokines interferon gamma (IFN- γ), interleukin 2 (IL-2), and tumour necrosis factor beta (TNF- β); whereas the latter produce inhibitory cytokines, IL-4, IL-5, and IL-10.

The CD8 molecule is a ligand for MHC class I antigen, expressed on most cell surfaces. IFN- γ is produced by one type of CD8⁺ T cell, which has a major function as a cytotoxic T cell (Kemeny 1994).

Both CD4 and CD8 molecules act as accessory molecules to increase the avidity and stability of the interaction between T cells and antigen presenting cells. However, this interaction alone is not sufficient for inducing T cell proliferation and differentiation into helper or effector lymphocytes. Costimulatory molecules expressed on the surface of activated antigen-presenting cells provide the second activation signal (Sayegh 1998).

Other graft infiltrating cells include B-lymphocytes, which differentiate into antibody-producing plasma cells, secreting both non-specific and specific antidonor antibodies (Garovoy 1982). Macrophages may act as antigen presenting cells that activate T cells and other macrophages, and as effector cells by cytokine secretion and antibody-dependent cellular cytotoxicity (Nathan 1980).

Natural killer cells are cytotoxic cells which do not express CD3 or the T-cell receptor. These cells may contribute to the damage produced by acute rejection by antibody-dependent cellular cytotoxicity (Moretta 1992). They may kill allogeneic cells selectively after failing to recognise self MHC Class I antigen on their surfaces.

1.3.1.3 Allorecognition and alloresponsiveness

The phenomenon of allorecognition was first defined after transplantation of incompatible tissues, and in turn led to the identification of the MHC and its products. The alloresponse is unusually strong, as evidenced by the vigorous primary immune responses observed in the mixed lymphocyte reaction. This may be related to the unusually high precursor frequency of T cells that recognise allogeneic MHC molecules. However, the reasons for this high frequency are less clear and are the focus of much discussion (Lechler 1990).

Two distinct pathways of allorecognition have been described. The “direct” pathway involves the recognition by T cells of intact allo-MHC molecules on the surface of donor stimulator cells. Peptides, derived from endogenous proteins such as other MHC molecules, bind into the groove of the donor MHC (antigen-presenting cell) and are recognised by recipient T cells (McPhaul 1981). Acute rejection might be mediated primarily by this direct allorecognition pathway.

In the “indirect “ pathway, T cells recognise processed alloantigen presented as allopeptides by self-antigen processing cells. The basic premise for indirect allorecognition as a mechanism for initiation and/or amplification of allograft rejection is that donor alloantigens are shed from the graft, taken up by recipient antigen presenting cells, and presented to T cells (Krensky 1994, Halloran 1997, Benham 1995).

1.3.1.4 Humoral responses

Although not completely delineated, host humoral activity may play a role in the process of acute allosensitiveness. Natural antibodies are involved in hyperacute rejection of organ xenografts. In allotransplantation, anti-MHC antibodies and anti-ABO antibodies can produce hyperacute rejection. Plasma cells and B-lymphocytes have been identified in large numbers within days of transplantation in both the graft and the recipient spleen. Antibodies eluted from rejected human renal allografts show specific antidonor reactivity, nonspecific reactivity and anti-HLA cross-reactivity (Garovoy 1982).

T cell and macrophage products, including IL-2, IL-4, IL-5, and IFN- γ , affect B cell differentiation and proliferation (Charpentier 1993). Donor specific antibodies appear quickly in the serum of grafted recipients, initially immunoglobulin M (IgM) and later, IgG types. These antibodies may bind to graft endothelial cells, activate complement and coagulation systems, and result in tissue injury.

1.3.1.5 Hyperacute, acute and chronic rejection

Allograft rejection can also be classified into the following three categories, which usually relate to the chronological pattern of occurrence.

a) Hyperacute rejection

As its name implies, hyperacute rejection is a rapidly developing process, which tends to occur early following implantation of the donor organ(s). It can develop from within minutes to hours post transplantation and results in loss of the graft. Histologically, it is characterised by thrombosis, haemorrhage, oedema and lack of a cellular infiltrate. The process is initiated by the binding of an antibody, which is already present in the recipient, to the graft endothelium and activation of the complement cascade. The immunopathology of hyperacute rejection has been well documented in the xenograft model (Platt 1991). Hyperacute rejection of the pulmonary allograft is now rarely observed due to ABO blood group matching of the donor and recipient and screening of the recipient for pre-formed antibodies.

b) Acute rejection

Despite advances in transplant management and immunosuppression methods, acute rejection remains a prominent entity (Tilney 1994). It is primarily a T-lymphocyte-mediated host event with cellular infiltration of the graft the most characteristic histopathological feature of the process (Halloran 1993).

The basic principles of the acute rejection process have been detailed above. In summary, once recognition of foreign MHC has occurred via a complex interaction involving cytokines, co-stimulatory, accessory and adhesion molecules, the T cell becomes “activated” eventually producing cell differentiation and clonal proliferation. These cells then induce inflammation within the graft leading to tissue damage. If unchecked this may develop into more extensive destruction of the graft.

Histologically, in the lung allograft, acute rejection is associated with perivascular and interstitial mononuclear cell infiltrates. With worsening rejection, mononuclear cells may extend beyond the vascular adventitia and percolate into the adjacent alveolar septa. Furthermore, there may be prominent alveolar pneumocyte damage usually associated with intra-alveolar necrotic cells, macrophages, hyaline membranes, haemorrhage, and neutrophils (Yousem 1996).

Many of the mechanisms of acute pulmonary rejection have been supported by observations in studies of human lung transplant recipients. De Blic *et al*, using immunohistochemical methods, demonstrated that cells infiltrating lung allografts during episodes of rejection were mainly of T cell origin with both CD4+ and CD8+ phenotypes (de Blic 1992). Other studies have shown that lymphocytes isolated from pulmonary allografts either by bronchoalveolar lavage or transbronchial biopsy are activated during acute rejection, as evidenced by their response to growth stimuli such as the cytokine, interleukin-2 (IL-2) and increased release of soluble IL-2 receptors (Rabinowich 1990, Lawrence 1989). In addition, other pro-inflammatory cytokine genes and their products have been shown to increase during acute rejection. Increases in IL-1, IL-2, tumour necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) have been shown to correlate with histological changes of rejection (Sundaresan 1995, Whitehead 1993a).

c) Chronic rejection

This form of rejection has been less well defined. It is common to all forms of solid organ transplantation and has also been termed “chronic allograft dysfunction”. It usually develops slowly over a period of months or years, although a more rapid onset may occur. Both alloantigen-dependent and alloantigen-independent factors appear to have a role in the aetiology of the process (Tullius 1995).

In lung transplantation, chronic allograft dysfunction is also described as “obliterative bronchiolitis” or “bronchiolitis obliterans” and is associated with high morbidity and a significant mortality. It has become the one most important

obstacle to long term success in lung transplantation. As it has assumed such a prominent position in the post lung transplant course, it will be discussed in greater detail in the next section.

1.3.2 Immunosuppression agents

Immunosuppression regimens comprise both induction and maintenance protocols. The former, used in the early post-transplant phase, often incorporates high dose glucocorticoids together with a cytolytic, or anti-lymphocytic, agent. Maintenance immunosuppression tends to be based on a triple therapy regimen combining cyclosporine or tacrolimus, azathioprine and a glucocorticoid.

Glucocorticoids and azathioprine are relatively non-selective immunosuppressive agents, affecting both T and B cell function. They can be considered as broad-spectrum agents. The former is used at higher doses to control acute rejection episodes and at lower maintenance doses to prevent rejection.

The efficacy of modern immunosuppressive protocols is derived from their ability to selectively inhibit different steps of the cell- and cytokine-mediated immune cascade. Cyclosporine and tacrolimus are both calcineurin inhibitors and both block IL-2 production by activated T cells, thereby blocking the rejection cascade (Vella 1998).

The newer agent rapamycin inhibits signal transduction through selected cytokine receptors. In contrast, mycophenolate mofetil, another relatively new drug, blocks the salvage pathway of purine metabolism upon which lymphocytes are particularly dependent (Vella 1998). Treatment with this latter agent has been associated with a reduction in the incidence of acute rejection of greater than 50% in renal transplantation (Halloran 1997).

Other antagonists of molecular targets are entering the clinical arena. One such agent is a humanised murine monoclonal antibody targeted against the 55kDa α chain of the IL-2 receptor used for induction therapy. In renal transplantation, when used with an azathioprine based triple therapy regimen, it reduces the risk of acute rejection from 40% to 20% within the first year (Vincenti 1998). However, there is not one immunosuppression regimen, which can guarantee freedom from rejection with no associated deleterious side effects. The Holy Grail would be to avoid immunosuppression completely by in some way modulating the allograft and so rendering it immunologically inert.

1.4 Infection in the lung allograft

Infections, both pathogenic and opportunistic, remain a major cause of morbidity and mortality in the lung transplant recipient. Although susceptible to infection at any anatomical site, the lung transplant recipient is at particular risk of developing pulmonary infection, by virtue of the lung's direct communication with the environment. Together with rejection, infection poses a significant threat to the integrity and function of the pulmonary allograft. However, with increasing experience, the burden of infection has lessened as clinicians become more aware of prevention, prompt identification and treatment of infective processes. The following details factors that influence the incidence of infection in the lung allograft and describes the various organisms encountered in the post transplant setting, their relative importance and treatment.

1.4.1 Predisposing factors

There are numerous factors predisposing the transplanted lung to infective processes. The most important early in the transplant process include surgical considerations and immunosuppression.

a) Surgical factors

Obviously, any invasive procedure, especially lung transplant surgery, increases the risk of infection. Together with this are the additional burdens of graft ischaemia, both between the time of organ retrieval and re-implantation, as well as the chronic ischaemia related to disruption of the bronchial arterial system. Other associated surgical factors include interruption of lymphatic drainage, loss of pulmonary innervation and impairment of mucociliary function leading to retention of secretions (Aeba 1993). Post-operative pain may inhibit the cough reflex, which can further predispose to sputum retention and pulmonary infection. The ill defined "reperfusion injury" which may accompany implantation of the pulmonary allograft

may also increase the risk of infection. This process produces diffuse lung damage with parenchymal oedema and a prominent influx of inflammatory cells - the ideal environment for the development of infection (Novick 1992).

b) Immunosuppression

The maintenance of immunosuppressive therapy renders the patient at increased risk of infection indefinitely. This may include infections by pathogenic organisms as well as those by opportunistic organisms. Any level of immunosuppression will impact upon this risk and, as lung transplant recipients receive appreciably more immunosuppression than other solid organ transplant recipients, they will be effectively more at risk of infectious complications (Gryzan 1988). As the most intense immunosuppression is attained early post-transplant, this is the period of greatest risk. Subsequently, the risk reduces as the degree of immunosuppression decreases. However, as a consequence of augmentation of immunosuppression for episodes of rejection occurring later, the risks of infection may once again rise.

c) Recipient related factors

Other factors that influence the onset of infection may relate to the recipient's original diagnosis, the state of the donor lungs and the adequacy of anti-microbial prophylaxis. Lung transplant recipients with cystic fibrosis are often colonised with multi-resistant or even pan-resistant organisms including *Pseudomonas aeruginosa* and *Burkholderia cepacia*. These difficult to treat organisms remain in the upper airways after transplantation and therefore may seed the newly transplanted lungs producing a pneumonic process (Egan 1995). This may also occur in those patients with other chronic suppurative lung diseases undergoing transplantation. This group may have a similar problem with colonisation of their upper airways with resistant pathogens. Patients with chronic lung disease, including those with and without cystic fibrosis, may be malnourished, and this in itself may predispose to infection post transplant.

d) Donor related factors

Donor related factors include the length of time of intubation and the presence of organisms in the donor airway. With the relative paucity of suitable donor organs, many transplant centres now accept lungs that were previously considered unsuitable. These may include donors with chest x-ray infiltrates and those with a positive tracheal aspirate on Gram stain. Early post-transplant pulmonary infections may be produced by these donor-transferred organisms (Zenati 1990, Low 1993).

1.4.2 Infectious agents

Many organisms have been implicated in producing infections in the pulmonary allograft. These may comprise both pathogenic and opportunistic organisms. The various types of microbial agents are detailed under the following categories.

1.4.2.1 Bacteria

Bacteria represent the commonest pathogens encountered and are responsible for most of the early post-transplant deaths due to infection (Kramer 1993, Bando 1995). In two separate series, gram-negative bacteria accounted for the majority of both pulmonary and non-pulmonary bacterial infections, with *Pseudomonas aeruginosa* the most common (Kramer 1993, Maurer 1992). Of the gram-positive organisms, *Staphylococcus* spp. were the commonest isolated. Other gram-negative and gram-positive bacteria isolated in these two comprehensive series, and their relative frequencies, are shown in Table 1.1.

Interestingly, both *Pseudomonas aeruginosa* and *Staphylococcus* spp. are typically nosocomial organisms, indicating a high incidence of hospital acquired infection in both of the series cited. Patients with cystic fibrosis may be colonised pre operatively with resistant bacteria including *Pseudomonas aeruginosa*, and these

may produce infection post transplant. More recently, *Burkholderia cepacia*, previously known as *Pseudomonas cepacia*, has become an important pathogen in the post transplant setting. It may cause overwhelming sepsis and be responsible for an associated high morbidity and mortality (Snell 1993). It has become so much of a concern that many transplant centres now consider that pre transplant colonisation with *Burkholderia cepacia* represents, in the least, a relative contraindication to lung transplantation. In addition, transplant patients who have established obliterative bronchiolitis often have an associated bronchiectasis and therefore may become chronically colonised with gram-negative bacilli such as *Pseudomonas aeruginosa* (Kramer 1993a).

Other bacteria implicated in producing infection in the pulmonary allograft include those that cause community-acquired pneumonia in the immunocompetent host (ATS statement 1993), *Mycoplasma pneumoniae* and mycobacteria, both tuberculosis and atypical (Kesten 1999). One case of post lung transplant tuberculosis was presumed to have resulted from reactivation of an old Gohn complex acquired from the donor (Kramer 1993). Atypical pulmonary mycobacterial infection has also been documented in a patient from one of the above series as well as in a patient with obliterative bronchiolitis (Trulock 1989).

Table 1.1 Bacterial organisms isolated from infected lung transplant recipients

Bacterial organism	Kramer <i>et al</i> (1993)	Maurer <i>et al</i> (1992)
	No. of subjects: 73	No. of subjects: 40
Gram-negative		
<i>Pseudomonas aeruginosa</i>	24	12
<i>Pseudomonas</i> spp.	0	5
<i>Serratia marcescens</i>	10	1
<i>Enterobacter</i> spp.	5	6
<i>Escherichia coli</i>	5	2
<i>Legionella</i> spp.	5	0
<i>Acinetobacter</i> spp.	4	2
<i>Haemophilus</i> spp.	4	1
<i>Klebsiella</i> spp.	3	5
<i>Citrobacter</i>	1	0
<i>Clostridium</i> spp.	2	2
<i>Bacteroides fragilis</i>	1	1
Gram positive		
<i>Staphylococcus</i> spp.	11	3
<i>Enterococcus</i>	8	3
<i>Streptococcus pneumoniae</i>	4	0
<i>Streptococcus</i> spp.	3	0
Total	90	43

1.4.2.2 Viruses

Viral infections of the lung are considered to be one of the major causes of pulmonary allograft dysfunction (Table 1.2). They account for a high percentage of morbidity and mortality, particularly in the early post transplant period.

Cytomegalovirus (CMV) remains the most important and frequent virus causing infection in the lung transplant population (Dauber 1990). In the early history of lung transplantation, prior to the availability of specific therapeutic agents, CMV infection frequently produced a severe pneumonitis, which often lead to respiratory failure and death. In a series from Pittsburgh, 56 lung transplant recipients with a positive history of CMV pulmonary infection showed a significant reduction in pulmonary function after six months compared to those subjects who did not. Moreover, there was an increased incidence of chronic allograft rejection (obliterative bronchiolitis) and a reduced survival in this group (Duncan 1992).

Table 1.2 Types of virus infection in the pulmonary allograft

Cytomegalovirus
Herpes simplex
Adenovirus
Respiratory syncytial
Influenzae
Epstein-Barr

Primary CMV infection was considered to be the most virulent and this lead to a policy in many centres of only transplanting organs from CMV seronegative donors into CMV seronegative recipients. The introduction of prophylaxis with CMV hyperimmune gammaglobulin and ganciclovir greatly reduced the incidence of post transplant CMV infection and has allowed the relaxing of CMV donor-recipient matching (Gould 1993).

Other herpes viruses, including herpes simplex and Epstein-Barr viruses, may also produce pulmonary and systemic infections. Prophylaxis with aciclovir or ganciclovir has relegated herpes simplex infections to the less severe category and now tends to produce only localised mucocutaneous disease (Smyth 1990). However, Epstein-Barr virus infection may have grave implications for the lung transplant recipient. Acute infection rarely produces major lung pathology, but may be responsible for the occurrence of Epstein-Barr virus related post transplant lymphoproliferative disease. This disease represents a process resembling B cell lymphoma, which has considerable morbidity and mortality (Walker 1995, Randhawa 1989). The mainstay of therapy for this condition is reduction of immunosuppression with its concomitant risk of rejection. High dose aciclovir has also been advocated although its efficacy remains questionable. With worsening clinical disease, anti-lymphoma chemotherapy may be required.

As will be discussed in greater detail in Chapter 3, adenovirus infection is emerging as another major viral disease entity within the lung transplant population (Otori 1995, Simsir 1998). It may produce a rapidly debilitating pneumonitis and predispose to the development of obliterative bronchiolitis. Currently, no specific treatment is available for adenoviral infection.

1.4.2.3 Fungi

Although relatively rare, fungal infections of the lung allograft are associated with an appreciable mortality and morbidity. Fungal infections may occur at any time in the post transplant period. *Candida albicans* is the most commonly isolated fungus in the early post-operative phase, but tends not to produce significant disease. However, *Aspergillus* spp. can induce a more invasive form of disease, with the development of a severe tracheobronchitis involving mucosal and cartilaginous invasion (Kramer 1991). This type of *Aspergillus* infection may be relatively resistant to anti-fungal therapy. Pre-transplant airway colonisation with *Aspergillus* spp. is not an uncommon occurrence, particularly in patients with cystic fibrosis, and

this may predispose to post transplant infection (Flume 1994). Other fungal infections are rare but cases of infection with *Histoplasma* and *Cryptococcus* have been reported (Dauber 1990).

1.4.2.4 Other agents

Other infective agents include *Pneumocystis carinii*. The peak incidence for *Pneumocystis carinii* pneumonitis (PCP) is at 4-6 months post transplant. In the absence of prophylaxis, the incidence of PCP may be as high as 88% (Gryzan 1988). However, PCP has now been virtually eliminated with the establishment of appropriate prophylaxis with trimethoprim-sulphamethoxazole (Kramer 1992).

Toxoplasma gondii can be acquired from the donor organ and produce myocardial, cerebral, and rarely pulmonary infections. Prophylaxis can be effectively achieved with pyrimethamine and is recommended if there is a donor-recipient mismatch (Wreghitt 1986).

A series from Stanford reported a 2% incidence of *Nocardial* infections (Kramer 1993). This infective process tends to occur late in the post transplant course (up to several years) and may present with a lung mass. With the introduction of trimethoprim-sulphamethoxazole prophylaxis, the incidence of nocardial infections has also decreased substantially.

1.5 Other factors

Ischaemia is one of the earliest injurious mechanisms that confronts the lung allograft. Despite improvements in organ preservation techniques (Chien 2000), lung allografts do not perform satisfactorily following prolonged ischaemia. Most lung transplant units attempt to limit graft ischaemic time to less than 240 minutes in order to optimise early graft function. In addition to this acute initial ischaemia, chronic lung ischaemia persists after the mandatory severing of the bronchial arterial supply, which accounts for approximately 15% of pulmonary blood flow. Some investigators have attempted to revascularise the lung allograft by attaching the internal mammary artery to the bronchial artery system, but this has led to only limited success (Yacoub 1997).

As well as interruption to the bronchial arterial supply, the pulmonary lymphatic drainage system is interrupted. This can lead to fluid retention in the lung allograft, which may produce direct injury and predispose the lung to infection. It is not known whether denervation has any direct injurious effect on the lung allograft, but suppression of the cough reflex below the level of the airway anastomosis produces retention of secretions and predisposes to infection (Higenbottam 1989).

Reperfusion injury of the lung allograft may occur. This involves an influx of inflammatory cells, initially neutrophils succeeded by other cell types including lymphocytes and macrophages. Methods of reducing this response have included modifications of the preservation fluid and treatment with nitric oxide (Macdonald 1995). Other injurious factors include gastro-oesophageal reflux and aspiration into the lung allograft (Reid 1990). This may result from damage to the vagus nerve during the transplant surgery. Some of these factors have been implicated in the aetiology of obliterative bronchiolitis

1.6 Obliterative bronchiolitis

The introduction of cyclosporine in the early 1980s revolutionised the approach to immunosuppression in solid organ transplantation and, in particular, enabled lung transplantation to become a reality (Reitz 1982). Unfortunately, the long-term success of lung transplantation was thwarted by the development of obliterative bronchiolitis in many recipients (Burke 1984). This condition was initially reported as a distinctive clinical syndrome associated with progressive dyspnoea, airflow obstruction and recurrent lower respiratory tract infections, ultimately leading to hypoxic respiratory failure and death.

1.6.1 Pathophysiology

Obliterative bronchiolitis involves a fibroproliferative process with the end result being complete obliteration of the bronchiolar lumens. There may be obliteration of accompanying vessels as well (Yousem 1985). This fibroproliferative process has been observed in other solid organ transplants producing chronic graft dysfunction. In the heart, it is manifested by the development of graft coronary artery disease (Gao 1987), in the liver by a syndrome described as the “vanishing bile duct syndrome” (Weisner 1991), and in the kidney, by chronic vascular and tubular fibrosis (Vella 1997). All of these syndromes appear to share a common pathogenesis thought to represent a form of “chronic rejection”. However, this description implies a pathophysiological process, which remains ill-defined (Tilney 1991).

An histological diagnosis of obliterative bronchiolitis may not always be possible, especially when employing the transbronchial biopsy technique (Chamberlain 1994). Moreover, histology in isolation does not encompass the associated functional abnormalities. To reconcile these two issues, a working group of the International Society for Heart and Lung Transplantation (ISHLT) formulated a staging schema for the evaluation of chronic lung allograft dysfunction. This incorporated both

functional and histological parameters and was termed “bronchiolitis obliterans syndrome” or “BOS” (Cooper 1993). The functional assessment was by formal pulmonary function measurement of the forced expiratory volume in one second (FEV₁). A summary of this schema is shown in Table 1.3.

Table 1.3 Bronchiolitis Obliterans Syndrome Staging Scheme

Stage 0	No significant abnormality: FEV ₁ 80% or more of baseline value
Stage 1	Mild bronchiolitis obliterans syndrome: FEV ₁ 66% to 80% of baseline value
Stage 2	Moderate bronchiolitis obliterans syndrome: FEV ₁ 51% to 65% of baseline value
Stage 3	Severe bronchiolitis obliterans syndrome: FEV ₁ 50% or less of baseline value

Each of the stages can be further categorised as: (a) without histological evidence of obliterative bronchiolitis; or (b) with histological evidence of obliterative bronchiolitis. FEV₁ = forced expiratory volume in one second.
Baseline value represents the highest FEV₁ recorded post transplant.

This grading schema may not be applicable in young children who are not able to perform spirometry reliably, nor in those who do not achieve a reasonable level of pulmonary function post transplant.

1.6.2 Incidence

The incidence of post transplant obliterative bronchiolitis or BOS remains high. Adult series have shown an overall incidence ranging from 24% (Scott 1991) to 50% (Burke 1986). As the incidence of obliterative bronchiolitis increases with time, a more appropriate method of describing the incidence is by actuarial freedom from the disease over time. In a paediatric series, the actuarial freedom from obliterative bronchiolitis in survivors was 76%, 59% and 37% at one, two and three years post

transplant respectively (Whitehead 1993). Another paediatric series demonstrated a greater age factor in the development of this complication. A higher incidence was reported in the younger child - 32% freedom from obliterative bronchiolitis at five years in those aged under eight years compared to 62% freedom from obliterative bronchiolitis in children aged over eight years (Radley-Smith 1995). Both these reports indicated a high incidence of obliterative bronchiolitis in children post transplant as compared to adults.

1.6.3 Aetiology

The aetiology of obliterative bronchiolitis remains obscure. No one factor has emerged as the primary cause of the condition. Rather, obliterative bronchiolitis is most likely the end result of numerous insults to the transplanted lung leading to a final common pathway of irreversible lung injury. Mechanisms, which have been implicated in the aetiology, include rejection, infection, lung ischaemia, interruption of pulmonary lymphatic drainage, and lung denervation (Yousem 1985, Scott 1991, Yousem 1991, Keenan 1991).

Numerous studies have demonstrated a strong association between acute rejection episodes and the later development of obliterative bronchiolitis. Scott *et al* showed that severe, frequent and persistent acute lung rejection was often a precursor of obliterative bronchiolitis (Scott 1991). Early, frequent, acute rejection episodes were also significantly associated with obliterative bronchiolitis in a paediatric cohort (Whitehead 1993). Rejection appears to be the most important alloantigen-dependent risk factor for the development of chronic allograft dysfunction, particularly episodes of acute rejection occurring after the first three months (Waaga 1997).

Some investigators believe that obliterative bronchiolitis is caused by a chronic form of rejection. However, the immunological mechanisms involved with chronic rejection have not been defined. Tilney *et al* described it as an “undefined

conundrum ... (and an) ill-understood process leading to the bulk of late graft failures” (Tilney 1991). It is unknown whether the pathophysiology is related to persistent host immunologic attack or progressive graft ischaemia, secondary to arterial insufficiency. The arteriosclerosis producing the arterial insufficiency could also be a consequence of immunological mechanisms.

As discussed previously, the interaction between the TCR and the MHC alloantigen in the presence of an appropriate costimulatory signal is now recognised as the central event that initiates immunological rejection (Sayegh 1998). The “direct” allorecognition pathway is thought to be associated with acute rejection, while the “indirect” pathway appears to play a major role in chronic rejection via Th2 CD4+ lymphocytes (Ciubotariu 1998). These in turn stimulate the production of a battery of cytokines, growth factors and alloantibodies.

Alloantigen-independent risk factors for the development of chronic graft dysfunction include: donor organ function prior to transplantation; effects of brain stem death on donor organ function; prolonged cold ischaemia and subsequent reperfusion injury; and surgical insults.

Evidence supporting the contribution of pulmonary infection in the development of obliterative bronchiolitis is not as convincing. Viral pneumonitis has certainly been implicated, as it has in the non-transplant situation. In particular, adenovirus infection is associated with post transplant obliterative bronchiolitis (Ohori 1995, Simsir 1998) while *Cytomegalovirus* pneumonitis has been shown to increase the risk of obliterative bronchiolitis (Keenan 1991).

Other factors implicated in the genesis of obliterative bronchiolitis have included chronic aspiration. Delayed gastric emptying and/or oesophageal dysmotility, presumably related to vagus nerve injury, may predispose to aspiration. In one series of five patients with significant aspiration, despite adequate anti-reflux therapy three proceeded to develop obliterative bronchiolitis and bronchiectasis (Reid 1990).

Non-compliance (or non-adherence) to the immunosuppression treatment regimen is another contributory factor to the development of obliterative bronchiolitis. This may be particularly apparent in the adolescent population, who wish to avoid the changes to body image associated with therapy, namely hypertrichosis, gingival hypertrophy, obesity and Cushingoid features (Serrano-Ikkos 1998).

1.6.4 Research aspects

Disappointingly, there has been little information produced which has led to greater understanding of the pathophysiological processes involved in the development of obliterative bronchiolitis. Recently, experimental animal models of obliterative bronchiolitis have been described. Hertz *et al* developed a heterotopic murine transplanted airway model using MHC matched and mismatched donor-recipient pairs. They were able to demonstrate that airway fibroproliferation was reduced by the administration of cyclosporine in a dose-dependent fashion (Hertz 1993).

A model of obliterative bronchiolitis has also been developed in miniature swine using whole-lung allografts with suboptimal immunosuppression (Al-Dossari 1994). Both these models may help produce greater understanding of the pathophysiological processes and enable new treatment modalities to be evaluated. For example, Cao *et al* have demonstrated a reduction in the fibroproliferative response in the heterotopic airway model with the use of rapamycin – a new immunosuppressive agent (Cao 1995).

Blockade of costimulatory pathways may induce graft tolerance and therefore prevent the development of chronic rejection. One such pathway is the CD28-B7 interaction. CD28 ligation facilitates naive T cell activation by recruiting membrane and intracellular kinase-rich raft microdomains at the site of TCR engagement (Viola 1999). This results in enhanced and sustained TCR signalling followed by increased IL-2 production and T cell proliferation. Recent studies have shown that blockade of the CD28-B7 reaction by CTLA-4-Ig, a recombinant protein that

contains the extracellular domain of soluble CTLA-4 fused to an IgG₁ heavy chain, prevents the development of chronic rejection in rat models of cardiac transplantation (Chandraker 1997).

Another co-stimulatory pathway, the CD40-CD40L, leads to pronounced immunostimulatory effects with the eventual production of cytotoxic T lymphocytes (CTLs) (Ridge 1998, Bennett 1998). In a rodent model, blocking the CD40-CD40L pathway with anti-CD40L antibodies results in long term graft acceptance (Gudmundsdottir 1999).

On the clinical stage, research strategies have been mainly directed towards the (early) detection of obliterative bronchiolitis. These have included the identification of various growth factors eg: platelet derived growth factor, fibroblast growth factor, transforming growth factor etc., from bronchoalveolar lavage samples (Al-Dossari 1995). Treatment research strategies have involved new immunosuppressive agents as well as topical immunosuppressive therapy. Inhaled corticosteroids have been used in established obliterative bronchiolitis and recently, inhaled cyclosporine has been trialed with promising results (Keenan 1997).

In conclusion, obliterative bronchiolitis remains a major obstacle to the long-term success of lung transplantation in both adults and children. It appears to have a primarily immunologically based aetiology, although numerous factors may be involved in its progression. Once established, it is greatly resistant to medical therapy with the only chance of cure being retransplantation, which is associated with poor results. Research strategies revolve around furthering the understanding of the pathophysiological processes and the development of effective treatment modalities.

Chapter 2 Clinical correlates of injury in the transplanted lung

2.1 Introduction

2.2 Rejection and infection

2.2.1 Clinical aspects of pulmonary rejection

2.2.2 Clinical aspects of pulmonary allograft infection

2.3 Obliterative bronchiolitis

2.4 Pulmonary function post lung transplantation

2.4.1 Post transplant pulmonary function in adults

2.4.2 Gas exchange

2.4.3 Donor – recipient size discrepancies

2.4.4 Effects of lung denervation

2.4.5 Effects of rejection and infection

2.4.6 Obliterative bronchiolitis

2.4.7 Pulmonary function following paediatric lung transplantation

2.5 Survival post lung transplantation

2.5.1 Survival in adults

2.5.2 Paediatric survival

2.1 Introduction

Of the injurious agents affecting the lung allograft, rejection and infection assume the greatest prominence in producing allograft dysfunction. Clinically, it may be difficult to differentiate between the two processes as both may present with relatively non-specific features including fever, general malaise and dyspnoea. Often, treatment is directed towards both processes before a specific diagnosis can be determined.

Repeated injury to the pulmonary allograft by these mechanisms may eventually result in the development of obliterative bronchiolitis – a form of chronic allograft dysfunction. Once established, this disease progresses irrevocably, leading ultimately to respiratory failure and death. Currently, there exists no successful treatment for this condition apart from re-transplantation.

One method of identifying lung allograft injury is by pulmonary function testing. This is non-invasive, easy to accomplish both in adults and children and a sensitive indicator of the clinical status of the lung graft. It lacks specificity in differentiating between acute processes such as pulmonary rejection and infection, but is a valuable adjunct in assessing the development and progression of the more chronic condition, obliterative bronchiolitis.

The above-mentioned injurious factors impact upon post transplant survival. Even though short-term survival has improved, long-term survival remains disappointingly low and is related mainly to the development of obliterative bronchiolitis in many of the recipients. Accordingly, this chapter describes some of the clinical implications, including alterations in pulmonary function, of the various injurious events complicating the lung allograft and details adult and paediatric survival post transplant.

2.2 Rejection and infection

2.2.1 Clinical aspects of pulmonary rejection

In the early experience of heart–lung transplantation, it was noted that the lungs underwent rejection more frequently than the cardiac component of the combined graft (McGregor 1985). Although the reasons for this phenomenon were not clearly understood, this differential rejection indicated a greater propensity for the lungs to undergo rejection. It may be related to the lung's inherent protective function in processing various antigens, toxins and infectious agents that can induce immune up-regulation thereby enhancing allo-immunity (Fujinami 1988). In addition, lung allografts are combined with a significant amount of donor lymphatic tissue containing large numbers of immune effector cells which may themselves predispose to a more aggressive form of rejection (Hasegawa 1999).

The diagnosis of acute pulmonary rejection may be made on clinical and/or histological grounds. The former are the most commonly used criteria early after transplantation and include dyspnoea, fatigue, pyrexia, decrease in pulmonary function, radiographic abnormalities and reduction in oxygenation. A prompt response to intravenous corticosteroid therapy usually confirms the presumptive diagnosis (Lawrence 1990).

However, for the unequivocal confirmation of a diagnosis of rejection and to exclude pulmonary infection, histological evaluation of the lung allograft is essential. This can be achieved with the aid of bronchoscopy and transbronchial biopsy. This method was initially described by Higenbottam and colleagues at the Papworth hospital, UK (Higenbottam 1987) and has been used extensively in most lung transplant centres worldwide, both in adults and children (Whitehead 1992).

The Lung Rejection Study Group, under the auspices of the International Society for Heart and Lung Transplantation (ISHLT), has produced a classification of the histology of pulmonary rejection. This defines the grading of acute rejection,

chronic rejection and chronic vascular rejection (Yousem 1996). The pathognomonic feature of acute rejection is the presence of a perivascular infiltration of lymphocytes. With increasing severity of rejection, these infiltrates extend to involve other regions of the lung parenchyma leading to further inflammation and eventually necrosis.

Despite the development of more effective induction and maintenance immunosuppression regimens, acute rejection episodes are frequently encountered following lung transplantation. Most units employ a multi-drug treatment regimen for maintenance, usually comprising cyclosporine or tacrolimus, azathioprine and corticosteroids. More recently, some centres have used mycophenolate mofetil *in lieu* of azathioprine. For induction, a cytolytic agent such as anti-thymocyte globulin or a T cell specific monoclonal antibody is administered. Even when “adequate” levels of immunosuppression are obtained as determined by blood levels etc, the majority of patients will experience one or more acute rejection episodes. The first line treatment is the delivery of high dose corticosteroid, usually in the form of intravenous methylprednisolone. This is often followed by a reducing course of oral prednisolone together with optimisation of other immunosuppressive therapy.

Some patients may progress to persistent or recurrent rejection, both of which are more difficult to manage. Further courses of corticosteroids are often tried, while repeated doses of cytolytic therapy may be required to control the rejection process. In some instances more aggressive therapy, including photophoresis and total lymphoid irradiation (Andreu 1995, Valentine 1996), may be instigated. However, these additional treatments carry a high risk of infection developing in the lung allograft, which may induce further lung injury.

2.2.2 Clinical aspects of pulmonary allograft infection

There are two important considerations in the management of pulmonary allograft infections. The first is prophylaxis and the second is accurate diagnosis and specific therapy. All lung transplant centres institute some form of an antimicrobial prophylactic regimen in newly transplanted patients. The details will vary from centre to centre and from patient to patient. Most regimens will incorporate a combination of broad-spectrum antibiotics, trimethoprim-sulphamethoxazole, an antifungal agent and possibly an antiviral agent if there are predisposing factors (eg donor-recipient CMV mismatch). In addition, specific anti-pseudomonal antibiotics are usually administered in those lung transplant recipients with cystic fibrosis.

Rigorous surveillance and prompt culture-directed antimicrobial therapy are paramount in limiting the morbidity and mortality associated with infection of the pulmonary allograft. The important role that bronchoscopy, bronchoalveolar lavage and transbronchial biopsy has to play in the management of lung rejection is paralleled by its role in the management of allograft infection. Accurate diagnosis of infective processes can be achieved by this means and help guide specific antimicrobial therapy (Penketh 1988).

2.3 Obliterative bronchiolitis

Clinically, obliterative bronchiolitis is characterised by progressive pulmonary dysfunction leading eventually to hypoxic respiratory failure. Recurrent lower respiratory tract infective exacerbations occur as a consequence of the associated central bronchiectasis, which accompanies this disease (Theodore 1990). The patient usually presents with an obstructive lung defect associated with hyperinflation of the chest and falling pulmonary function. The commonest time of onset is some two or three years post transplant, but it may present much earlier – even within months of operation. Radiologically, there is often evidence of

hyperinflation and attenuation of vascular markings with bronchiectatic changes appearing subsequently. High resolution computerised tomography of the chest may elucidate these changes more clearly (Skeens 1989, Lentz 1992).

The treatment of obliterative bronchiolitis has been frustrated by its recalcitrant behaviour to all therapeutic modalities. Once the fibroproliferative process becomes established, it is extremely difficult to reverse with current medical therapies. The most often used approach is to augment immunosuppression in order to abrogate any ongoing rejection. This usually comprises increasing steroid therapy although other agents have been tried (Glanville 1987). Unfortunately, augmentation of immunosuppression increases the risks of infective complications, which may induce further lung damage thereby furthering disease progression. Naturally, all intercurrent pulmonary infections need to be aggressively treated with appropriate antimicrobials. Other general measures including maintenance of an adequate nutritional status, physiotherapy, bronchodilator therapy and psychosocial support are required to optimise the patient's survival and quality of life. With disease progression and associated hypoxaemia, oxygen therapy will often be required.

The only possible cure for obliterative bronchiolitis is retransplantation with a single lung, double lung or combined heart-lung block. Despite some recent improvement, survival following retransplantation remains poor and inferior to primary transplantation (Novick 1994). The issue of retransplantation raises the serious ethical dilemma of whether scarce donor organs be directed towards those patients who may have an inferior outcome. It is the onus of each transplant centre to decide on their individual approach to this question.

2.4 Pulmonary function post lung transplantation

The advent of successful clinical heart-lung transplantation in the early 1980s led to a unique opportunity to study the physiology of the transplanted lung and respiratory system (Theodore 1990a). Despite transplant-related events including cardiopulmonary denervation, disruption of the pulmonary lymphatic and bronchial arterial systems and transient ischaemia of the donor organs at the time of surgery, the function of the transplanted lungs clearly remains capable of supporting activities of normal life. Long-term pulmonary function may remain well preserved with essentially normal gas exchange. The following discussion applies equally to both combined heart-lung transplantation and bilateral lung transplantation. Some of the aspects may also be applicable to single lung transplantation, but due to the contribution of the remaining native lung, many of the physiological parameters will be non-interpretable.

2.4.1 Post transplant pulmonary function in adults

The earliest observed and significant alteration in pulmonary function following lung transplantation is the development of a mild to moderate restrictive ventilatory defect. This is almost certainly related to alterations in chest wall mechanics following surgery (Braun 1978) and appears to be related primarily to a reduction in inspiratory capacity (IC – see Table 2.1 for glossary of terms and abbreviations of pulmonary function parameters). There is a resultant decrease in all static lung volumes, as compared with predicted normal values, with the exception of residual volume (RV) and functional residual capacity (FRC). These latter two parameters remain essentially normal (Theodore 1984). The overall decrease in total lung capacity (TLC) correlates highly with reductions in maximum inspiratory pressure and transpulmonary pressure.

Table 2.1 Glossary of pulmonary function terms

(Report working party 1993)

Pulmonary function parameter	Abbreviation	Description
Forced vital capacity	FVC	Volume of pulmonary gas forcefully and completely expired from full inspiration.
Forced expiratory volume in one second	FEV ₁	Maximum lung volume expired during the first second from full inspiration.
Ratio of forced expiratory volume in one second over forced vital capacity	FEV ₁ /FVC	Ratio expressed as percentage. Identifies restrictive and obstructive defects.
Total lung capacity	TLC	Volume of gas in the lungs at the end of a full inspiration.
Residual volume	RV	Volume of gas remaining in the lungs at the end of full expiration.
Functional residual capacity	FRC	Volume of gas in the lungs and airways at tidal end-expiratory level.
Inspiratory capacity	IC	Volume of gas inspired from tidal end-expiratory level (FRC) to total lung capacity (TLC).
Peak expiratory flow rate	PEFR	Maximum instantaneous air flow during a forced expiratory manoeuvre from full inspiration.
Mean forced expiratory flow between 25% and 75% of vital capacity	FEF ₂₅₋₇₅	Average flow rate measured over the middle half of forced expiration.
Lung diffusing capacity for carbon monoxide	D _L CO	Measures the surface area of lung available for gas exchange.
Forced expiratory flow at 50% of vital capacity	FEF ₅₀	Forced expiratory flow rate at 50% of remaining vital capacity. A measure of smaller airway flow.
Maximum expiratory flow with 25% of vital capacity to be expired	MEF ₂₅	Maximum expiratory flow rate at 25% of remaining vital capacity. A measure of smaller airway flow.

Early pulmonary dynamic function remains relatively normal in the transplanted lung, although flow rates tend to be more variable than expected. There are reductions in those values that are directly related to the reduction in vital capacity. These include forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁). Measurements that are highly effort-dependent, including peak

expiratory flow rate (PEFR), are also reduced. However, the FEV₁/FVC ratio is usually increased in keeping with the restrictive defect.

Effort-independent flow rates are usually normal if corrected for lung volume.

These include mean forced expiratory flow between 25 percent and 75 percent of the FVC (FEF₂₅₋₇₅) and forced expiratory flow at 50 percent of the FVC (FEF₅₀). If these are reduced, it can usually be directly related to decreased volumes.

Measurements of airway resistance and specific airway conductance are also normal in the transplanted lung (Theodore 1984).

Long-term pulmonary function may also remain near normal levels if complications do not intervene. The early restrictive ventilatory defect usually persists but does not progress, while the other functional indices are capable of remaining essentially normal over time. Recognising the “normal” pattern of pulmonary function following lung transplantation is important so that abnormal patterns may be discernible. These abnormal patterns may indicate the onset of complications such as rejection, infection or obliterative bronchiolitis (see below).

2.4.2 Gas exchange

In the absence of complications, gas exchange in the resting transplanted lung remains normal. Moreover, the distribution of ventilation within the lung and the lung diffusing capacity for carbon monoxide (D_LCO) remain normal. Arterial blood gas estimations confirm the ability of the transplanted lung to sustain normal gas exchange. The mean arterial partial pressure of oxygen (PaO₂) in a group of heart-lung transplant recipients over a three year period ranged from 88.0 to 92.8 mmHg (normal range: 80 – 103 mmHg) (Burke 1984). In the same cohort, the mean arterial partial pressure of carbon dioxide (PaCO₂) ranged from 34.2 to 36.3 mmHg (normal range: 37 – 45 mmHg) indicating the presence of mild alveolar hyperventilation.

2.4.3 Donor – recipient size discrepancies

With regard to donor and recipient size matching and post transplant pulmonary function, it appears that chest wall compliance is the major determinant of post operative lung volume rather than donor lung size or compliance. Otulana *et al* described the adaptation of the donor lung to a different thoracic cavity following heart-lung transplantation (Otulana 1989). They confirmed the previously reported fact that TLC falls immediately after surgery with recovery over the succeeding 2-3 months. The observation of most physiological interest was that after 6-12 months, the TLC of the recipient approximated their pre transplant value, irrespective of the donor lung size.

In another study it was shown that, within limits, larger donor lungs appear to adapt to the constraints of the recipient chest without apparent adverse effects (Lloyd 1990). Obviously, this is a major consideration in paediatric lung transplantation, where marked size discrepancies between donor and recipient may occur. Smaller donor lungs function well within larger thoracic cavities and readily expand to an appropriate size. Lungs too large for the thoracic cavity may become atelectatic and thus be predisposed to infection. Reduction in size of the donor lung has been one method used to avoid this problem (Artemiou 1997, Couetil 1997).

2.4.4 Effects of lung denervation

Lung denervation results in a diminished cough reflex, which leads to retention of secretions, and a concomitant increased risk of superimposed infection. Numerous studies have investigated the transplanted lung in relation to its denervation.

Evaluation of the cough response to nebulised distilled water is one method of assessing lung denervation. Higenbottam *et al* showed that there was diminished coughing when distilled water was distributed by aerosol to the central airways (ie: below the airway anastomosis), with an intact laryngeal response (Higenbottam 1989). They concluded that this observation supported the theory that vagal afferent nerves do not reinnervate the lungs after heart-lung transplantation. Another study

suggested that heart-lung transplant recipients had a reduced ventilatory response to carbon dioxide, although not to progressive hypoxia, resultant from a blunted augmentation of breathing (Sanders 1989).

Finally, bronchial hyper-responsiveness to inhaled methacholine was noted to be significantly elevated in heart-lung transplant recipients compared to normals (Glanville 1987a). The investigators speculated that this could result from hypersensitive muscarinic receptors deprived of tonic vagal tone and that their results were consistent with the possibility that human heart-lung transplantation produces longstanding denervation. The implications of these findings are that lung transplant recipients may have a predisposition to developing reactive airways disease or asthma.

2.4.5 Effects of rejection and infection

So far, the discussion has related to the transplanted lung devoid of major complications. However, it is recognised that this is a rare situation and that most lung allografts will undergo a degree of rejection and infection. Assessment of pulmonary function has become an important adjunct in the non-invasive monitoring of the lung allograft and characteristic changes in lung function may be observed when the transplanted lung is affected by these events.

When compared with transbronchial lung biopsy results, the gold standard for the diagnosis of rejection and infection (Higenbottam 1987), FEV₁ was observed to decrease significantly with both lung rejection and lung infection (Otulana 1990). In addition, vital capacity and lung diffusing capacity (D_LCO) were also reduced during these acute lung complications. In this study, lung function testing was shown to have a sensitivity of 86% in detecting lung rejection in the first three months post transplant and 75% in the subsequent period. Its sensitivity for detecting lung infection was 75%. Although not able to distinguish between the two events, lung function had an overall specificity of 84% for detecting an acute lung complication.

As a consequence, daily home spirometry has been employed extensively as a useful adjunct in the monitoring of the lung allograft.

In another evaluation of pulmonary function monitoring in single lung transplantation, pathological changes associated with rejection and infection phenomena were reflected by acute falls in lung function parameters, particularly FEF₂₅₋₇₅ and PaO₂ (Marshall 1991). A fall of 10% or greater in either of these values was associated with a high predictive value of a pathological abnormality on transbronchial lung biopsy.

2.4.6 Obliterative bronchiolitis

Pulmonary function testing has assumed a prominent role in the diagnosis and grading of obliterative bronchiolitis or bronchiolitis obliterans syndrome (Cooper 1993). This condition has been addressed in a previous section and is the major long-term complication confronting the lung transplant recipient. Decreases in flow rates, which are not completely accounted for by reductions in volume, may be the first indication of the development of airway obstruction, thus heralding the onset of obliterative bronchiolitis.

Burke *et al* were one of the first groups to report this phenomenon. They presented data from six long-term heart-lung transplant recipients, which showed that initial airflow obstruction and impaired oxygenation occurred at a mean of 14 months after surgery. Once established, the obstructive defect followed a progressive course (Burke 1985). Histological evidence of obliterative bronchiolitis was found at autopsy in five of these patients, while transbronchial lung biopsy showed peribronchial inflammation in the sixth. Reductions in TLC, FVC, FEV₁, FEV₁/FVC, and FEF₂₅₋₇₅ were observed in all patients, with the greatest fall being in FEF₂₅₋₇₅.

In a more recent review, a comparison was made of FEF_{25-75} and FEV_1 for the detection of obstructive airway disease as an early manifestation of obliterative bronchiolitis (Patterson 1996). This study concluded that FEF_{25-75} was more sensitive than FEV_1 for the early detection of obliterative bronchiolitis and suggested that for epidemiological studies or for vital statistics, a decline in FEF_{25-75} to less than 70% be used to define the onset of obliterative bronchiolitis. However, FEV_1 currently remains the parameter of choice for defining this condition.

2.4.7 Pulmonary function following paediatric lung transplantation

Despite the increasing number of published reports of successful heart-lung and lung transplantation in children, few have detailed pulmonary function in this population. Starnes *et al* presented limited serial pulmonary function data in children who had received heart-lung transplants (Starnes 1991). They measured serial FEV_1 values and expressed these as a percentage of predicted normal ($FEV_1\%$). There was a significant deterioration in this parameter over the first year followed by stabilisation. The mean $FEV_1\%$ was 72.6% at three months falling to 44.8% at 12 months. By two years post transplantation, the mean $FEV_1\%$ had fallen further to 34.4%.

Another report has shown a similar deterioration in airflows and volumes over a relatively short period of follow up post transplant (Whitehead 1994). In 16 patients receiving heart-lung transplants for cystic fibrosis, the mean FEV_1 (expressed as percentage of predicted normal, $FEV_1\%$) fell from 72.8% at 12 months post transplant to a mean of 60.2% and 52.4% at two and three years post transplant respectively. The maximum forced expiratory flow at 25% of remaining vital capacity (expressed as percentage of predicted normal, $MEF_{25}\%$), a sensitive measure of smaller airway flows, showed a more dramatic decrease from 60.7% at 12 months post transplant to 40.5% and 31.9% at two and three years post transplant respectively.

The same group used pulmonary function criteria to aid in the diagnosis of obliterative bronchiolitis in a cohort of paediatric heart-lung transplant recipients (Whitehead 1993). In this study, obliterative bronchiolitis was defined either histologically or in the presence of irreversible airway obstruction, documented by a FEV₁ of less than 40% of predicted normal present for more than three months. Using these criteria, a high incidence of obliterative bronchiolitis was observed with an actuarial freedom from this complication of 76%, 59% and 37% at 12, 24 and 36 months post transplant respectively.

In Chapter 4 of this thesis, a detailed description of pulmonary function following paediatric lung transplantation is presented. Both short term and long term results are illustrated and the correlation with injurious events is explored.

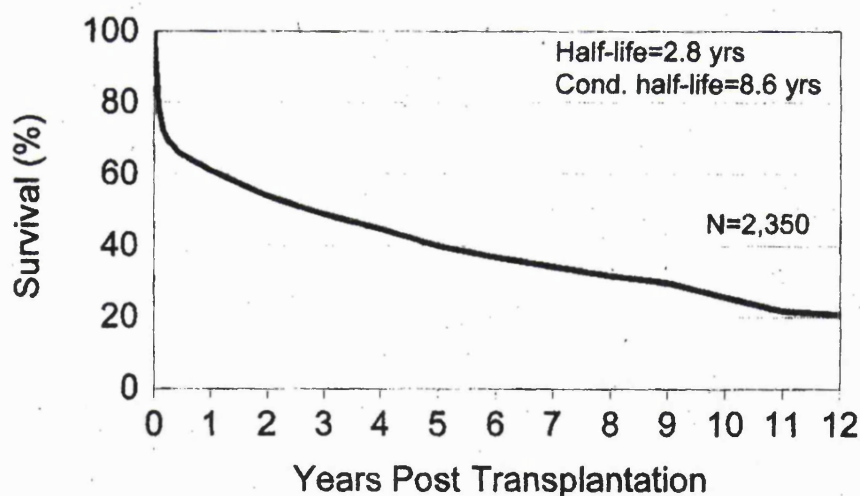
2.5 Survival post lung transplantation

2.5.1 Survival in adults

The Registry of the International Society for Heart and Lung Transplantation: Sixteenth Official Report – 1999 gives details on 2,510 heart-lung transplants reported from 124 programmes and 5,347 single lung and 3,751 double lung transplants from a total of 153 lung transplant programmes, since 1982 (Hosenpud 1999). These include both adult and paediatric lung transplant recipients.

In the adult population, the three most common indications for combined heart-lung transplantation were pulmonary hypertension, congenital heart disease and cystic fibrosis. Actuarial survival for this group has been calculated to 12 years post transplant (Figure 2.1). One-year survival was approximately 61%, while the 12 year survival was 21%. The survival half-life for the entire curve was 2.8 years. The conditional half-life for those surviving the first year was 8.6 years.

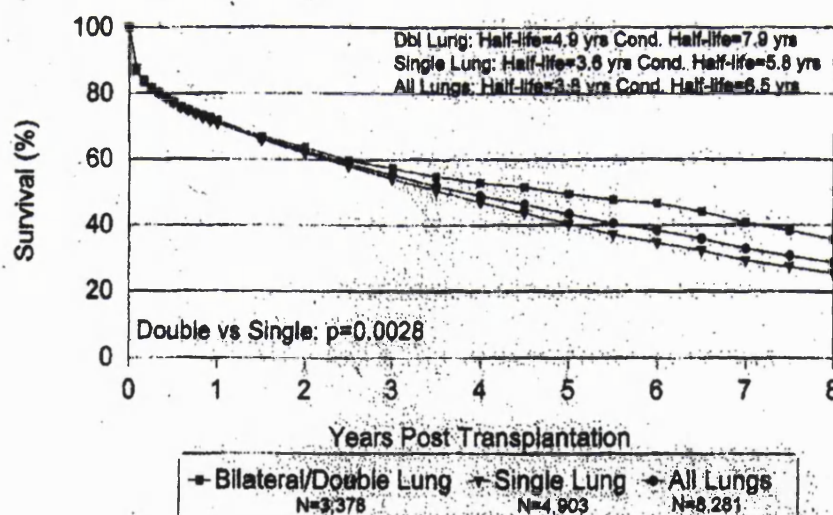
Figure 2.1 Actuarial survival following adult heart-lung transplantation
(Adapted from Hosenpud 1999)



As previously reported, retransplantation, ventilator dependency, and advanced donor and recipient ages are statistically significant risk factors for mortality at one and three years post transplant by multivariate logistic regression analysis. Infection remains an important cause of death at all time points up to five years post transplant. After the first year, obliterative bronchiolitis contributes as strongly to mortality as does infection.

Isolated lung transplantation has been used increasingly in both adults and children over the 1990s. Cystic fibrosis is one of the main indications for double lung transplantation across all age groups, while emphysema, alpha-1-antitrypsin deficiency, pulmonary hypertension and idiopathic pulmonary fibrosis represent the majority of the other indications in the adult population. The eight-year actuarial survival curve for all lung transplantation (adult and paediatric) is shown in Figure 2.2.

Figure 2.2 Total lung transplant actuarial survival by procedure
(Adapted from Hosenpud 1999)



As demonstrated, double lung transplantation survival is superior to single lung transplantation by post operative year three. The half-life for double lung transplantation is approximately 4.9 years, while that for single lung transplantation

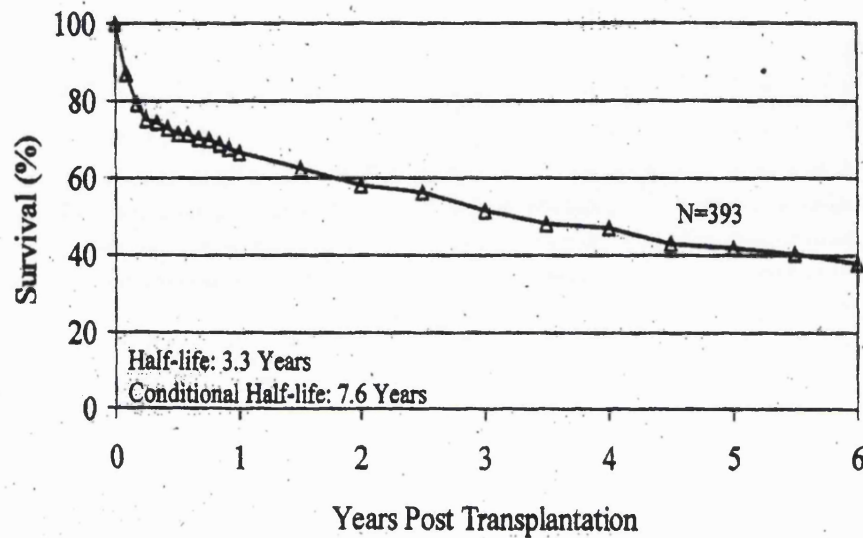
is 3.6 years. Multivariate logistic regression analysis of risk factors for mortality post-isolated lung transplantation is similar to that for combined heart–lung transplantation. Pre-operative ventilator support, retransplantation and recipient age are all implicated, while an indication other than emphysema is associated with a significantly greater risk of mortality. As with heart-lung transplantation, the commonest causes of death following lung transplantation feature infection and obliterative bronchiolitis.

2.5.2 Paediatric survival

The Registry of the International Society for Heart and Lung Transplantation has now produced three separate paediatric reports – the last published in 1999 (Boucek 1999). This report contains international data on 411 heart-lung and 525 lung (single and double) transplantations performed between 1983 and 1998.

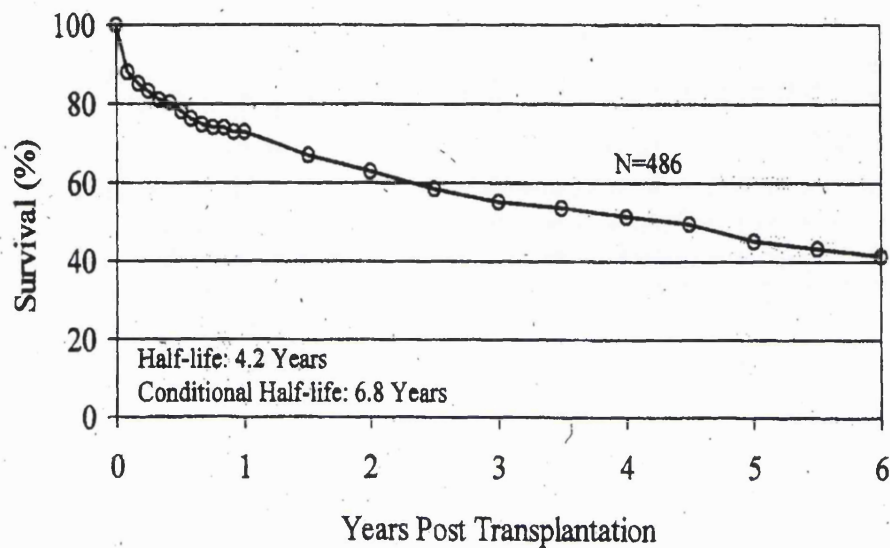
The number of heart-lung transplants being performed continues to decrease as isolated lung transplantation assumes greater application. The three main indications for the combined heart-lung procedure include congenital lung abnormalities, primary pulmonary hypertension and cystic fibrosis. The overall patient survival half-life is 3.3 years (Figure 2.3). Primary graft failure and infection are the two commonest causes of death in the early post transplant period and first transplant year. After this period, obliterative bronchiolitis becomes the major cause of mortality.

Figure 2.3 Actuarial survival following paediatric heart-lung transplantation (1982-1999)
(Adapted from Boucek 1999)



As mentioned above, cystic fibrosis dominates as the main indication for isolated lung transplantation in the paediatric age group. Actuarial survival for the entire paediatric cohort is shown in Figure 2.4. The survival half-life is 4.2 years, with a 40% six-year survival. When comparing survival from different eras, there is a significant increase in survival in the most recent period (1995-1998) compared to earlier epochs. Once again, major causes of mortality include infection and obliterative bronchiolitis, with the latter becoming the dominant factor beyond three years post transplant.

Figure 2.4 Actuarial survival following paediatric lung transplantation (1982-1998)
(Adapted from Boucek 1999)



In conclusion, survival following all types of lung transplantation has shown continued improvement over the past decade. The early post operative phase remains a high-risk period for all recipients due to the complications of primary graft dysfunction and acute rejection. Later, infection and obliterative bronchiolitis emerge as major causes of mortality. Hence, alleviation of these complications could have a major impact on the success of pulmonary transplantation.

Chapter 3 The development of novel therapeutic approaches

3.1 Introduction

3.2 Gene transfer in the lung

3.2.1 Gene transfer - a review

3.2.2 Gene vectors

3.2.2.1 Viral gene vectors

3.2.2.1.1 Retroviral vectors

3.2.2.1.2 Adenoviral vectors

3.2.2.1.3 Adeno-associated viral vectors

3.2.2.2 Non-viral gene vectors

3.2.2.2.1 Cationic lipid based vectors

3.2.2.2.2 Ligand-polylysine based vectors

3.2.2.2.3 Other non-viral methods of gene delivery

3.2.3 Endosomal release

3.2.4 Gene transfer in the lung

3.2.5 Transplant applications

3.2.7.1 Gene vector systems

3.2.7.2 Modulation of transplanted organs

3.3 Antiviral therapy with antisense oligodeoxynucleotides

3.3.1 Adenovirus infection in the lung

3.3.2 Antisense oligodeoxynucleotides

3.3.3 Antiviral applications

3.3.4 Antisense approach to adenovirus infection

3.1 Introduction

As discussed previously, rejection is one of the principal complications affecting the pulmonary allograft. This can lead to acute graft dysfunction or to the more insidious chronic dysfunction associated with obliterative bronchiolitis. Most methods to control rejection relate to the use of immunosuppressive therapy. This approach is not universally successful and, due to the very nature of treatment, may produce additional complications including infection and dysfunction of other organ systems.

Another theoretical approach is to prevent rejection by rendering the transplanted organ immunologically inert or at least resistant to the rejection process. This might be achieved by utilising gene transfer techniques. For example, a therapeutic gene could be transferred to the donor organ designed to downregulate expression of MHC molecules on the surface of its cells. Other methods might involve selective inhibitory cytokine expression (Drazen 1995) or local production of immunosuppressive proteins such as CTLA4-Ig (Lenschow 1992). The donor organ could be treated at the time of transplantation by injecting the therapeutic gene, combined with an effective gene vector, into the arterial supply of the graft. In the case of the lung allograft, both the circulation and the airways could be targeted. With lung allografts “top up” gene therapy could, in principle, readily be administered topically via the airways. Accordingly, in Section 3.2, the current status of gene transfer and its application in lung transplantation is reviewed.

Although therapies now exist for many of the infectious processes afflicting the lung transplant recipient, there remain some that have no specific treatment available. One of these includes adenovirus infection of the lung allograft. Adenoviral pneumonitis, although relatively rare, is assuming a notable position in the post transplant setting due to its associated high morbidity and mortality. None of the current antiviral treatments is effective in the management of this disease, and therefore a specific therapy is sought.

One approach, which has gained considerable interest recently, has been the use of antisense oligodeoxynucleotides to inhibit viral replication. In the setting of lung transplantation, this approach would be particularly attractive as the oligodeoxynucleotides could be administered topically to the lung. Section 3.3 reviews the impact of adenovirus infection in the lung transplant recipient, the fundamentals of antisense oligodeoxynucleotide technology and its application in the treatment of viral infections.

3.2 Gene transfer in the lung

3.2.1 Gene transfer - a review

Although not a new concept, gene therapy unites pharmacotherapeutic with genetic principles, implicating the use of a polynucleotide to treat a disease state (Wolff 1994). Prior to the recombinant DNA era, some investigators predicted that viruses could be used to transduce genes and be used in the treatment of cancer (Tatum 1966). It was further speculated that polynucleotide sequences could be grafted onto virus DNA and this theory was enhanced by Kornberg's successful replication of DNA in a test tube (Kornberg 1968). By the late 1960s and early 1970s, gene therapy had become the subject of an increasing number of articles and meetings. Aposhian advocated the use of pseudoviruses derived from the mouse polyoma virus and raised gene therapy to a pharmaceutical level (Aposhian 1970):

"If one considers the purpose of a drug to be to restore the normal function of some particular process in the body, then DNA would be considered to be the ultimate drug."

Over the following decade, further enthusiasm for the development of gene therapy ensued (Anderson 1984). It was not until early transfection techniques such as the calcium phosphate technique (Graham 1974), and selection systems for cultured cells were combined that real progress was made in gene transfer. Another major advance in the field at this time was the ability to produce large quantities of single genes using recombinant DNA techniques.

There are three considerations involved in the process of gene therapy. The first is the formulation and route of administration of the gene. The nature of the DNA construct, especially the control of gene expression by its promoter, can be critical. Second is the delivery of the gene to the desired cell and its nucleus and third is expression of the therapeutic gene.

3.2.2 Gene vectors

Vectors are required to deliver the gene of interest to the target cell. Delivery involves attachment of the gene to the target cell, the uptake of the gene into the cell and the intracellular trafficking of the gene to the nucleus (Greber 1996). Viral infection remains the most important paradigm for gene delivery, and has highly evolved processes. All these processes need to be replicated in a non-viral gene delivery system. Gene vectors can be categorised into viral and non-viral vectors and will be discussed separately under these headings below.

3.2.2.1 Viral gene vectors

Viral gene vectors were first described in the late 1960s. Some of the early viral vectors used included tobacco mosaic virus (Rogers 1968), polyoma viral capsid proteins (Friedmann 1971) and papilloma simian virus (SV40) (Jackson 1972). Herpes simplex virus (HSV) vectors have also been developed. HSV has a number of advantages as a vector for delivering specific genes to the nervous system. These include its wide host range and ability to establish long-lived asymptomatic infections in neuronal cells in which a specific region of the viral genome continues to be expressed (Latchman 1994). However, its ability to replicate lytically in the brain, with the potential of producing an encephalitis under some circumstances, has produced fears about its potential use in humans.

Currently, the viral vectors considered to be of greatest potential include retroviral, adenoviral, and adeno-associated viral vectors and therefore will be discussed separately below.

3.2.2.1.1 Retroviral vectors

Retroviral vectors are derived from RNA viruses possessing the main feature of reverse-transcribing their viral RNA genome into double stranded viral DNA, which is then stably inserted into the host DNA. Members of this class of RNA viruses include the murine leukaemia viruses and the lentiviruses, which are used extensively for virus vector engineering.

Retroviral vectors were initially developed by three different groups (Shimotohno 1981, Tabin 1982, and Wei 1981) and numerous clinical trials have evolved subsequently. These have mainly comprised *ex vivo* retroviral-mediated gene transfer transduction of target cells (Salmons 1993).

The advantages of retroviral vectors are determined by their characteristics of stable integration into the host genome, infectivity of the recombinant viral particles for a broad variety of target cell types and ability to carry foreign genes of reasonable sizes (<8kb). These properties are essential for persistence of the transgene in transduced cells and their progeny cells, and for the long term and high level expression of the transgene (Walther 2000). However, the disadvantages of retroviral vectors include their instability, possible insertional mutagenesis by random viral integration into host DNA and, for some, the requirement of cell division for their integration (Miller 1990).

Lentiviruses, which include the human immunodeficiency virus (HIV), are of particular interest as a gene vector as they possess the ability of infecting and integrating into non-dividing cells. During the last few years, HIV-based vectors have been established and tested for efficacy of gene transfer and their biological safety to exclude possible reconstitution of the pathogenic replication-competent HIV-1. *In vivo* studies have been encouraging (Naldini 1996), but there has been no direct evidence that the HIV-based vector was stably integrated into the host genome. Naturally, there are concerns with the use of this type of virus particularly

those of potential cell growth dysregulation, mutagenesis, and recombination, which could have a dramatic impact on their potential clinical use.

3.2.2.1.2 Adenoviral vectors

Adenoviral vectors constitute a commonly used viral vector system for gene transfer. They can be used for nondividing cells (Graham 1991) and represent an attractive option for gene delivery in various tissues including the lung.

Adenoviruses are non-enveloped DNA viruses with 49 serotypes distinguishable. Adenovirus serotypes 2 and 5 were used for engineering the first adenoviral vectors as these types are not associated with severe disease in humans and therefore suitable for *in vivo* administration (Karlsson 1986). Modifications of the adenoviral genome are based on deletion of the early gene 1 (E1A) to create replication-incompetent vectors providing sufficient space for gene insertions. The packaging capacity of these vectors is 7-8kb, which is not the maximum insertion capacity. These vectors can be produced at high titres, which is an advantage for *in vivo* gene transfer.

The disadvantage of adenoviral vectors is their episomal status in the host cell allowing only transient expression of the therapeutic gene. Furthermore, expression of early (E2) viral protein provokes inflammatory reactions and toxicities that limit the repeated application of the vector (Yang 1996). Newer second-generation vectors were established which lacked E1A and E4 gene functions. More recently, defective adenovirus vectors were created in which all viral coding regions were removed. These were successfully used to transfect the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Fisher 1996). Such vectors are less immunogenic, and as a consequence may be more efficient for gene delivery.

3.2.2.1.3 Adeno-associated viral vectors

A number of adeno-associated virus vector systems have been developed (Adesanya 1996, McElvaney 1996). The adeno-associated virus (AAV) is a single stranded DNA dependo-virus and belongs to the family of Parvoviruses. A broad range of cell types are susceptible to AAV infection, and there has been no pathology observed with AAV infection in humans. The AAV requires an adenovirus or a herpes virus for viral replication and difficulty with complete removal of helper viruses during their preparation is still hindering application of recombinant AAV vectors. In contrast to adenoviruses, AAV has low immunogenicity, but only a limited capacity for insertion of foreign genes (4.1-4.9kb).

AAV vectors can infect both dividing and non-dividing cells and have been used in a Phase I clinical trial for CFTR gene transfer in the maxillary sinus of ten cystic fibrosis patients (Wagner 1998). In these patients there was persistence of the transgene for up to 10 weeks with nil or only minor immune responses observed. This study indicated that rAAV vectors may be of value for gene correction therapies requiring long-term and high level gene expression in targeted tissues.

3.2.2.2 Non-viral gene vectors

In order to gain clinical acceptance gene therapy needs to be, above all else, safe. With regard to this requirement, many investigators have shown increasing interest in the development of non-viral gene delivery systems (Ledley 1994a). Although sometimes referred to as “artificial viruses”, non-viral gene delivery systems differ fundamentally in their composition, manufacture, characterisation and therapeutic profile (Ledley 1994b). Some recent animal studies have shown that non-viral gene delivery can be less efficient than viral systems for introducing genes into large numbers of cells *in vivo*. These same studies indicate that the non-viral systems used were relatively safe, although it is likely that different non-viral systems will

have different safety profiles. Non-viral gene vector systems can be further categorised and are discussed separately under these headings.

3.2.2.2.1 Cationic lipid based vectors

Cationic lipids and liposomes have become important reagents for gene transfer *in vitro* (Felgner 1987). Formulations of DNA with cationic lipid complexes are capable of transferring DNA into some cells at high efficiency (>90%). The use of these cationic complexes enhances entry of DNA into the cell. The net positive charge of the complex is important for attachment to the cell surface, presumably through electrostatic interactions with anionic structures on the cell surface, and for endocytosis (Ledley 1994b). Commercially available cationic lipid mixtures (Lipofectin and Lipofectamine) are used routinely in basic research. Conventional liposomes, in which compounds are encapsulated within a lamellar structure, have proven to be less effective than cationic liposomes (Bertling 1991). Some investigators have combined liposomes with viral proteins to aid in DNA delivery (Kato 1991).

3.2.2.2.2 Ligand-polylysine based vectors

Another method for introducing genes into cells is to complex DNA with ligands capable of binding to cell surface receptors of the target cells. Such complexes are commonly formed by covalently coupling the ligand to polylysine and then forming a ligand-polylysine-DNA complex by the ionic interaction between the positively charged polylysine and the negatively charged DNA (Wu 1988, Wagner 1991). This method allows receptor-mediated internalisation of the complex into the cell.

Many different ligands have been used to target cells. These have included transferrin, asialoglycoprotein, sugars and insulin (Wagner 1990, Wu 1987, Midoux 1993, Rosenkrantz 1992). More recently, cell surface integrins have become

popular choices as targets for DNA vectors as multivalent binding to the integrin results in ligand internalisation (Isberg 1994). Integrins are heterodimeric cell surface glycoproteins consisting of α and β subunits, which are expressed on many cells (Newham 1996). Their physiological functions relate to cell-cell and cell-matrix interactions. However, microorganisms including *Yersinia pseudotuberculosis* (Isberg 1991) and adenovirus (Wickham 1993) use cell surface integrins for internalisation. Thus, it is feasible that non-viral vectors could be developed to target cell surface integrins, thereby enabling cell attachment and internalisation.

The components of adhesive proteins responsible for integrin binding have been the subject of active research. Pierschbacher and Rouslahti demonstrated a recognition determinant on fibronectin for $\alpha_5\beta_1$, consisting of arginine, glycine and aspartic acid (Arg-Gly-Asp, RGD) amino acid sequence (Pierschbacher 1984,). Subsequent studies have established that this sequence is an integrin recognition determinant on many adhesive proteins. Peptides containing the conserved arginine-glycine-aspartic acid (RGD) domain bind integrins with a high affinity and enter cells via an integrin-mediated manner (Hart 1994).

Using this principle, Hart *et al* described the use of a non-viral gene vector in the form of a synthetic peptide, which comprised two essential domains. One, the DNA binding domain of a sequence of 16 lysine and the other, a targeting domain of a cyclic RGD-containing sequence (Hart 1995). This *in vitro* study demonstrated that an RGD containing peptide-DNA complex transformed Caco-2 cells and significantly increased luciferase activity compared with naked plasmid DNA and polylysine-DNA complexes lacking the RGD domain. They also demonstrated that gene delivery was truly integrin-mediated by performing competitive inhibition studies.

Using a natural peptide of 15 amino acids corresponding to a segment of the integrin-binding venom molossin from the American pit viper *Crotalus molossus molossus* (northern black-tailed rattlesnake), Shewring *et al* demonstrated that up to

30% of rabbit corneal endothelial cells could be transfected by a similar method (Shewring 1997). Molossin was chosen as it had been shown to bind with high affinity to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Scarborough 1993). In this study, transfection was only observed in the presence of chloroquine to aid endocytic exit. It was this polylysine-molossin peptide which was used in the experiments reported in Chapter 5 of this thesis.

3.2.2.2.3 Other non-viral methods of gene delivery

In vitro transfection of cultured cells with plasmid DNA represents an important model for non-viral gene delivery. Even though they have proven to be imperfect predictors of *in vivo* function, they are still utilised for the initial validation of most gene delivery systems. Some of the *in vitro* transfection methods used include calcium phosphate-mediated transfection (Graham 1973) and DEAE-mediated transfection (Ishikawa 1992). Direct microinjection of DNA into the nucleus of cultured cells represents another method of gene transfection (Capecchi 1980). Particle bombardment and electroporation are other methods of DNA delivery (Yang 1990, Chu 1987).

3.2.3 Endosomal release

Most methods of gene delivery *in vitro* involve entry of DNA into the cell by endocytosis. One of the factors that limits the efficiency of gene delivery to the nucleus after endocytosis is the rapid degradation of DNA within the endosome and following fusion of endocytic vesicles with lysosomes. Several methods have been developed to enhance the release of DNA from the endosome before degradation by nuclease ensues. Viral particles have been incorporated to enhance endosomal release, but the non-viral approach incorporates the use of drugs such as chloroquine (Cotten 1990). However, chloroquine can be toxic at high dosages (August 1995), while prolonged exposure reduces the viability of cells in culture (Shewring 1997).

Other agents that facilitate endocytic exit include polycationic lipids (Walker 1996, Hart 1998), but these too can be toxic.

Another approach is the use of peptides designed from naturally occurring proteins, which disrupt endosomes. One such peptide is the fusogenic peptide of the influenza virus haemagglutinin. Synthetic peptides containing 16-20 amino acids of the influenza HA-2 amino terminus have been reported to fuse phosphatidylcholine liposomes and also cause leakage of aqueous liposomal contents at pH values lower than 6 (Wharton 1988, Takahashi 1990). Using similarly acting synthetic peptides, Plank *et al* demonstrated substantial augmentation of receptor-mediated gene transfer in a series of cell lines (Plank 1994). In the experiments reported in Chapter 5, a fusogenic peptide was also used with the vector-DNA complex.

3.2.4 Gene transfer in the lung

Gene therapy may be of high potential in the field of lung disease, in particular cystic fibrosis. Numerous clinical trials of gene transfer in cystic fibrosis populations using different techniques have been reported. Adenovirus-mediated gene transfer has been the most commonly employed. A phase I study using a third generation adenoviral vector containing human cystic fibrosis transmembrane conductance regulator (CFTR) gene was recently reported (Zuckerman 1999). The results of this study demonstrated that gene transfer to the epithelium of the lower respiratory tract can be achieved, but was of low efficiency and of short duration.

In another study utilising cationic lipid-mediated CFTR gene transfer into the lungs of cystic fibrosis patients, there was a correction of the chloride abnormality as assessed by *in vivo* potential difference and chloride efflux. It was noted that bacterial adherence was also reduced. The investigators concluded that this method of gene transfer was effective with a significant influence on the underlying cystic fibrosis lung chloride defect (Alton 1999).

There have been no reports of clinical studies using integrin-targeted, non-viral vector systems, although there has been a recent report on *in vivo* delivery of genes to lungs in rodents, using an integrin-targeted vector. Jenkins *et al* demonstrated that a vector consisting of a cationic lipid and an integrin-binding peptide with a polylysine tail transfected rat bronchial and alveolar cells *in vivo* with high efficiency (Jenkins 2000). They compared the non-viral vector transfection with an adenoviral vector and found it to be comparable, but with a greatly reduced inflammatory response. They concluded that this vector might provide an alternative to current gene delivery systems.

3.2.7 Transplant applications

Gene therapy is a relatively new approach in transplantation. It offers the possibility of delivering molecules with immunomodulating activity to either the graft itself or to defined sites in the recipient. The established gene transfer methods have not been fully evaluated nor specifically optimised for application to transplantation. The following reviews the current status of gene therapy in transplantation, addressing the type of vectors being studied and the methods of modulating transplanted organs.

3.2.7.1 Gene vector systems

Fundamental to the application of gene therapy to facilitate transplantation is the delivery of exogenous DNA to the cells of various tissues. Some success has been reported with the uptake of “naked” DNA. Wang *et al* reported the ability of rat cardiac allografts to stably express reporter genes after direct injection of DNA into the apex of the heterotopically transplanted hearts (Wang 1992).

However, in order to optimise gene transfer, vector systems, either viral or non-viral, need to be employed. These vector systems have been reviewed in a preceding

section. The preferred delivery strategy may vary between organs depending upon their individual susceptibility to gene transduction. One of the advantages of using viral vectors in transplantation is that the concomitant immunosuppression therapy may reduce the immune response generated by the vector itself.

Adenoviral vectors have been used to perfuse a rat heart allograft *ex vivo*, with resultant expression of the reporter gene (Wang 1996). However, Merrick *et al* demonstrated that adenovirus did not appear to be an effective DNA vector for the *ex vivo* genetic manipulation of the vascular endothelium of transplanted organs (Merrick 1996)

Another of the experimental models developed involved an *ex vivo* strategy with a retroviral vector to transfer allogeneic MHC class I or II genes into recipient bone marrow cells in order to establish chimerism (Sykes 1993). The investigators demonstrated relatively long-term expression of the transgenes in the recipients. They also showed that expression of allogeneic MHC class I by autologous bone marrow cells conferred specific hyporesponsiveness to allogeneic skin grafts.

3.2.7.2 Modulation of transplanted organs

Direct modification of the transplanted organ or tissue has been shown to have potential as a strategy for modifying the immune response to allo- or xenografts. Qin *et al* compared the ability of the cytokines transforming growth factor (TGF)- β and viral IL-10 to modulate the rejection response following the delivery of these cytokines directly to mouse myoblast and non-vascularised heart grafts (Qin 1995, Qin 1996). The delivery of viral IL-10 and TGF- β using a retrovirus and plasmid delivery system resulted in prolongation of graft survival as compared with the vector only controls. In another study using an adenovirus mediated system, Drazan *et al* demonstrated that gene transfer of TGF- β 1 into rat liver grafts *ex vivo* resulted in the expression of biologically active protein, which downregulated the production of TNF α and IFN γ production early after orthotopic transplantation (Drazan 1996).

In a further study, Liu *et al* demonstrated that adenoviral gene transfer of CTLA4-Ig in a pancreatic transplant model induced permanent allograft survival and donor-specific tolerance (Liu 1999).

Endothelial cells express a number of targets that might be used to inhibit endothelial cell activation, prevent reperfusion injury and promote graft acceptance. For example, inhibition of NF- κ B, an inducible activator of transcription of a large array of genes, including some required for inflammatory and immune responses, may prevent endothelial cell activation. Using a recombinant adenovirus to transfer the gene for an inhibitor known as A20 to aortic endothelial cells, Cooper *et al* demonstrated the inhibition of upregulation of NF- κ B sensitive reporter genes and endothelial cell activation (Cooper 1996).

Non-viral gene transfer strategies have also been documented in experimental transplant models. *In vivo* transfection of the liver with plasmid-liposome complexes encoding the Bcl-2 gene has been shown to prevent the death of hepatocytes induced by hypoxia (Yamabe 1997). However, the efficiency of gene transfer was very low. Plasmid-liposome complexes have also been shown to be capable of transferring genes to donor hearts before transplantation (Dalesandro 1996). These non-viral strategies may also provide approaches for expressing genes that would disrupt the inflammatory response after transplantation.

It is clear from the above discussion that there is great potential for the development of gene therapy in the transplant setting. Its future application depends upon refinements in gene transfer technology, coupled with a better understanding of the molecular basis of ischaemic/reperfusion injury and allogeneic and xenogeneic immune responses.

3.3 Antiviral therapy with antisense oligodeoxynucleotides

3.3.2 Adenovirus infection in the lung

Adenoviruses are frequent causes of fevers, upper respiratory tract symptoms and conjunctivitis in young children and produce infections, which are usually mild, self-limiting and associated with no significant sequelae (Brandt 1969). Adenoviral lower respiratory infections are infrequent and occur sporadically. The most commonly encountered types associated with pneumonia are adenovirus types 3, 5 and 7 (Murtagh 1993). Rarely, more severe forms of adenoviral respiratory disease, including acute necrotising bronchitis and bronchiolitis, may occur in children or debilitated and immunocompromised patients (Ruuskanen 1985, Edwards 1985, Zahradnik 1980). These may in turn lead to long term complications including pulmonary fibrosis, recurrent wheezing, bronchiectasis and obliterative bronchiolitis (Simil 1981, Sly 1984, Hardy 1988, Macek 1994).

Respiratory adenoviral infections in lung transplant recipients, although relatively rare, may demonstrate a rapidly progressive course leading to premature death (Kramer 1993, Ohori 1995, Simsir 1998, Bridges 1998). These infections may be acquired in the community or transferred via infected donor lungs. In the series described by Ohori *et al*, four lung transplant recipients, out of a total of 308, were identified as having developed adenoviral pneumonia (Ohori 1995). Three of the four were children and all four had a progressive and rapidly fatal course within 45 days following transplant. On histology, the lungs showed necrotising bronchocentric pneumonia with a tendency to spread diffusely to produce alveolar damage and organising pneumonia. They suggested that the infection was primary to the recipient.

With the high level of immunosuppression required for lung transplantation, adenovirus infection can be devastating and has a high associated mortality of 60%-80% (Hierholzer 1992). If not fatal, it is often implicated in the early development

of obliterative bronchiolitis (Burke 1984). This complication may be more evident in paediatric lung transplant recipients (Whitehead 1993).

Unlike other viral pneumonitides (eg: those caused by herpes simplex virus, cytomegalovirus, etc.), no specific treatment for adenovirus pneumonitis exists. Apart from anecdotal reports of treatment with ribavirin (Jurado Chacon 1998, Wulffraat 1995, Maslo 1997), the current therapeutic approach for this condition comprises general supportive measures only. Accordingly, an adenoviral specific therapeutic option would be highly desirable, particularly in the setting of high level immunosuppression encountered following lung transplantation.

3.3.2 Antisense oligodeoxynucleotides

Antisense methodology utilises Watson-Crick base pairing specificity to inhibit gene transcription and/or translation and thus protein production. Normally, when a gene is transcribed, the corresponding portion of the DNA helix opens with the “sense” and its complementary “antisense” strands separating. The latter strand then acts as a template for a chain of sense messenger RNA (mRNA) to be produced, which is in turn translated into the corresponding protein. The antisense approach incorporates the use of an antisense oligodeoxynucleotide (ODN) to target the sense mRNA. This leads to the formation of a mRNA/DNA duplex, which is then degraded by RNase H. The ODN might also physically block mRNA function or gene transcription. This approach may prevent the expression of specific proteins within cells (Gibson 1994).

Antisense ODNs are synthesised as single-stranded DNA and are usually 15-28 bases in length. In theory, an ODN of 15 nucleotides in length has the specificity necessary to inhibit the expression of a single target gene through complementary hybridisation with a cellular mRNA (Helene 1990). The first report of inhibition of gene expression by an exogenously added ODN was in 1978 (Zamecnik 1978) and

since then numerous reports of the use of ODNs both *in vitro* and *in vivo* have been published (Heidenreich 1995).

Numerous studies have been performed to elucidate ODN activity. It has been shown that after entry into the cell, the ODN hybridises to the target mRNA in the cytoplasm, nucleus or both, forming a mRNA-DNA duplex. At least two mechanisms in the inhibition of gene expression are implicated. Firstly, the enzyme RNase H cleaves the mRNA strand of the complex. Once cleaved, the mRNA is no longer competent for translation and is rapidly degraded. In the other mechanism, gene expression may be inhibited through simple steric blocking consequent upon the high-affinity binding of the ODN to the target mRNA (Milligan 1993).

Other factors to be considered in the development of antisense ODN therapy include the entry of the ODN into the cell cytoplasm and nucleus and its stability once there. As for the latter consideration, ODNs containing phosphodiester linkages are rapidly degraded by nucleases with an intracellular half-life of only around 20 minutes (Fisher 1993). Modification of the ODN by using phosphorothioate, phosphoramidate or methylphosphonate linkages at the 3' end of the ODN will render it nuclease resistant and thus "protected" with a consequent increase in the half-life (Hoke 1991, Tidd 1989, Shaw 1991).

As for entry into cells, ODNs are bound non-specifically (by electrostatic interactions) to cell surfaces, internalised by receptor-mediated endocytosis and have been shown to rapidly accumulate in endocytic vesicles within cells in tissue culture (Iverson 1992). However, entry into the nucleus is not efficient due to the poor release of ODNs from endocytic vesicles. Co-administration with cationic lipids alleviates this problem to some extent and enhances the nuclear uptake of ODNs and hence their antisense activity (Bennett 1992). Other potential advantages of using liposomes include the protection from enzyme degradation and the control of oligomer-release kinetics, in addition to the improved biocompatibility and ultimate biodegradability of the delivery system (Akhtar 1991).

Fluorescence microscopy has been used to ascertain the intracellular location of ODNs tagged with fluorescent labels. Two groups demonstrated that ODNs rapidly concentrate into the nucleus after microinjection into cells (Leonetti 1991, Chin 1990). However, when fluorescently labelled ODNs were placed in tissue culture media, the fluorescence accumulated only in vacuoles within the cell. Co-administration with cationic lipids enhanced the nuclear uptake of the labelled ODNs as judged by nuclear localisation of fluorescence (Bennett 1992).

Another aspect to be considered with ODN therapy is related to their non-specific effects. Unintended side effects could occur through a number of mechanisms. For example, sequence-specific cross-reactions with unrelated mRNAs and non-specific binding of ODNs to small molecules and proteins have been described (Ellington 1990, Gao 1992). Inhibition of viral infection by ODNs may include non-specific mechanisms, which interfere with viral absorption, penetration or uncoating (Yakubov 1993, Yao 1993, Azad 1993). Accordingly, Wagner recommends that well controlled assays should be performed in an attempt to alleviate these difficulties and to demonstrate specific antisense effects (Wagner 1994).

3.3.3 Antiviral applications

Considerable interest has been generated recently by the potential use of antisense ODNs in the treatment of some viral infections. As the life cycle of viruses is dependent upon the expression of specific proteins unique to the virus, the antisense approach has considerable potential as a form of virus-specific therapy. Lisziewicz and colleagues demonstrated specific inhibition of human immunodeficiency virus type 1 (HIV-1) replication by antisense ODNs *in vitro*. Using a culture system which simulated *in vivo* conditions of HIV-1 infection, they showed that five ODN phosphorothioates (28-mers), complementary to different regions of HIV-1 RNA, blocked replication of the virus in a sequence-specific manner at 1 μ M concentration (Lisziewicz 1994). In another study, Cowser *et al* demonstrated inhibition of transactivation of the E2 mRNA of papillomavirus by phosphorothioate ODNs in an

in vitro assay (Cowser 1992). Some of the other viruses studied with this method have included herpes simplex virus 1 (Vinogradov 1994); Epstein-Barr virus (Roth 1994); and hepatitis C virus (Wakita 1994).

3.3.4 Antisense approach to adenovirus infection

The replication cycle of human adenovirus type 5 (AdV5) can be divided into two phases: early, corresponding to events occurring before the onset of viral DNA replication; and late, corresponding to the period after initiation of DNA replication (Ginsberg 1984). Early (E) genes are situated in the E1 to E3 regions of the genome. The E1 domain, which is subdivided into E1A and E1B, is of special interest. This region codes for the transformation function of the adenoviruses (Sambrook 1974). Although the E1A region can transform in the absence of E1B, it may be the latter that is normally responsible for adenovirus transformation. However, this cannot be proven unequivocally as the E1A gene exerts an important control function on all the other early adenovirus mRNAs (Lewis 1980). It follows that inhibition of one, or both, of these gene products may induce a reduction in adenovirus replication.

The late phase of viral replication is associated with the production of virion structural proteins or their precursors. These late viral polypeptides are synthesised at a maximum rate 20 hours post-infection, by which time host-cell protein synthesis has been inhibited (Anderson 1973). One of these late polypeptides is protein IX (PIX) and appears at the “young virion” stage. It is essential for the completion of virus assembly (Ghosh-Choudhury 1987). Thus, targeting this gene also presents as a reasonable approach.

There have been few reported studies on the use of the antisense approach against adenovirus replication or infection. Using an anti-sense *gene* method, which represents a very different approach to ODNs, Miroshnichenko and colleagues

showed that antisense DNA constructs cloned into a plasmid could inhibit adenoviral replication (Miroshnichenko 1989).

Quinlan demonstrated that antisense ODNs could inhibit cellular adenoviral DNA synthesis (Quinlan 1993). Her technique involved an *in vitro* culture of 293 cells, a cell line that had been immortalised and transformed by adenovirus E1A and E1B (early) genes. With the addition of synthetic E1A antisense ODNs, there was an inhibition of 293 cellular DNA synthesis. In addition, transfecting 293 cells with an antisense E1A expression vector led to cells with altered morphologies, which did not survive.

These two reports indicated that the antisense approach in treating adenovirus infection was feasible, although apart from the above two, there have been no other published studies. The study detailed in Chapter 6 of this thesis was designed to assess the efficacy of antisense ODNs directed towards the E1A, E1B and polypeptide IX gene regions of adenovirus 5 in reducing viral replication in an *in vitro* assay. Various ODNs were assessed at different concentrations and by utilising different delivery methods. In addition, Chapter 7 details the evaluation of ODN entry into the nuclei of cells utilising fluorescence techniques.

Section B
Experimental results

Chapter 4 Correlation of pulmonary function with injurious events following lung transplantation

4.1 Introduction

4.2 Patients and methods

4.3 Results

4.3.1 Dynamic and static pulmonary function test results

4.3.2 Area under the curve results

4.4 Discussion

4.1 Introduction

Pulmonary function testing is a useful adjunct in the monitoring of the lung transplant recipient. It is a sensitive, although relatively non-specific, measure of the status of the lung allograft and may prompt further investigations and therapeutic interventions. Pulmonary function has been well delineated in the adult lung transplant population, but less so for the paediatric group. In addition, little is known of the relationship between early injurious events and long-term pulmonary function.

This chapter describes pulmonary function in a cohort of paediatric lung transplant recipients over an extended post transplant period. Both dynamic and static lung function parameters are detailed and compared with pre transplant values. A novel method of assessing pulmonary function over a prolonged period is described. Using this technique, the relationship between pulmonary function and various lung injurious events is explored.

The principal research questions were as follows:

- What is the pattern of dynamic and static pulmonary function following paediatric lung transplantation?
- Does this pattern differ significantly from the adult cohort?
- Is there a correlation between pulmonary function and peri or post transplant lung injurious events including: graft ischaemia; rejection; and infection?
- Can pulmonary function testing aid in predicting the development of obliterative bronchiolitis?

4.2 Patients and methods

Subjects for this study were recruited from a cohort of children who had undergone either combined heart-lung or bilateral lung transplantation at the Great Ormond Street Hospital for Children, London UK between June 1988 and July 1998.

Inclusion criteria were as follows:

1. age under 16 years at time of transplant
2. original diagnosis of cystic fibrosis
3. capable of performing formal pulmonary function tests
4. no evidence of significant airway stenosis
5. post transplant survival of at least one year

Recipient and donor selection, surgical techniques, post-operative management and immunosuppression regimen are detailed in Appendix I. In addition to daily home spirometry, all subjects performed formal pulmonary function testing (at a minimum of every three months) at the respiratory function laboratory of the Great Ormond Street Hospital for Children, London during each outpatient and inpatient attendance. The latter often incorporated bronchoscopy, bronchoalveolar lavage and transbronchial lung biopsy, in which event pulmonary function testing was performed prior to the procedure. Formal dynamic pulmonary function testing comprised spirometry performed on a compact spirometer (Vitalograph Ltd., Buckingham, UK). This measured forced vital capacity in litres (FVC), peak expiratory flow rate in litres per minute (PEFR), forced expiratory volume in one second in litres (FEV₁), and maximum expiratory flow at 25% of remaining vital capacity in litres per second (MEF₂₅). From the FEV₁ and FVC values, a percentage ratio for FEV₁/FVC was derived.

Static pulmonary function included measurements of total lung capacity in litres (TLC) and residual volume in litres (RV). These were estimated either by the helium dilution method (PK Morgan, Chatham, Kent, UK) or by whole body plethysmography using the respiratory body box (Jaeger, Coventry, UK). Lung

diffusing capacity for carbon monoxide (D_LCO) was also measured (PK Morgan, Chatham, Kent, UK) and expressed as a ratio of available lung volume in millimoles of carbon monoxide per minute per millimetre of mercury per litre (KCO).

Spirometric measurements were recorded pre-operatively (pre), and at six-monthly intervals post transplant until death, the end of the study period or 60 months, whichever occurred sooner. Plethysmographic (TLC and RV) and diffusing capacity (KCO) measurements were performed at less frequent intervals and as tolerated by the patients.

In order to standardise results, both dynamic and static pulmonary function measurements are expressed as a percentage of predicted normal, using height and gender matched control data derived at the Brompton Hospital, London, UK (Rosenthal 1993a, Rosenthal 1993b). Thus data are described as FVC%, FEV₁%, PEFR%, MEF₂₅%, TLC%, RV% and KCO%. The ratio of FEV₁/FVC is also described as a percentage, but not as a percentage of predicted normal.

Pulmonary function data were compared with other variables including graft ischaemic time in minutes, both cold (GIT-C) and total (GIT-T). These times were recorded from the time of aortic cross clamping in the donor to the removal of the donor graft from ice, and removal of the aortic cross clamp in the recipient, respectively. In addition, the number of rejection episodes (REJ) and the number of pulmonary infection episodes (INF) occurring in the first 12 months post transplant were compared with the pulmonary function data.

Acute rejection was defined as a clinically or histologically diagnosed episode requiring treatment with augmented immunosuppression, which usually comprised a pulse of corticosteroids – either intravenously or, less commonly, orally. Thus, a rejection episode was confirmed in the presence of pathognomonic histological changes of rejection on transbronchial lung biopsy as defined by the International Society for Heart and Lung Transplantation (ISHLT) (Yousem 1996) and/or with

clearing of chest X-ray infiltrates, or reversal of an acute fall in pulmonary function following treatment.

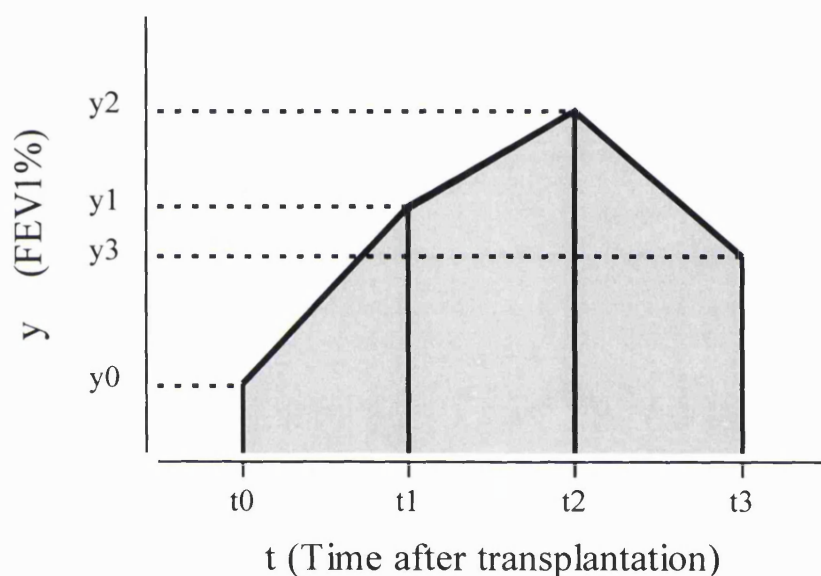
Pulmonary infection was established in the presence of clinical symptoms and signs, pulmonary infiltrates on chest X-ray, together with a positive sputum or bronchoalveolar lavage culture, or a rise in serological titres, or an histological identification of an infectious process and reversal of findings after appropriate anti-microbial therapy (Whitehead 1995).

Median values for the study cohort for each of the parameters were calculated and presented together with minimum and maximum values. Changes from pre to post transplantation values were calculated for each time point post-transplantation, each parameter and each patient. A two-tailed Wilcoxon's signed rank test was used to determine, for each parameter, at each time point post-transplantation, whether these changes were significantly different from zero, for the population of all patients. Calculations were performed on the statistics package Minitab version 11 for Windows (Minitab Inc, USA) for personal computers. A p value of less than 0.05 was considered significant. Graphs of pulmonary function parameters were also generated using the same software package.

In order to compare the effects of various factors on overall post transplant pulmonary function, the area under the curve (AUC) was calculated from the FEV₁% graphs for each of the study subjects (FEV₁% AUC). The AUC is a useful way of summarising the information from a series of measurements on one individual. Data points are joined by straight lines to produce a "curve". The AUC is then calculated by adding the areas under the curve between each pair of consecutive observations (Altman 1991). FEV₁ was chosen as this parameter is recognised as the most sensitive, single measure of overall pulmonary function. It was also recorded at regular intervals for all individual subjects. For calculation purposes, the baseline FEV₁ axis was taken as zero and values from three months post operatively were used to avoid any pre operative baseline variation or immediate post surgical effect. Thus, calculations of the FEV₁% AUC were derived

for the periods three to 12, 18, 24, 30, and 36 months after grafting. In addition, FEV₁% AUC was derived separately for the second and third years, that is the epochs 12 – 24 months and 24 – 36 months respectively. Figure 4.1 illustrates the method for calculating AUC.

Figure 4.1 Method for calculating area under the curve (AUC)



The area under the curve is calculated using the formula:

$$AUC = \frac{1}{2} \sum_{i=0}^{n-1} (y_i + y_{i+1})(t_{i+1} - t_i)$$

In the diagram above, AUC is calculated by the formula:

$$AUC \text{ (from } t_0 \text{ to } t_3) = \frac{1}{2} ((y_0 + y_1)(t_1 - t_0) + (y_1 + y_2)(t_2 - t_1) + (y_2 + y_3)(t_3 - t_2))$$

An absolute value for AUC can then be derived and thus enable comparison between subjects. The units of the AUC are arbitrary, and in this instance will be percentage of predicted FEV₁ x months (%. months).

The FEV₁% AUC to varying post transplant time points (12, 24, and 36 months) were then examined for associations with age and gender of recipient and donor respectively, donor graft ischaemic times – both cold and total, and number of rejection and infection episodes in the first 12 months post transplant. FEV₁% AUC for the second and third post transplant years were also compared with the above parameters. All comparisons were made using Spearman's rank correlation on the statistics package Minitab version 11 for Windows (Minitab Inc, USA) for personal computers. The test was two-tailed to identify either a positive or negative correlation. A p value of less than 0.05 was considered significant.

4.3 Results

Twenty-four patients satisfied the inclusion criteria and participated in the study. Age at transplant ranged from 5.8 to 15.6 years (median, 12.8 years). There were 16 males and 8 females, all of whom had received transplants (22 heart-lung and 2 bilateral lung) for cystic fibrosis, which represented the major indication for lung transplantation at this institution (Whitehead 1995). Post transplant survival of the group ranged from 17.6 months to 120.7 months (median, 45.5 months), with 15 of the group dying by the end of the study. The demographic data of the cohort, including those of their respective donors, together with post transplant survival and outcome are shown in Table 4.1. The median donor age was 9 years (range, 4 - 41 years), while there was a gender mismatch between donor and recipient in 6 of the 18 cases with complete data.

The cold graft ischaemic time (GIT-C) ranged from 56 to 291 minutes (median, 159 minutes), while total ischaemic time (GIT-T) ranged from 94 to 393 minutes (median, 201.5 minutes). The median number of rejection episodes in the first 12 months post transplant was three (range, 1 - 8), while for the same time period, there was a median of two pulmonary (lower respiratory) infection episodes (range, 0 - 6) (Table 4.2).

Table 4.1 Demographic data of paediatric lung transplant recipients and their respective donors, post transplant survival and outcome.

Subject No.	Donor gender	Recipient gender	Donor age (years)	Recipient age (years)	Post Tx survival (months)	Outcome (alive/dead)
1	N/A	F	7	14.0	79.7	D
2	M	M	11	14.3	120.7	A
3	F	F	4	5.8	116.3	A
4	F	M	9	11.9	63.8	D
5	N/A	M	N/A	15.3	52.1	D
6	M	M	9	10.0	31.8	D
7	M	F	9	10.0	39.2	D
8	M	F	9	12.3	33.4	D
9	M	M	5	10.3	86.4	D
10	N/A	F	N/A	7.1	17.6	D
11	M	M	15	15.6	34.7	D
12	M	M	10	10.3	88.0	A
13	F	F	14	14.6	21.0	D
14	N/A	M	N/A	13.1	46.4	D
15	N/A	F	N/A	8.3	36.4	D
16	M	M	15	15.3	48.8	D
17	M	F	6	8.0	66.2	A
18	M	M	10	13.3	63.6	A
19	M	M	7	9.8	41.8	D
20	N/A	M	7	11.4	57.2	A
21	M	M	9	13.6	44.5	A
22	F	M	13	14.8	31.2	D
23	M	M	19	14.9	33.1	A
24	F	M	41	14.1	25.2	A
Median	-	-	9	12.8	45.5	-

N/A, not available

Table 4.2 Graft ischaemic times and number of rejection and pulmonary infection episodes in paediatric lung transplant recipients

Subject No.	Graft ischaemic time – cold (GIT-C) (minutes)	Graft ischaemic time – total (GIT-T) (minutes)	Acute rejection episodes in 1st 12 months (REJ)	Lower respiratory infections in 1st 12 months (INF)
1	N/A	170	5	3
2	N/A	170	5	1
3	56	94	2	1
4	100	151	8	1
5	159	204	5	1
6	120	145	3	2
7	N/A	211	4	2
8	N/A	158	6	2
9	180	209	3	3
10	147	185	5	6
11	100	160	1	3
12	N/A	200	3	1
13	142	202	1	0
14	157	201	2	1
15	208	248	1	5
16	213	252	3	3
17	177	211	N/A	N/A
18	139	186	1	4
19	169	204	5	1
20	177	209	3	2
21	120	160	3	1
22	229	274	2	3
23	291	393	1	0
24	225	345	2	1
Median	159	201.5	3	2

N/A, not available

4.3.1 Dynamic and static pulmonary function test results

Pulmonary function data for each of the subjects have been tabulated for FVC%, FEV₁%, FEV₁/FVC, PEFR%, MEF₂₅%, TLC%, RV%, and KCO% (Tables 4.3 – 4.10). As demonstrated in the tables, the data sets for the spirometric values (dynamic lung function) were more complete than for the static lung function parameters. This was related to the impracticality of performing the static lung function tests on such a frequent basis. At 12 months post transplant, spirometric data were available on all 24 subjects, but by 60 months, data were available on only seven.

In order to produce a more informative description of pulmonary function, data are presented graphically in Figures 4.2 – 4.6. These graphs depict the median value of the group for each of the parameters assessed, together with the range, over the study period. The number of subjects contributing data at each time point is indicated.

The graphs of dynamic pulmonary function all show a similar pattern of improvement in the early post transplant period, albeit with evidence of a persistent minor restrictive defect. This initial improvement was followed by a gradual decrease over the course of the study, with median values returning to pre transplant levels.

Following lung transplantation, median pulmonary function measures (FVC%, FEV₁%, FEV₁/FVC, and PEFR) had increased significantly ($p < 0.001$) by three months post transplantation and peaked by 15 months post transplantation. These had all declined to pre transplantation levels by 60 months post-transplantation. Individually, the effects of lung transplantation on pulmonary function were highly variable, although all patients in the study showed initial improvement in lung function.

The p value of the differences between pre and post transplant measurements have been summarised for each of the parameters in Table 4.11. For the cohort as a whole, there was a statistically significant increase in FVC% at 12 months post transplant compared with pre transplant values ($p < 0.001$). This difference became non-significant by 42 months post transplant. FEV₁% was also higher at 12 months post transplant ($p < 0.001$), the increase becoming non-significant by 36 months. FEV₁/FVC, PEFR%, and MEF₂₅% were also increased at 12 months post transplant ($p < 0.001$, $p < 0.001$, and $p < 0.001$ respectively) with loss of this difference by 30, 30, and 36 months post transplant respectively.

Due to the relatively small number of data points, graphs for static lung function were not formulated. However, the median TLC% value was higher at 12 months post transplant compared with the pre transplant value ($p < 0.01$). RV% was also reduced compared to the abnormally elevated pre transplant values at 6 months ($p < 0.05$) and at 12 months ($p < 0.01$) post transplant (Table 4.10). Although too few in number to enable formal statistical evaluation, values for KCO% appeared to remain relatively stable throughout the post transplant period.

Table 4.3 Forced vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (FVC%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	36	88	96	84	98	96	91	90	77	53	42	39
2	35	73	78	93	80	59	60	50	44	35	35	N/A
3	N/A	133	119	165	149	124	123	127	139	N/A	125	N/A
4	59	74	91	83	78	72	72	69	59	42	32	20
5	21	78	92	62	56	41	41	40	30	16	D	D
6	48	85	123	121	124	114	31	D	D	D	D	D
7	36	98	96	54	77	58	55	57	D	D	D	D
8	28	73	70	110	60	46	39	D	D	D	D	D
9	57	60	72	69	62	73	N/A	58	62	76	59	64
10	41	68	62	35	D	D	D	D	D	D	D	D
11	25	28	38	53	57	57	59	D	D	D	D	D
12	54	103	70	100	80	48	58	77	68	80	76	65
13	36	84	84	86	80	D	D	D	D	D	D	D
14	43	49	70	67	75	78	60	60	29	D	D	D
15	37	70	69	55	48	37	29	21	D	D	D	D
16	49	97	94	76	85	77	46	37	N/A	N/A	D	D
17	26	N/A	69	88	87	85	94	81	102	101	97	88
18	20	71	86	90	86	74	43	36	40	40	46	39
19	38	103	105	105	121	97	90	48	D	D	D	D
20	47	63	77	86	83	53	38	28	23	28	20	19
21	41	91	84	92	103	97	108	84	52	75	N/A	N/A
22	27	52	53	37	34	30	26	D	D	D	D	D
23	33	54	62	72	57	45	43	52	N/A	N/A	N/A	N/A
24	33	76	85	93	103	94	N/A	N/A	N/A	N/A	N/A	N/A
Median	36	74	81	85	80	72.5	56.5	57	55.5	47.5	46	39

N/A, not available; D, deceased

Table 4.4 Forced expiratory volume in one second in paediatric lung transplant recipients expressed as percentage of predicted normal (FEV₁%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	21	91	100	52	62	68	57	53	41	24	19	18
2	17	75	87	103	84	48	24	18	13	14	13	N/A
3	N/A	90	90	130	122	91	133	122	137	N/A	117	N/A
4	39	72	97	84	74	70	72	69	60	43	32	20
5	13	68	81	44	32	25	24	25	17	12	D	D
6	21	90	116	132	121	119	23	D	D	D	D	D
7	31	97	92	49	57	39	37	33	D	D	D	D
8	16	76	57	80	41	36	22	D	D	D	D	D
9	41	49	58	63	46	69	N/A	47	62	64	58	61
10	22	69	39	23	D	D	D	D	D	D	D	D
11	23	29	45	58	59	52	58	D	D	D	D	D
12	49	87	55	106	79	33	36	41	40	37	40	38
13	29	87	85	92	89	D	D	D	D	D	D	D
14	21	51	61	61	65	72	52	43	14	D	D	D
15	25	54	52	39	29	23	17	14	D	D	D	D
16	28	89	97	48	53	53	34	26	26	N/A	D	D
17	21	N/A	70	75	85	89	94	71	95	101	91	85
18	14	78	91	95	85	55	23	19	22	20	22	18
19	28	102	81	99	101	99	52	25	D	D	D	D
20	37	63	62	78	80	45	27	20	15	19	13	N/A
21	25	75	56	64	59	65	78	43	34	N/A	N/A	N/A
22	19	51	30	19	21	17	14	D	D	D	D	D
23	27	43	50	56	47	34	28	N/A	N/A	N/A	N/A	N/A
24	27	64	79	87	101	90	N/A	N/A	N/A	N/A	N/A	N/A
Median	25	75	74	69.5	65	54	35	37	34	24	32	29

N/A, not available; D, deceased

Table 4.5 Percentage ratio of forced expiratory volume in one second to forced vital capacity (FEV₁/FVC) in paediatric lung transplant recipients

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	56	92	92	55	56	62	55	52	47	41	41	40
2	42	85	93	91	86	67	34	29	25	33	30	N/A
3	N/A	61	68	72	74	67	97	86	88	N/A	83	N/A
4	56	83	91	90	81	83	86	84	87	87	84	84
5	53	73	73	59	48	51	49	52	48	60	D	D
6	37	91	81	93	84	90	65	D	D	D	D	D
7	78	87	85	81	65	59	60	52	D	D	D	D
8	52	93	73	64	60	68	50	D	D	D	D	D
9	62	72	69	79	63	80	N/A	70	85	71	83	79
10	49	91	57	57	D	D	D	D	D	D	D	D
11	77	86	99	90	85	74	82	D	D	D	D	D
12	77	72	68	91	84	59	52	49	49	38	44	48
13	70	91	89	94	97	D	D	D	D	D	D	D
14	42	89	75	78	74	79	73	62	40	D	D	D
15	60	69	68	64	54	57	53	58	D	D	D	D
16	49	78	88	53	52	57	63	59	62	N/A	D	D
17	71	N/A	91	76	87	94	89	79	83	89	84	86
18	60	N/A	90	89	84	63	46	45	47	42	40	39
19	64	N/A	66	81	72	88	49	45	D	D	D	D
20	67	N/A	69	77	82	73	60	60	54	57	53	54
21	51	N/A	57	60	49	57	61	44	56	46	N/A	N/A
22	61	84	47	43	50	48	45	D	D	D	D	D
23	70	67	68	66	69	63	54	57	N/A	N/A	N/A	N/A
24	70	71	78	78	82	79	N/A	N/A	N/A	N/A	N/A	N/A
Median	60	84	74	77.5	74	67	57.5	57	54	51.5	53	54

N/A, not available; D, deceased

Table 4.6 Peak expiratory flow rate in paediatric lung transplant recipients expressed as percentage of predicted normal (PEFR%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	38	88	99	38	54	73	54	55	44	23	34	32
2	34	74	102	110	98	65	42	25	22	24	27	N/A
3	N/A	60	100	102	118	83	100	137	135	N/A	127	N/A
4	71	84	104	105	101	104	109	100	109	95	58	44
5	42	63	87	47	47	42	23	43	28	24	D	D
6	40	94	125	111	92	114	47	D	D	D	D	D
7	51	102	86	65	68	61	57	56	D	D	D	D
8	30	69	62	62	52	40	26	D	D	D	D	D
9	57	53	100	110	67	107	D	79	87	99	97	97
10	28	57	43	16	D	D	D	D	D	D	D	D
11	37	48	77	81	80	72	83	D	D	D	D	D
12	47	88	81	96	96	38	58	60	61	53	53	47
13	57	115	114	116	97	D	D	D	D	D	D	D
14	33	59	77	93	82	105	88	66	25	D	D	D
15	46	45	64	69	58	57	43	34	D	D	D	D
16	50	126	118	76	73	83	47	34	41	N/A	D	D
17	34	N/A	68	100	79	99	95	62	101	94	80	79
18	30	67	92	88	84	61	32	23	34	29	35	32
19	34	118	120	66	114	112	109	25	D	D	D	D
20	60	102	100	109	101	70	43	30	28	29	26	N/A
21	46	72	56	79	75	88	78	65	54	50	N/A	N/A
22	58	76	53	45	44	40	35	D	D	D	D	D
23	71	68	88	84	76	63	53	58	N/A	N/A	N/A	N/A
24	79	67	85	92	96	93	N/A	N/A	N/A	N/A	N/A	N/A
Median	46	72	87.5	86	80	72.5	53.5	56	44	39.5	53	45.5

N/A, not available; D, deceased

Table 4.7 Maximum expiratory flow at 25% of remaining vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (MEF₂₅%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	9	118	47	18	17	26	25	15	19	10	9	9
2	10	76	113	62	75	31	11	12	9	13	9	N/A
3	N/A	79	97	110	100	74	159	83	109	N/A	65	N/A
4	12	65	119	99	32	44	63	50	52	38	18	9
5	3	63	88	24	19	14	13	17	10	8	D	D
6	10	92	112	148	109	147	11	D	D	D	D	D
7	15	83	69	26	16	17	14	13	D	D	D	D
8	5	89	24	30	14	17	11	D	D	D	D	D
9	11	19	26	26	23	43	N/A	33	54	24	52	45
10	10	63	11	10	D	D	D	D	D	D	D	D
11	12	40	96	73	68	20	64	D	D	D	D	D
12	24	60	25	126	54	15	14	17	14	12	12	15
13	10	151	149	161	113	D	D	D	D	D	D	D
14	5	50	34	25	16	38	22	10	14	D	D	D
15	11	22	14	16	8	9	7	4	D	D	D	D
16	7	45	72	18	21	22	19	13	11	N/A	D	D
17	7	N/A	94	32	73	95	77	41	64	85	60	69
18	5	105	104	102	71	17	11	8	9	9	11	8
19	20	79	40	70	50	83	19	11	D	D	D	D
20	15	38	20	38	47	27	14	9	6	9	7	5
21	10	37	17	17	12	23	14	16	14	14	N/A	N/A
22	11	46	13	10	10	7	D	D	D	D	D	D
23	11	13	20	13	16	13	11	26	N/A	N/A	N/A	N/A
24	8	40	44	65	92	68	N/A	N/A	N/A	N/A	N/A	N/A
Median	10	63	45.5	31	32	24.5	14	15	14	12..5	12	9

N/A, not available; D, deceased

Table 4.8 Total lung capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (TLC%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	72	101	99	110	105	106	102	107	N/A	N/A	N/A	N/A
2	65	105	96	100	98	85	97	107	101	101	N/A	N/A
3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	122	99	100	97	77	87	81	80	N/A	51	N/A	N/A
5	46	78	N/A	76	78	81	N/A	69	N/A	N/A	D	D
6	N/A	113	127	127	N/A	N/A	N/A	D	D	D	D	D
7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	D	D	D	D
8	61	81	81	88	N/A	60	N/A	D	D	D	D	D
9	76	76	78	70	N/A	76	N/A	65	N/A	N/A	65	N/A
10	106	99	81	N/A	D	D	D	D	D	D	D	D
11	56	57	N/A	61	N/A	64	N/A	D	D	D	D	D
12	76	86	N/A	100	N/A	90	N/A	97	N/A	93	N/A	N/A
13	66	90	N/A	96	N/A	D	D	D	D	D	D	D
14	78	49	N/A	88	N/A	N/A	83	72	N/A	D	D	D
15	60	N/A	N/A	N/A	79	N/A	57	N/A	D	D	D	D
16	77	87	103	107	N/A	98	N/A	55	N/A	N/A	D	D
17	74	N/A	N/A	N/A	105	121	101	N/A	N/A	N/A	N/A	N/A
18	41	N/A	N/A	93	N/A	78	N/A	76	N/A	62	N/A	59
19	79	N/A	N/A	104	N/A	103	N/A	N/A	D	D	D	D
20	101	N/A	N/A	104	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	74	N/A	N/A	102	N/A	113	N/A	N/A	N/A	N/A	N/A	N/A
22	52	N/A	N/A	N/A	65	N/A	N/A	D	D	D	D	D
23	N/A	51	N/A	79	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	N/A	N/A	N/A	107	N/A	115	N/A	N/A	N/A	N/A	N/A	N/A
Median	74	86.5	97.5	98.5	79	88.5	90	76	101	77.5	65	59

N/A, not available; D, deceased

Table 4.9 Residual volume in paediatric lung transplant recipients expressed as percentage of predicted normal (RV%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	165	130	100	168	115	124	124	141	N/A	N/A	N/A	214
2	167	187	136	108	135	155	202	273	268	303	N/A	N/A
3	N/A	75	131	73	136	N/A	72	N/A	107	N/A	N/A	N/A
4	275	160	118	129	70	124	102	105	N/A	74	N/A	N/A
5	113	73	133	110	135	188	N/A	144	N/A	N/A	D	D
6	153	183	137	140	N/A	N/A	N/A	D	D	D	D	D
7	218	75	61	117	81	N/A	135	142	D	D	D	D
8	137	97	106	N/A	N/A	89	N/A	D	D	D	D	D
9	120	115	78	73	N/A	82	N/A	82	N/A	N/A	77	71
10	240	208	123	N/A	D	D	D	D	D	D	D	D
11	143	136	N/A	75	N/A	76	N/A	D	D	D	D	D
12	128	N/A	N/A	96	N/A	200	N/A	162	N/A	124	N/A	N/A
13	139	97	N/A	114	N/A	D	D	D	D	D	D	D
14	163	N/A	N/A	141	N/A	N/A	140	101	N/A	D	D	D
15	109	N/A	N/A	N/A	149	N/A	123	N/A	D	D	D	D
16	150	N/A	120	173	N/A	147	N/A	102	N/A	N/A	D	D
17	175	N/A	N/A	N/A	142	200	144	N/A	N/A	123	N/A	N/A
18	90	N/A	N/A	98	N/A	86	N/A	179	N/A	116	N/A	110
19	171	N/A	N/A	101	N/A	114	N/A	N/A	D	D	D	D
20	231	N/A	N/A	145	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	156	N/A	N/A	126	N/A	153	N/A	N/A	N/A	N/A	N/A	N/A
22	117	N/A	N/A	N	147	N/A	N/A	D	D	D	D	D
23	99	N/A	N/A	94	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	136	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Median	150	122.5	120	112	135	124	129.5	141.5	187.5	123	77	110

N/A, not available; D, deceased

Table 4.10 Standardised lung diffusing capacity of carbon monoxide in paediatric lung transplant recipients expressed as percentage of predicted normal (KCO%)

Subject No.	Time post transplantation (months)											
	pre	3	6	12	18	24	30	36	42	48	54	60
1	N/A	N/A	79	80	81	90	74	86	N/A	N/A	N/A	N/A
2	N/A	60	49	52	51	N/A	58	72	79	N/A	N/A	N/A
3	N/A	N/A	53	60	56	N/A	67	N/A	72	N/A	N/A	N/A
4	N/A	67	60	63	70	73	70	N/A	N/A	N/A	N/A	N/A
5	N/A	81	70	87	84	94	N/A	N/A	N/A	N/A	D	D
6	N/A	60	51	56	N/A	N/A	N/A	D	D	D	D	D
7	N/A	93	78	78	94	N/A	N/A	N/A	D	D	D	D
8	N/A	57	85	N/A	N/A	N/A	N/A	D	D	D	D	D
9	N/A	N/A	74	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A	D	D	D	D	D	D	D	D
11	N/A	N/A	N/A	78	N/A	83	N/A	D	D	D	D	D
12	N/A	N/A	N/A	67	N/A	N/A	N/A	N/A	N/A	116	N/A	N/A
13	N/A	N/A	N/A	71	N/A	D	D	D	D	D	D	D
14	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	D	D	D
15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	D	D	D	D
16	127	N/A	67	N/A	N/A	75	N/A	N/A	N/A	N/A	D	D
17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
18	N/A	N/A	N/A	78	N/A	73	N/A	N/A	N/A	N/A	N/A	N/A
19	N/A	N/A	N/A	57	N/A	N/A	N/A	N/A	D	D	D	D
20	N/A	N/A	N/A	81	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	N/A	N/A	N/A	85	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
22	N/A	N/A	N/A	N/A	N/A	N/A	N/A	D	D	D	D	D
23	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Median	127	63.5	68.5	74.5	75.5	74	68.5	79	75.5	116	-	-

N/A, not available; D, deceased

Figure 4.2 Graph of median and range of forced vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal (FVC%)

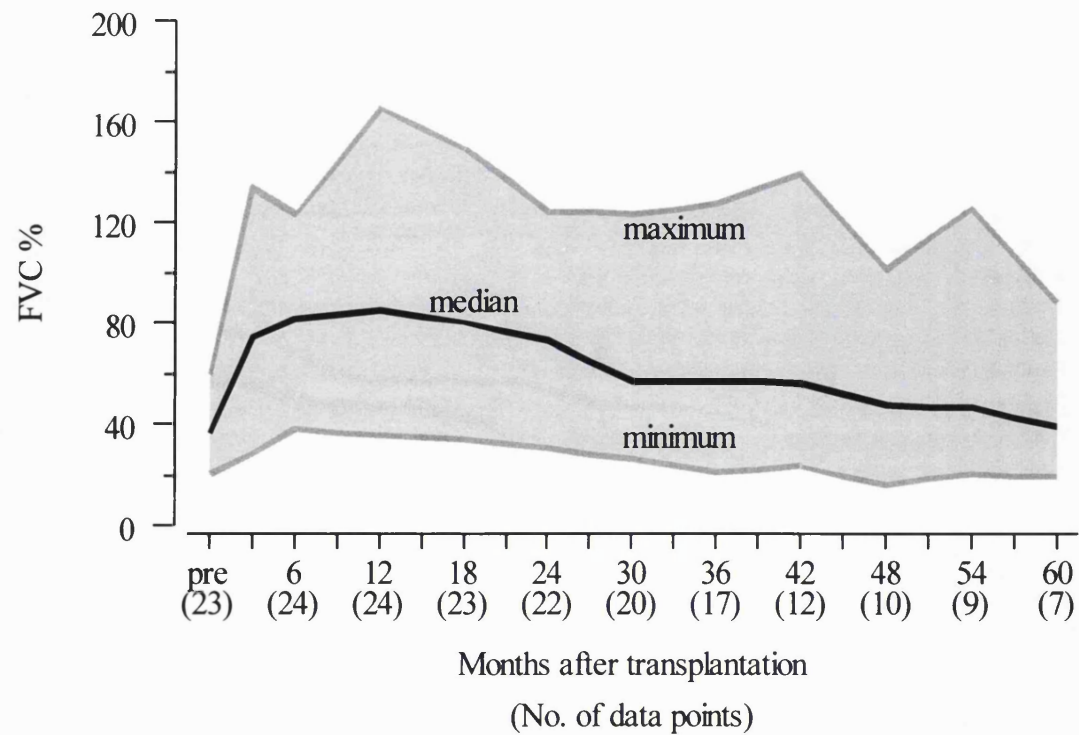


Figure 4.3 Graph of median and range of forced expiratory volume in one second in paediatric lung transplant recipients expressed as percentage of predicted normal (FEV₁%)

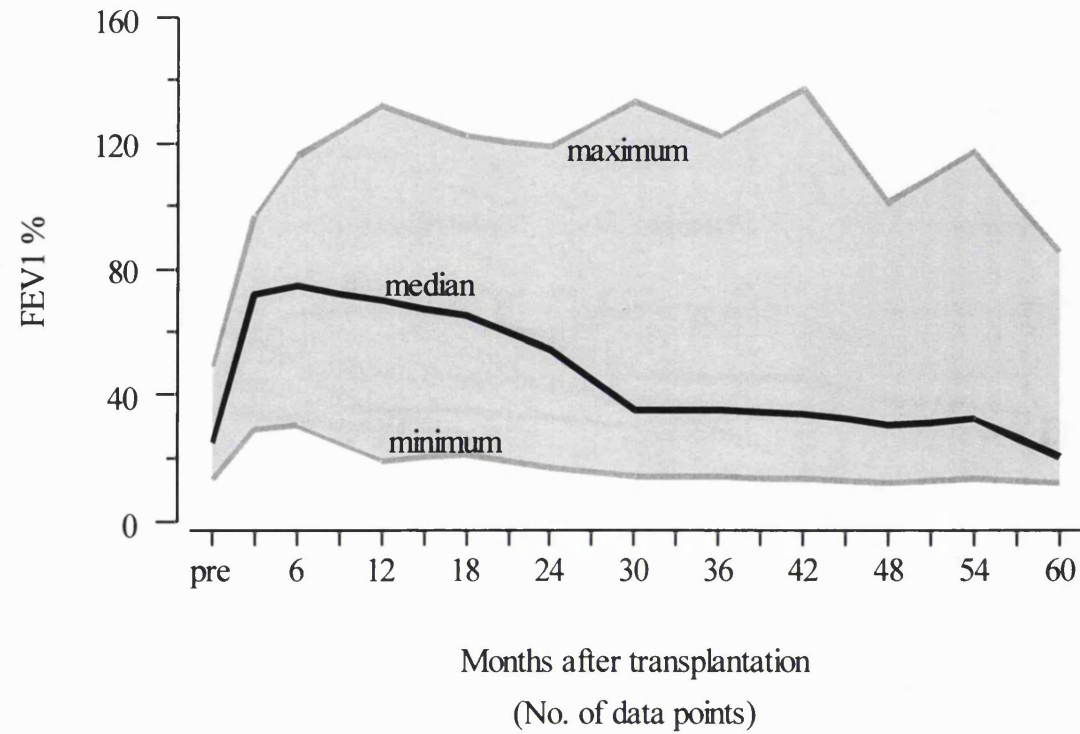


Figure 4.4 Graph of median and range of percentage ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC) in paediatric lung transplant recipients

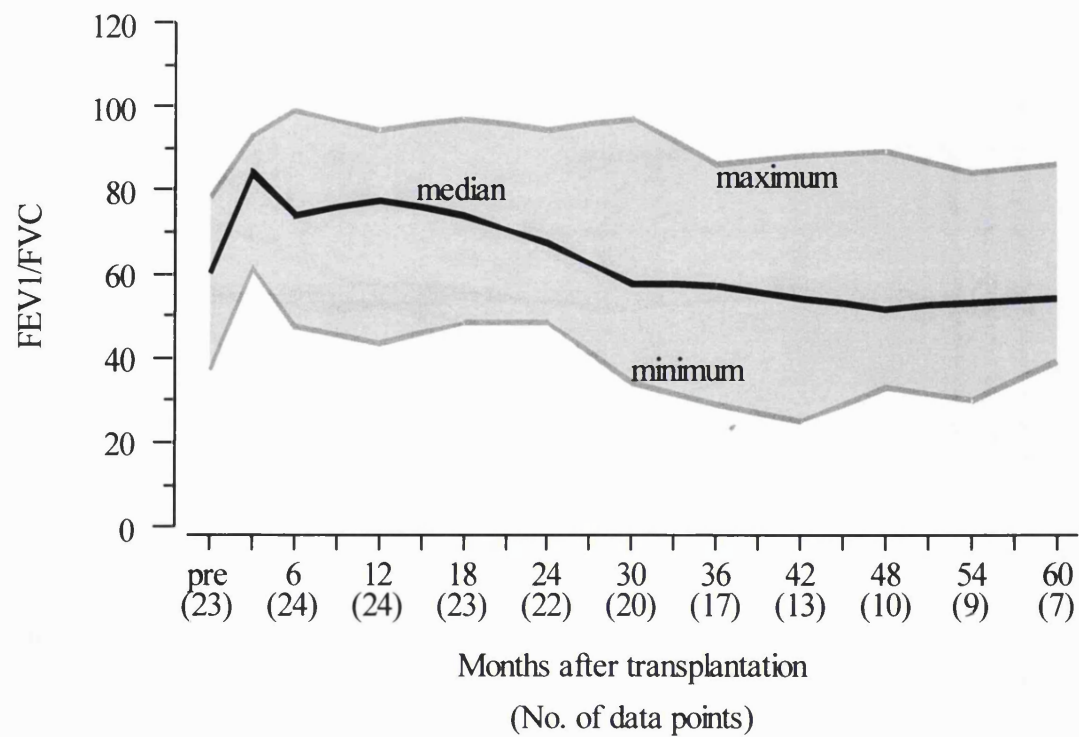


Figure 4.5 Graph of median and range of peak expiratory flow rate in paediatric lung transplant recipients expressed as percentage of predicted normal (PEFR%)

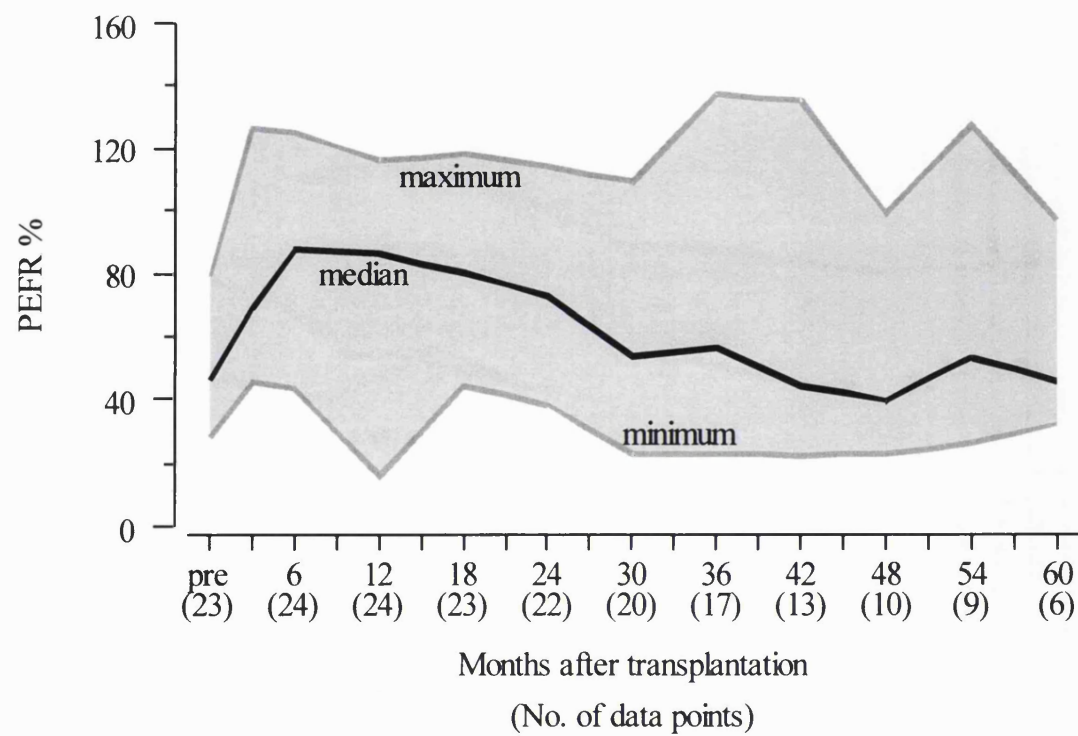


Figure 4.6 Graph of median and range of maximum expiratory flow at 25% of remaining vital capacity in paediatric lung transplant recipients expressed as percentage of predicted normal ($MEF_{25}\%$)

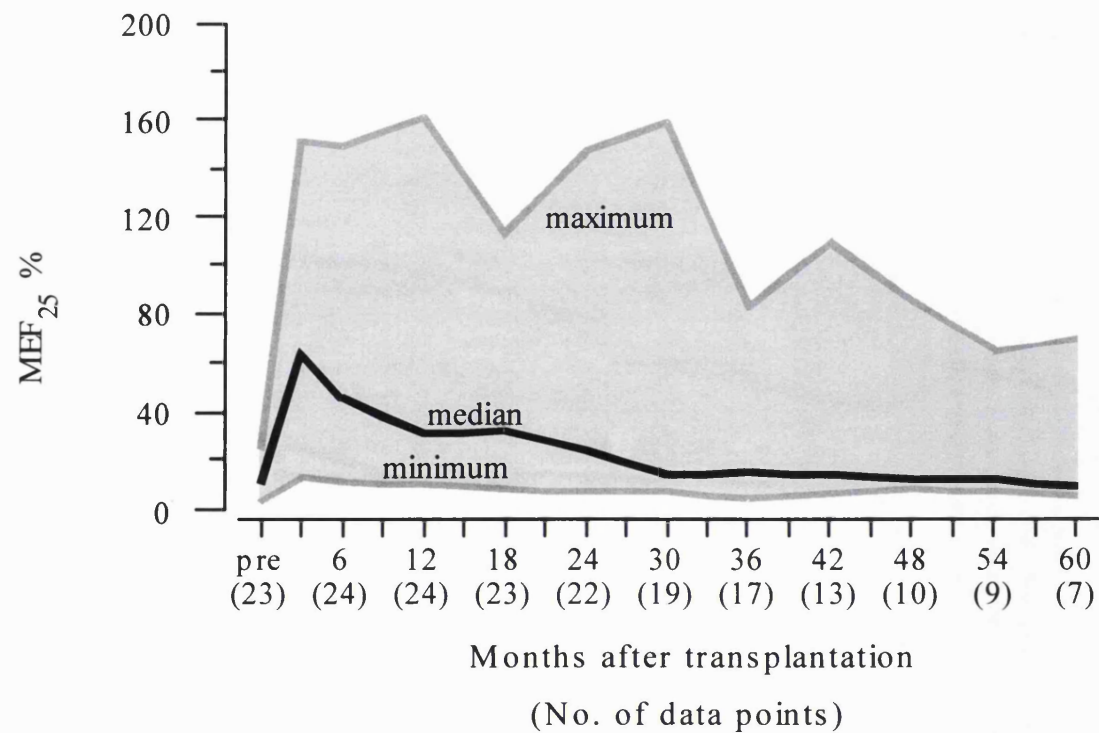


Table 4.11 **Significance values for differences in pulmonary function measurements at 6 – 60 months post transplant, compared with pre transplant values**

Pulmonary function parameter	Months post transplantation									
	6	12	18	24	30	36	42	48	54	60
FVC%	p<0.001	p<0.001	p<0.001	p<0.001	P<0.005	P<0.05	NS	NS	NS	NS
FEV ₁ %	p<0.001	p<0.001	p<0.001	p<0.001	p<0.05	NS	NS	NS	NS	NS
FEV ₁ /FVC	p<0.001	p<0.001	p<0.01	p<0.05	NS	NS	NS	NS	NS	NS
PEFR%	p<0.001	p<0.001	p<0.001	p<0.001	NS	NS	NS	NS	NS	NS
MEF ₂₅ %	p<0.001	p<0.001	p<0.001	p<0.001	p<0.05	NS	NS	NS	NS	NS
TLC%	NS	p<0.01	NS	NS	NS	NS				
RV%	p<0.05	p<0.01	NS	NS	NS	NS				

Significance values for differences assessed by Wilcoxon signed rank test.

NS, not significant

There were too few values for TLC% and RV% beyond 36 months post transplantation to enable statistical evaluation.

4.3.2 Area under the curve results

Area under the curve of FEV₁% measurements (FEV₁% AUC) from 3 months to 12, 18, 24, 30, and 36 months post transplant for each patient are detailed, together with FEV₁% AUC values for the second and third post transplant years (Table 4.12).

The FEV₁% AUC measurement was shown to be a useful instrument in comparing pulmonary function between patients following lung transplantation. Two examples of this are shown in Figures 4.7 and 4.8. In the first, two subjects (Subjects 4 and 18) were identified as having similar maximum FEV₁% values during the first year post transplant. However, there was a major difference when comparing their respective FEV₁% AUC values from 3 to 36 months post transplant (Figure 4.7). The second example shows two subjects (Subjects 5 and 19) with similar low FEV₁% measurements at 36 months post transplant. But again there was a substantial difference in their respective FEV₁% AUC values from 3 to 36 months post transplant (Figure 4.8). Both of these observations indicate two quite different post transplant clinical courses, which may not be apparent when only reviewing isolated FEV₁% measurements.

There was a trend in the correlation between FEV₁% AUC values and survival after the third post-transplant year (Figure 4.9), although there were too few data for formal statistical analysis. In essence, the data suggested that the higher the FEV₁% AUC value to 36 months, the longer the survival will be after 36 months post transplant. Interestingly, the subjects remaining alive tended to be clustered in the upper right hand end of the graph, indicating both increased FEV₁% AUC values and survival.

Significance values for the correlation of FEV₁% AUC with lower respiratory infections in the first 12 months post transplant, acute rejection episodes in the first 12 months post transplant, graft ischaemic time – cold and total, age of recipient at transplant, and pre transplant FEV₁% are shown in Table 4.13. Cold graft ischaemic time was negatively correlated with FEV₁% AUC from 3 to 18, 30, and 36 months

as well as with FEV₁% AUC for the second year post transplant. There was also a negative correlation observed for total graft ischaemic time and FEV₁% AUC from 3 to 12, 18, 30, and 36 months respectively, in addition to that for the second post transplant year.

Graphs depicting the correlation between FEV₁% AUC from 3 to 36 months and graft cold ischaemic time and graft total ischaemic time are shown in Figures 4.10 & 4.11 respectively. Graphs depicting the correlation between FEV₁% AUC in the second post transplant year and graft cold ischaemic time and graft total ischaemic time are shown in Figures 4.12 & 4.13 respectively.

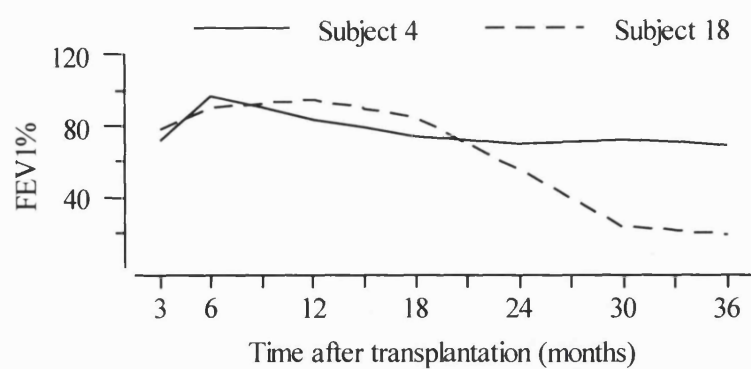
There was a significant negative correlation between age of recipient at time of transplant and FEV₁% AUC from 3 to 30 months and the second post transplant year (Figure 4.14). There was no correlation between FEV₁% AUC and the number of lower respiratory infections in the first 12 months, acute rejection episodes in the first 12 months or pre-transplant FEV₁% at any time point or epoch post-transplant (Table 4.13).

Table 4.12 **Area under the curve of FEV₁% measurements (FEV₁% AUC)**
for each subject from 3 months to 12, 18, 24, 30, and 36 months
post transplant and for the second and third post transplant
years

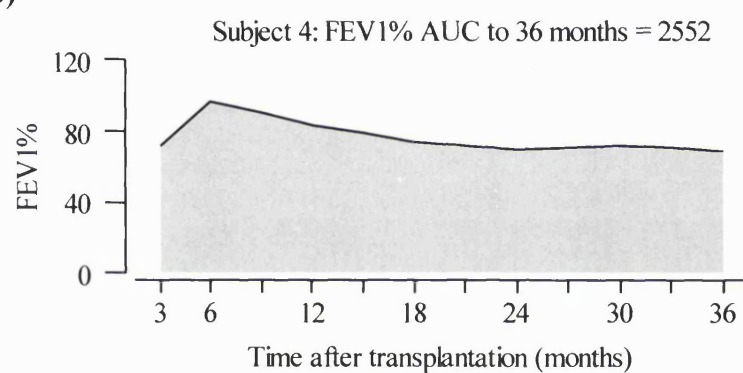
Subject No.	Total FEV ₁ % AUC from 3 months post transplantation to...						Total FEV ₁ % AUC from:	
	12 months	18 months	24 months	30 months	36 months	42 months	12 to 24 months post tx	24 to 36 months post tx
1	743	1085	1476	1850	2180	2462	732	705
2	813	1374	1770	1986	2112	2205	957	342
3	930	1686	2325	2997	3762	4539	1395	1437
4	797	1271	1703	2129	2552	2939	906	849
5	599	827	998	1145	1292	1418	399	294
6	1053	1812	2532	2958	D	D	1479	D
7	707	1025	1313	1541	1751	D	606	438
8	611	974	1205	1379	D	D	594	D
9	524	851	1196	1577	1892	2219	672	696
10	348	D	D	D	D	D	D	D
11	420	771	1104	1434	D	D	684	D
12	696	1251	1587	1794	2025	2268	891	438
13	789	1332	D	D	D	D	D	D
14	534	912	1323	1695	1980	2151	789	657
15	432	636	792	912	1005	D	360	213
16	714	1017	1335	1596	1776	1932	621	441
17	608	1088	1610	2159	2654	3152	1002	1044
18	812	1352	1772	2006	2132	2255	960	360
19	815	1415	2015	2468	2699	D	1200	684
20	608	1082	1457	1673	1814	1919	849	357
21	557	926	1298	1727	2090	2321	741	792
22	269	389	503	596	D	D	234	D
23	458	767	1010	1196	1385	N/A	552	375
24	713	1277	1850	N/A	N/A	N/A	1137	N/A

N/A, not available
D, deceased

(a)



(b)



(c)

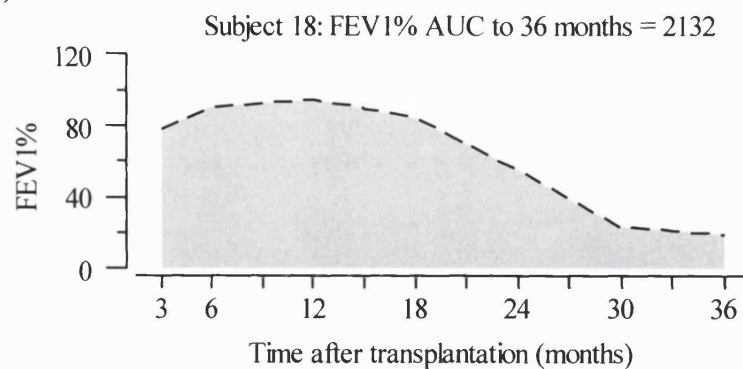


Figure 4.7 (a) Graph of FEV₁% for two subjects (4 and 18) during the first 36 months post transplant. Despite similar FEV₁% values over the first 12 months, FEV₁% AUC values from 3 to 36 months were markedly different for each subject (b) and (c)

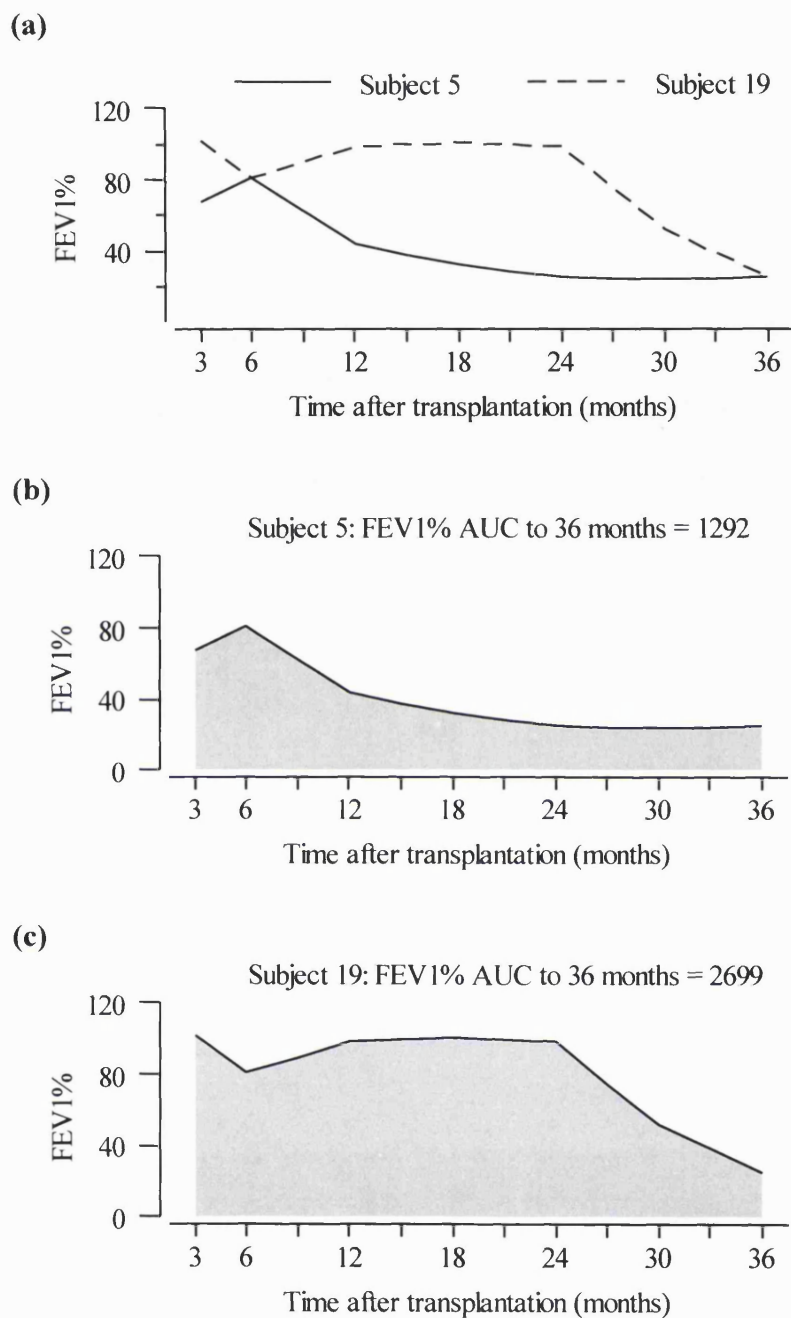
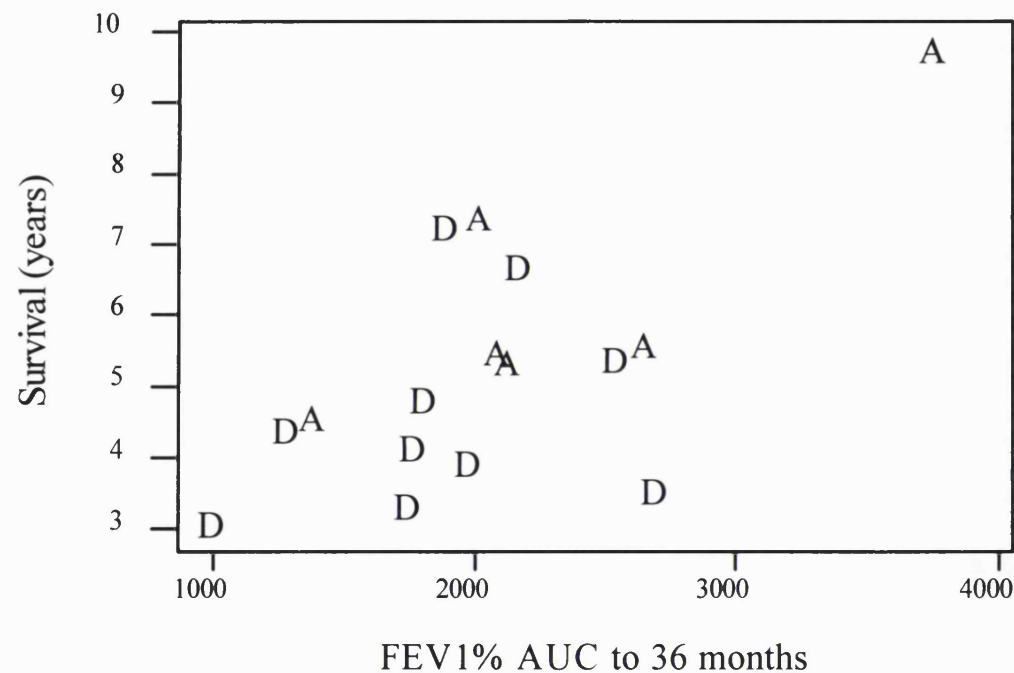


Figure 4.8 (a) Graph of FEV₁% for two subjects (5 and 19) during the first 36 months post transplant. Despite similar FEV₁% values at 36 months, FEV₁% AUC values from 3 to 36 months were markedly different for each subject (b) and (c)

Figure 4.9 Relationship between post transplant survival (from 3 years) and FEV₁% AUC from 3 to 36 months post transplant



D indicates deceased patients, while A indicates those still alive at the end of the study. There is a trend between survival and FEV₁% AUC although formal statistical evaluation was not possible. Note the clustering of live subjects with higher FEV₁% AUC values (in the upper right hand section of the graph).

Table 4.13 Significance values for correlations between FEV₁% AUC and other parameters

	Total FEV ₁ % AUC from 3 months post- transplant to:					Total FEV ₁ % AUC from	Total FEV ₁ % AUC from
	12 months	18 months	24 months	30 months	36 months	12 to 24 months	24 to 36 months
INF	NS	NS	NS	NS	NS	NS	NS
REJ	NS	NS	NS	NS	NS	NS	NS
GIT-C	NS	p<0.05*	NS	p<0.05*	p<0.05*	p<0.05*	NS
GIT-T	p<0.05*	p<0.05*	NS	p<0.05*	p<0.01*	p<0.05*	NS
Age	NS	NS	NS	p<0.05*	NS	p<0.05*	NS
Pre-Tx FEV₁%	NS	NS	NS	NS	NS	NS	NS

FEV₁% AUC, area under the curve for FEV₁% (see text for details of derivation).

INF, number of lower respiratory infections in first 12 months post transplant.

REJ, number of rejection episodes in first 12 months post transplant.

GIT-C, cold graft ischaemic time.

GIT-T, total graft ischaemic time.

Age, recipient's age at time of transplant.

NS, not significant

* Negative correlation

Figure 4.10 Relationship between graft cold ischaemic time (GIT-C) and FEV₁% AUC from 3 to 36 months post transplantation with regression line ($p < 0.05$)

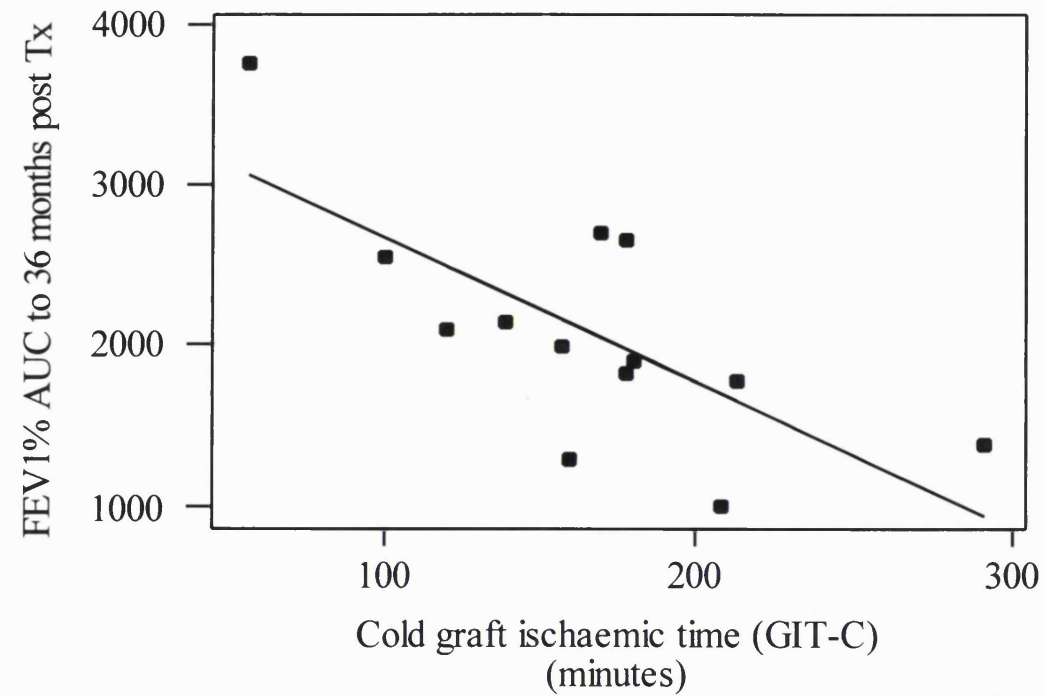


Figure 4.11 Relationship between graft total ischaemic time (GIT-T) and FEV₁% AUC from 3 to 36 months post transplantation with regression line ($p < 0.01$)

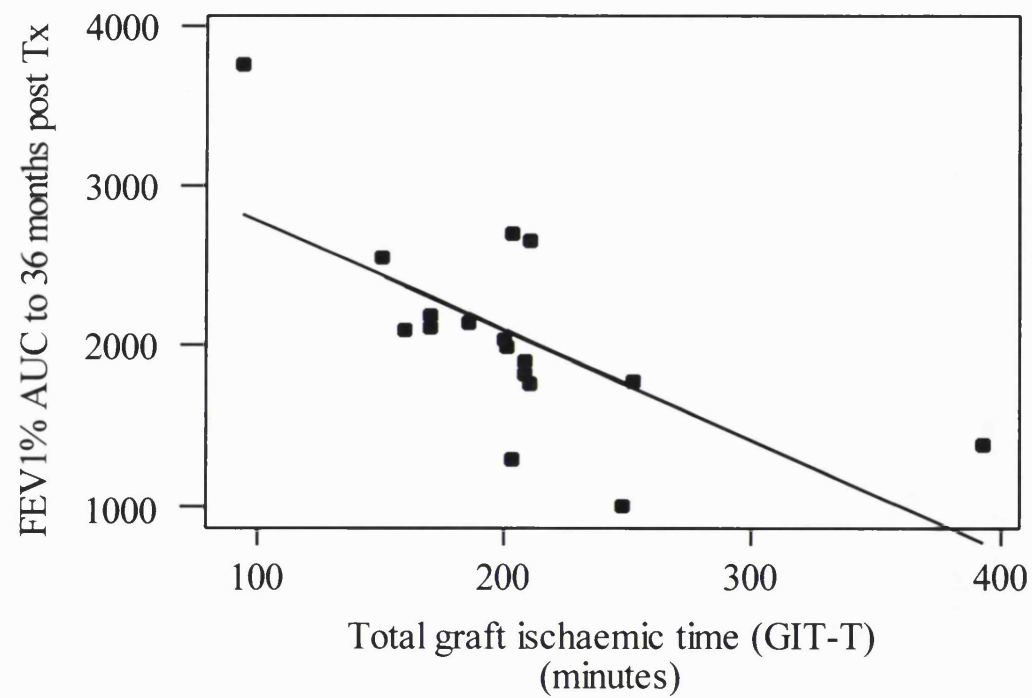


Figure 4.12 Relationship between graft cold ischaemic time (GIT-C) and FEV₁% AUC in second year post transplantation with regression line ($p < 0.05$)

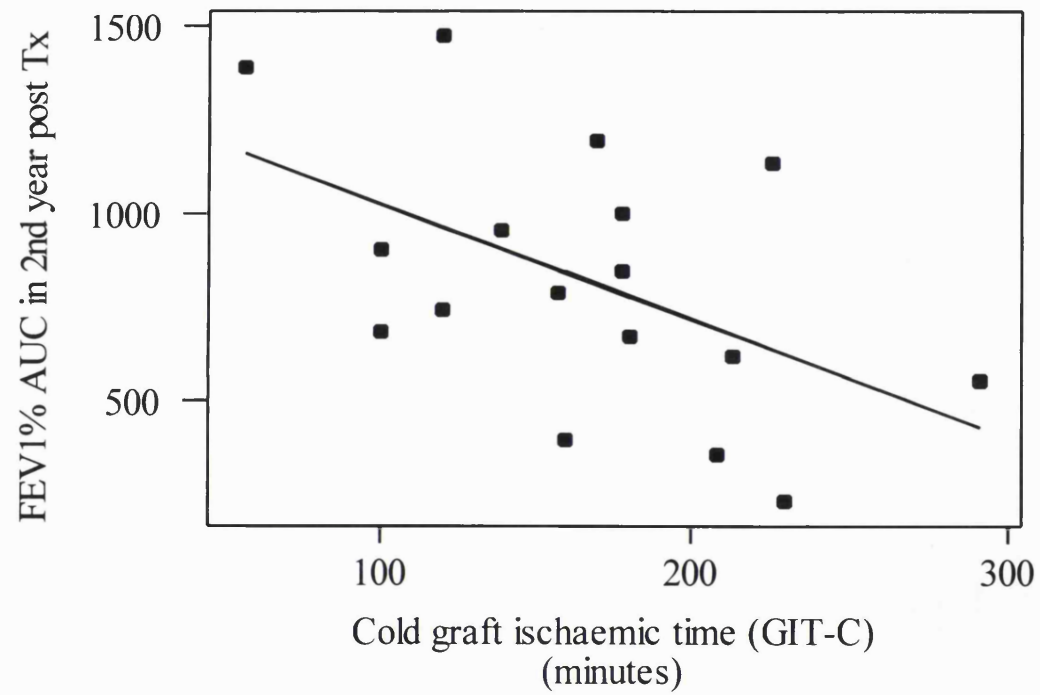


Figure 4.13 Relationship between graft total ischaemic time and FEV₁% AUC in the second year post transplantation with regression line($p<0.05$)

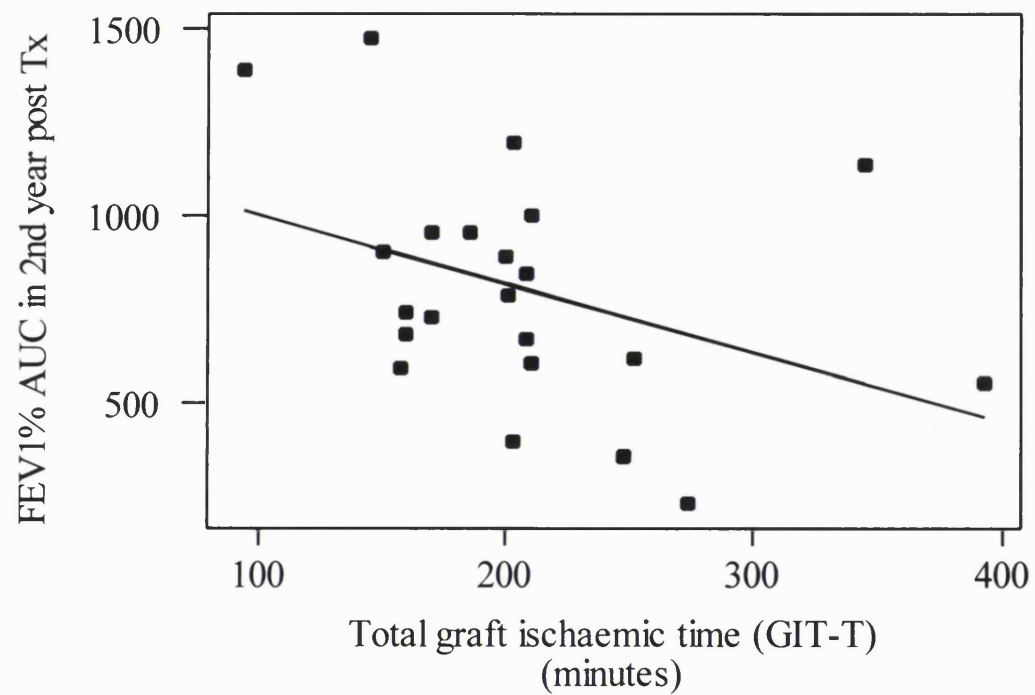
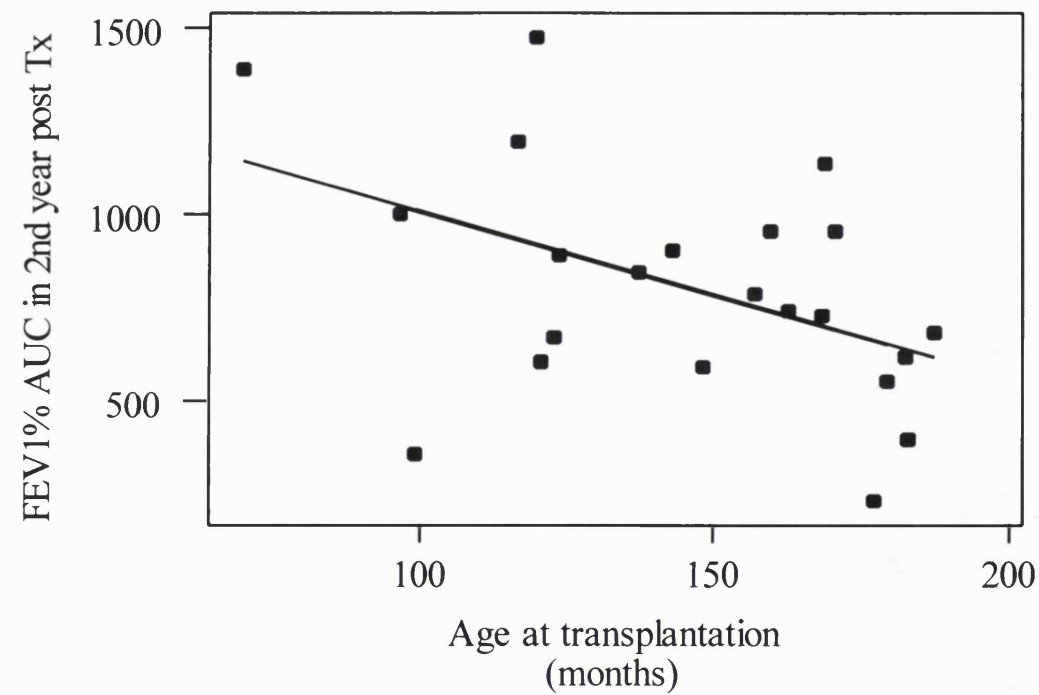


Figure 4.14 Relationship between recipient age at time of transplantation and FEV₁% AUC in the second year post transplantation with regression line ($p < 0.05$)



4.4 Discussion

This study represents one of the first comprehensive reviews of pulmonary function in children following lung transplantation. It not only describes lung function in the early post transplant period, but also that found in the longer-term paediatric lung transplant survivor. Although similarities in patterns of post transplant lung function exist between adults and children, the latter are a unique group in that other issues are involved, such as somatic and lung growth, and these may influence pulmonary function.

In order to standardise the study, relatively stringent entry criteria were developed. An age of less than 16 years at time of transplant was considered appropriate to incorporate a true paediatric cohort. Obviously, subjects were required to be capable of performing formal pulmonary function tests with reproducible results. A post transplant survival of at least one year was thought necessary to enable the development of an established pattern of pulmonary function. Patients with significant airway stenosis were excluded to avoid the confusion that this structural defect might generate on airflow values. Only those subjects with an original diagnosis of cystic fibrosis were included. This allowed standardisation of pre-transplant baseline lung function and the avoidance of confounding factors that could be related to the original disease process. In reality, only two non cystic fibrosis patients, who fulfilled the other entrance criteria, were excluded from the study.

Consistent with previously reported adult data (Theodore 1984), a restrictive defect was evident in this paediatric cohort as demonstrated by an overall reduction in forced vital capacity (FVC%) measurements. Maximum values were attained by 12 months post transplantation, well after the expected surgical effects on the alteration of chest wall mechanics had passed. However, there was a persistent mild restrictive defect for the group as indicated by a median FVC% of 85% at 12 months post transplant. In fact, this restrictive defect showed evidence of progression to the

severe category with a median FVC% of only 39% in subjects at 60 months post transplant.

Commensurate with the mild restrictive pattern early post transplant, was a reduction in forced expiratory volume in one second ($FEV_1\%$). The peak median value (75%) was attained at three months post transplant. However, there was an even greater deterioration in this parameter over time leading to a median nadir of 24% by 48 months post transplant. The falling FEV_1/FVC ratio thus produced indicated an increasing obstructive defect. Therefore, the end result was a severe mixed restrictive and obstructive defect.

Smaller airway flows, as defined by the $MEF_{25}\%$ values, remained at generally low levels, with a maximum attained at 3 months post transplant (63%). Following this there was a persistent decrease in values with a median value of only 9% at 60 months. These results indicate that obstruction of the smaller airways became a prominent feature in the transplanted lungs in this paediatric cohort.

All dynamic pulmonary function measurements remained significantly different to pre-transplant values up to 24 months post transplant. Subsequently, with the development of further obstruction and, to a lesser extent, restriction, these differences became non-significant. Disappointingly, by 42 months post transplant, all parameters had fallen to pre-transplant levels.

This pattern of progressive airflow obstruction appears to denote the development of obliterative bronchiolitis in the transplanted lung (Burke 1985, Patterson 1996). The pulmonary function abnormality may be the only clinical manifestation of this complication due to difficulties associated with obtaining an histopathological diagnosis (Kramer 1993b). As described above, obliterative bronchiolitis has been further classified as bronchiolitis obliterans syndrome, which incorporates staging by reductions in FEV_1 from baseline values (Cooper 1993). It is difficult to apply this classification in children, as with somatic growth there is a concomitant increase in baseline FEV_1 . Thus, it may be more correct to use sequential FEV_1

measurements as a percentage of predicted normal ($FEV_1\%$) to define the development of obliterative bronchiolitis. An $FEV_1\%$ value which had fallen to less than 40% has been used previously to define obliterative bronchiolitis (Whitehead 1994, Scott 1991).

Although FEF_{25-75} , as a measure of smaller airway flows, has been proffered as a more sensitive measure for diagnosing obliterative bronchiolitis (Patterson 1996), this appears to be a difficult measurement to interpret in children, as evidenced by this study. As the median $MEF_{25}\%$ value was only 63% at three months post transplant, decreasing to less than 40% at 12 months, there may be an overestimation of the incidence of obliterative bronchiolitis using the criteria developed by Patterson and colleagues. By the above-mentioned $FEV_1\%$ criteria, the incidence of obliterative bronchiolitis would be approximately 50% at 30 months post transplant.

The method of assessing serial lung function by the use of area under the curve of $FEV_1\%$ ($FEV_1\%$ AUC) estimation has not previously been evaluated. As demonstrated in the results section, the FEV_1 AUC is a useful means of determining an overall value of post transplant lung function and aids in inter-subject comparisons. Given that pulmonary function, namely FEV_1 , is now used to determine the onset and severity of obliterative bronchiolitis, $FEV_1\%$ AUC values may be another method of determining this disease process. In children, this latter method may in fact be superior given the problems discussed above. This value may also “smooth out” some of the inconsistencies in lung function, which have been observed, eg: the graph of $FEV_1\%$ (Figure 4.3).

There were some interesting observations when comparing $FEV_1\%$ AUC with other parameters previously associated with the development of obliterative bronchiolitis. Early recurrent pulmonary allograft rejection episodes have featured as one of the major associations with the onset of obliterative bronchiolitis in many reports (Scott 1991, Yousem 1991, Whitehead 1994). However, in this current analysis, no correlation was found between $FEV_1\%$ AUC and the number of rejection episodes in

the first post transplant year. Similarly, the number of pulmonary infective episodes in the first post transplant year showed no correlation with FEV₁% AUC.

It has been proposed that pre-transplant clinical status has little impact upon post transplant outcome (Gassas 1999). This proposal has been supported by the current data with the observation of a lack of correlation between FEV₁% AUC and pre-transplant FEV₁%. In effect, this indicates that children with a greater reduction in lung function pre-transplant do not have a discernibly worse post transplant outcome, at least as measured by pulmonary function.

One published paper has indicated an increased incidence of obliterative bronchiolitis in the younger aged paediatric lung transplant recipient (Radley-Smith 1995), while another showed no difference in the incidence of obliterative bronchiolitis between those recipients aged over and under 10 years (Balfour-Lynn 1997). In the current analysis younger recipients demonstrated superior pulmonary function, as measured by FEV₁% AUC during the second transplant year, indicating an improved outcome for the younger child.

Of greater surprise was the correlation between graft ischaemic time, both total and cold, with post transplant lung function as assessed by FEV₁% AUC (Table 4.13). There have been no previous reports documenting an association between graft ischaemic time and post transplant lung function or the development of obliterative bronchiolitis. This correlation is an important observation, made even more fascinating by the fact that the correlation persists well into the post transplant course. It suggests that early lung injury caused by graft ischaemia has long term effects on pulmonary function, possibly by injury to the smaller airways. This observation may have considerable implications, especially as graft ischaemic times are being pushed to the limit in the hunt for elusive donor organs.

Chapter 5 *In vivo* gene transfer in the rat lung

5.1 Introduction

5.2 Methods

- 5.2.1 General overview
- 5.2.2 Peptide gene vector
- 5.2.3 Reporter genes
- 5.2.4 Chloroquine, fusogenic peptide, and Lipofectamine
- 5.2.5 Preparation of reporter gene and vector
- 5.2.6 Tracheal intubation and pulmonary instillation in the rat
- 5.2.7 Harvesting of rat lungs
- 5.2.8 Processing of rat lungs
- 5.2.9 X-GAL staining of whole lungs and small lung cubes
- 5.2.10 Preparation of lungs for frozen section
- 5.2.11 X-GAL staining of frozen sections
- 5.2.12 Antibodies for immunostaining
- 5.2.13 Immunohistological staining

5.3 Results

- 5.3.1 Intratracheal instillation of the rat lung
- 5.3.2 Phase 1 experimental results
 - 5.3.2.1 Studies with small cubes of lung tissue
 - 5.3.2.2 X-GAL stained histological and frozen sections
- 5.3.3 Phase 2 experimental results
 - 5.3.3.1 Human Thy-1 studies
 - 5.3.3.2 High dose β -GAL studies
 - 5.3.3.3 Vector distribution studies
 - 5.3.3.4 Inflammatory responses

5.4 Discussion

5.1 Introduction

To enable gene therapy to be successfully applied to solid organ transplantation, the first priority is the development of an efficient vector system capable of transferring genes to the organ of interest. In the case of lung transplantation, direct application of the desired gene and vector complex to the transplanted lung can be achieved, relatively simply, via the airway. However, many vector systems, particularly viral vectors, have inherent problems including immunogenic activity, which can induce inflammatory reactions within the lung. These reactions can be detrimental in their own right as well as leading to reduced gene transfer efficiency.

As discussed in Chapter 3, integrin-targeted non-viral vectors show promise as an efficient and less toxic system than the viral vectors in current use. The peptide vectors containing the arginine-glycine-aspartic acid (RGD) motif have generated considerable interest. In our own laboratory, the development of the polylysine-molossin vector, which contains the RGD motif, has produced exciting results *in vitro*, with efficient gene transfection demonstrated in a number of cell lines (Shewring 1997, Collins 2000). In view of the success of *in vitro* gene transfection modulated by the polylysine-molossin vector, similar methodology was developed for an *in vivo* experimental lung model as a prelude to its application in lung transplantation.

The study plan comprised the instillation of reporter gene with vector into the native lungs of rats in various combinations. The main research questions were:

- Can an RGD containing synthetic peptide gene vector transfer DNA to intact respiratory epithelium and alveoli in the rat lung?
- What is the distribution of the vector/DNA complexes within the rat lung?
- Do the vector/DNA complexes produce an inflammatory response in the lung?

5.2 Methods

5.2.1 General overview

Utilising methods developed in the *in vitro* experimental protocol, reporter genes were directly administered into the lungs of male DA rats in combination with the polylysine-molossin vector. Variations in the amount of DNA administered were assessed as well as different ratios of DNA to vector. Other factors were also evaluated, including pre-treatment with chloroquine.

Evaluation of gene expression was made after *en-bloc* staining and immunohistological staining, depending on the reporter gene used. The initial reporter gene used was the β -galactosidase gene. However, due to difficulties in differentiating between endogenous and exogenous β -galactosidase in the lung, the marker gene was changed to the human Thy-1 gene. Thy-1 gene expression could then be identified by using a specific monoclonal antibody to human Thy-1.

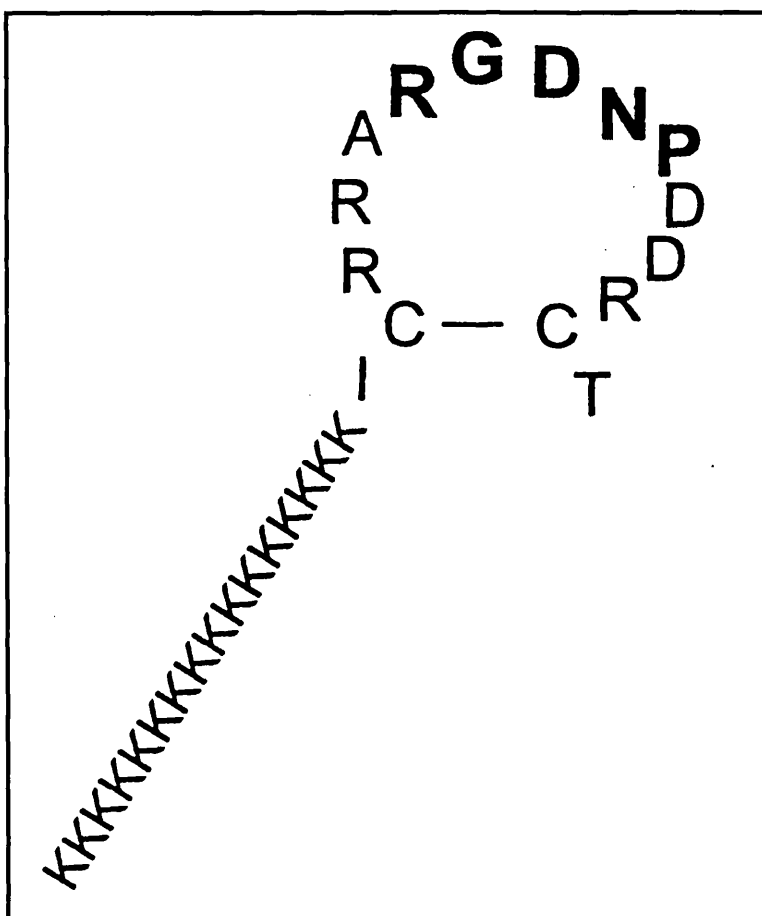
Studies of the distribution of the gene vector within the lung were also performed. These incorporated immunostaining with a specific anti-vector (anti-molossin) antibody.

5.2.2 Peptide gene vector

A synthetic 31-amino acid peptide vector was used in this study. This comprised a 15-amino acid peptide encompassing the RGDNP motif of the integrin-binding domain of the venom, molossin (from the American Pit Viper *C. molossus molossus*), which was synthesised with a 16-lysine chain at the amino terminus as illustrated in Figure 5.1. This has been referred to as polylysine-molossin (Poly-Mol) and has a molecular weight of 3,797 Daltons (Collins 2000). Synthesis, cyclisation, and purification were performed by Cambridge Research Biochemicals (Northwich, Cheshire, UK).

Figure 5.1 Polylysine-molossin (Poly-Mol) gene vector

The integrin-binding loop of molossin (targeting moiety, 13 amino acids) was synthesised with two flanking amino acids and a 16-lysine chain at the amino terminus (DNA-binding moiety). The RGDNP motif is shown in bold.



5.2.3 Reporter genes

In initial experiments, the CMV- β plasmid containing the β -galactosidase gene of *Escherichia Coli* under the control of the CMV promoter (Clontech, Palo Alto, CA, USA) was used. This was propagated and purified under endotoxin-free conditions, as previously described (Shewring 1997). In later experiments, the human Thy-1 gene was used in a reporter system used in our laboratory.

5.2.4 Chloroquine, fusogenic peptide, and Lipofectamine

In phase 1 experiments, chloroquine was freshly prepared and added to the DNA and vector complex at a concentration of 500 μ M in the tracheal instillate (rats P8, P9, P13). In phase 2 experiments, chloroquine was administered intra-peritoneally at a dose of 17mg three hours prior to the tracheal instillation of DNA and vector (rats P20 and P21).

Fusogenic peptide was synthesised by Cambridge Research Biochemicals (Northwich, Cheshire, UK). The sequence of the peptide was: NH₂-gly-leu-phe-glu-ala-leu-leu-glu-leu-leu-glu-ser-leu-trp-glu-leu-leu-leu-glu-ala-CO₂H. It was used at a concentration of 10 μ g/mL in phase 1 experiments and 20 μ g/mL in phase 2 experiments.

Lipofectamine (Gibco, Paisley, UK) was only added to the DNA/vector complex in phase 2 experiments at a concentration of 20 μ g/mL.

5.2.5 Preparation of reporter gene and vector

Instillation of the plasmid β -galactosidase (β -GAL) reporter gene was used in the Phase 1 experiments. As discussed in Chapter 3, β -GAL is a commonly used reporter gene in studies assessing gene delivery systems. However, endogenous β -galactosidase is expressed in some tissues including the gut and lung and it can be

difficult to differentiate between exogenously and endogenously produced β -galactosidase activity. Although methods have been developed to enhance identification of the bacterial form, mainly by adjustment of pH during processing (Weiss 1997), an element of doubt often remains. Accordingly another reporter gene, human Thy-1, was used in latter (Phase 2) experiments.

Various combinations of the gene vector, polylysine-molossin (Poly-Mol), and Lipofectamine, fusogenic peptide and chloroquine were evaluated. The combinations and quantities used reflected those that yielded the optimum conditions for gene transfection in previously performed *in vitro* experiments (Collins 2000a).

Tables 5.1 and 5.2 identify the components of tracheal instillate for each of the experiments performed. These have been separated into the different treatment groups. Table 5.1 relates to Phase 1 experiments using β -GAL as the gene marker, while table 5.2 relates to Phase 2 experiments using Thy-1 gene marker (except for two cases). Rats used in the experiments are identified by the letter P (for pulmonary) and a number (1-13, Phase 1 and 20-31, Phase 2).

In Phase 1 experiments, rats P1 and P2 were instilled with plasmid DNA only, P3-P6 plasmid DNA plus Poly-Mol vector, P7 and P12 were negative controls with normal saline only administered. P8, P9 and P13 received plasmid DNA, Poly-Mol vector and chloroquine, while P10 and P11 received plasmid DNA, Poly-Mol vector and fusogenic peptide.

The ratio of plasmid DNA to Poly-Mol vector in this group of experiments was 1:3, representing the optimum ratio determined in the *in vitro* experiments. The required amount of DNA was diluted to 10 μ g/mL in 199 medium (without foetal calf serum) in a 15mL Falcon tube. The appropriate volume of Poly-Mol, from 1mg/mL stock solutions in normal saline, was added dropwise to the DNA solution while vortexing, to yield the 1:3 ratio. The resulting solution was allowed to stand at room

temperature for 30 minutes. Where relevant, the fusogenic peptide or chloroquine was added and the final solution instilled into the rat lung within one hour.

In Phase 2 experiments, in addition to the change in reporter gene used (human Thy-1), the ratio of DNA to vector was also altered to 1:5. This ratio also yielded good results in the *in vitro* arm of the study. Lipofectamine was also added to the instillate, as it had been shown to promote gene delivery (Hart 1998). Animals P20 and P21 were pretreated with chloroquine, which was administered intraperitoneally three hours prior to the tracheal instillation of plasmid DNA and Poly-Mol vector. P22 and P23 were given plasmid DNA and Poly-Mol vector only, while P24, P25, P31 and P32 received a suspension of plasmid DNA, Poly-Mol vector, fusogenic peptide and Lipofectamine in the ratio of 1:5:2:2 respectively.

Rat P26 was given normal saline only as another negative control. High dose Thy-1 plasmid DNA, in combination with Poly-Mol vector, Lipofectamine and fusogenic peptide, was given to rats P27 and P28. P29 and P30 received high dose β -GAL together with the other listed components.

The protocol for combining the DNA and vector was similar to that for the Phase 1 experiments, although on this occasion the required amount of DNA (10 μ g/mL) was mixed with Poly-Mol in a ratio of 1:5. When Lipofectamine was used, it was added to the solution after mixing with a portion of the DNA and prior to the addition of the fusogenic peptide.

Table 5.1 Components of tracheal instillate for *in vivo* gene transfer – Phase 1

Rat number	Gene marker: β-GAL^a	Poly-Mol	Fusogenic peptide	Chloroquine	Total volume (N/S)^b
P7, P12 Negative control	-	-	-	-	300 μ L
P1, P2	3 μ g (10 μ g/mL) ^c	-	-	-	300 μ L
P3, P4, P5, P6	3 μ g	9 μ g (30 μ g/ml)	-	-	300 μ L
P8, P9, P13	3 μ g	9 μ g	-	500 μ M ^d	300 μ L
P10, P11	3 μ g	9 μ g	3 μ g (10 μ g/mL)	-	300 μ L

^a β -galactosidase (β -GAL) gene.

^b Total volume of instillate prepared with normal saline (N/S).

^c Concentration of compound in tracheal instillate.

^d Chloroquine administered at a concentration of 500 μ M in the tracheal instillate.

Table 5.2 **Components of tracheal instillate for *in vivo* gene transfer – Phase 2**

Rat number	Gene marker: Thy-1^a	Poly-Mol	Fusogenic peptide	Chloro-quine	Lipo-fectamine	Total volume (N/S)^b
P26 Negative control	-	-	-	-	-	300µL
P20, P21	3µg (10µg/mL) ^c	15µg (50µg/mL)	-	17mg I/P ^d	-	300µL
P22, P23	3µg	15µg	-	-	-	300µL
P24, P25 P31, P32	3µg	15µg	6µg (20µg/mL)	-	6µg (20µg/mL)	300µL
P27, P28	30µg (100µg/mL)	150µg (150µg/mL)	6µg	-	6µg	300µL
P29, P30	β-GAL ^e 30µg	150µg	6µg	-	6µg	300µL

^a Human Thy-1 gene marker.

^b Total volume of instillate prepared with normal saline (N/S).

^c Concentration of compound in tracheal instillate.

^d Chloroquine given by intraperitoneal (I/P) injection 3 hours prior to intratracheal instillation.

^e β-GAL gene marker used in place of Thy-1 marker.

5.2.6 Tracheal intubation and pulmonary instillation in the rat

Male DA rats, aged between 8-12 weeks, were supplied by Harlon Olac (Bicester, Oxon, UK). Adapting previously reported techniques of tracheal intubation in the rat (Thet 1983, Costa 1986), a suitable and practical method was developed. Firstly, a paediatric laryngoscope was modified by removal of the blade, leaving the fiberoptic light source intact. The latter was used as a surrogate blade to enable visualisation of the larynx. Intubation was effected with the use of a plastic intravenous cannula, which facilitated continuous administration of gaseous anaesthesia as well as instillation of liquid test substances. The sheath of a 16 or 18 gauge Abbocath (Abbot, UK) was used and inserted through the vocal cords under direct vision afforded by the laryngoscope.

The method was first assessed on an euthanased rat to evaluate positioning of the endotracheal cannula. A young female DA rat was culled by inhalation of carbon dioxide (Schedule 1). The deceased rat was placed supine on a metal plate, with all four limbs taped to the plate. A rubber band attached to the top-end undersurface of the plate was then passed under the upper incisors of the rat to hold the mouth open, while an assistant held the tongue to one side with blunt forceps. The larynx and vocal cords were then directly visualised by the operator with the use of the laryngoscope and the cannula inserted. Three hundred microlitres of water were instilled followed by 200 μ L of air to expel the fluid in the dead space of the cannula. There appeared to be a small amount of efflux of this fluid up past the cannula.

The thorax of the rat was then dissected and the position of the cannula assessed. Even with the 51mm long cannula fully inserted through the mouth, the tip of the cannula remained above the bifurcation of the trachea, indicating that any instilled fluid would be distributed to both lungs.

Under experimental conditions with live animals, the procedure was as follows:

1. DA rat placed in anaesthetic chamber and anaesthetised with 4% isoflurane and 4L/min oxygen.
2. Rat removed from chamber and placed supine onto metal plate (with underlying warming bed), all limbs taped down onto plate while maintained under gaseous anaesthesia (isoflurane 4% and 0.2 L/min oxygen) via a face mask.
3. Mouth immobilised with rubber band and tongue held clear (as above), laryngoscope inserted into mouth and positioned to visualise vocal cords.
4. 16 or 18 gauge Abbocath cannula inserted through vocal cords and into trachea.
5. Intratracheal position of cannula assessed by insufflating the lungs with a small (5mL) air filled rubber bung attached to the cannula.
6. Maintenance of gaseous anaesthesia by attachment of adapted connector with tubing carrying anaesthetic gases (isoflurane 4% and 0.2 L/min oxygen) to cannula.
7. Test fluid (300µL) instilled, followed by 200µL of air, with rat positioned head up at an angle of approximately 45° for one minute to aid in distribution of the instillate throughout the lungs.
8. Rat identified with ear markings, anaesthesia ceased, cannula removed and rat returned to recovery incubator.

5.2.7 Harvesting of rat lungs

Depending upon the experimental protocol, lungs were harvested from treated rats at different time points and using different lung preservation methods. However, the basic methodology for removal of the lungs remained the same and was as follows:

1. DA rat placed in anaesthetic chamber and anaesthetised with 4% isoflurane and 4L/min oxygen.
2. Rat removed from chamber and placed supine onto metal plate with warmer, all limbs taped down onto plate while rat maintained under gaseous anaesthesia (isoflurane 4% and 0.2 L/min oxygen) via a face mask.

3. Fur over abdomen and chest moistened with normal saline to keep clear of wound.
4. After confirmation of adequate level of anaesthesia, abdomen incised longitudinally and abdominal cavity entered.
5. Aorta located, transected with scissors to effect rapid exsanguination with concomitant cessation of respiration.
6. Further dissection into thoracic cavity with excision of diaphragm and exposure of thorax.
7. Thymus removed, blunt dissection to locate trachea, which was directly intubated with a 16 G Abbocath cannula through a small anterior tracheal incision.
8. Intratracheal cannula secured by two 4/0 silk sutures placed around trachea.
9. Lungs individually insufflated with preservation fluid (either 50% OCT in phosphate buffered saline or 1% glutaraldehyde – see below for further details).
10. Lungs removed from thorax *en-bloc*, after heart dissected away, and placed in normal saline for transfer to laboratory for further processing.

5.2.8 Processing of rat lungs

Processing of lungs from the experimental animals varied depending upon the reporter gene used. The X-GAL staining method (see below) was used for identifying the β -GAL gene product in rat lungs from Phase 1 and in two rats from Phase 2 experiments. Staining was performed on whole lung samples, small lung cubes, and frozen sections (F/S) of lung.

For the Thy-1 gene, an anti-Thy-1 monoclonal antibody was used to identify gene expression in histological sections of the lung. In addition, an anti-molossin monoclonal antibody was used to assess vector distribution within the lung. Table 5.3 lists the lung preservation techniques and processing/staining methods used in each of the experimental animals, together with the time after gene administration that harvesting of the lungs occurred.

Table 5.3 Preservation and staining methods used for rat lungs

Rat number	Time post-instillation	Lung preservation	Type of section, staining method
P1, P3, P8, P10	48 hours	L - 1% glutaraldehyde R - Nil	Whole lung, X-GAL Lung cubes, X-GAL
P2, P4	72 hours	L - 50% OCT R - Nil	F/S, X-GAL Lung cubes, X-GAL
P7	4 hours	L - 1% glutaraldehyde R - Nil	Whole lung, X-GAL Lung cubes, X-GAL
P5, P6, P31, P32	1.5-2 hours	50% OCT	F/S, anti-Poly-Mol mAb
P9, P11, P12, P13, P29, P30	72 hours	50% OCT	F/S, X-GAL
P20, P22, P23 - P28	72 hours	50% OCT	F/S, anti-Thy-1 mAb

L, left lung. R, right lung.

F/S, frozen section

mAb, monoclonal antibody

5.2.9 X-GAL staining of whole lungs and small lung cubes

(P1, P3, P7, P8, P10)

Preservation for whole lung staining was by insufflation of the left (L) lung with 1% glutaraldehyde in phosphate buffered saline (PBS), while the right lung was removed without preservation for staining of small lung cubes. After culling of the rat, dissection of the thorax, direct cannulation of the trachea and removal of the right (R) lung into normal saline, 1.5-2 mL of 1% glutaraldehyde in PBS were slowly instilled into the left lung. The latter was then submerged *in toto* into a container of 1% glutaraldehyde in PBS and fixed for one hour, prior to X-GAL staining.

Cubes, approximately 2mm x 2mm x 2mm in size, were excised from the upper, middle and lower regions of the right lung and placed into separate wells of a 24-well plate. These were fixed in 1% glutaraldehyde in PBS for 10 minutes at room temperature, followed by two washes with PBS plus magnesium chloride (MgCl₂).

As previously described (Weiss 1997, Mastrangeli 1993), whole lung and small lung cubes were stained overnight with filtered X-GAL solution (5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆ · 3 H₂O and 0.2 mM MgCl₂ in Tris at pH 5.5, 7.5 and pH 8.5) containing 1mg/mL X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Melford Laboratories, Ipswich, UK). These were then washed twice with PBS/ MgCl₂ and fixed in 10% formalin/normal saline. The small lung cubes were assessed macroscopically for the characteristic blue staining, while histological sections from the whole lung specimens, prepared after embedding in paraffin wax, were evaluated microscopically.

5.2.10 Preparation of lungs for frozen section

(P2, P4, P5, P6, P9, P11-13, P20, P22-31)

Rat lungs destined for frozen sectioning were perfused with 50% OCT in PBS at time of harvesting (Table 5.3). Small cubes (approx. 5mm x 5mm x 5mm) were excised by scalpel from the upper and lower parts of the lung, as well as the middle part in the Phase 1 experiments. These sections were then placed onto a small amount of OCT on a cork tile, snap frozen in liquid nitrogen and placed in a -80°C freezer. Cryosections 8 to 20 microns thick were placed onto Vectabonded slides (Vector Laboratories, Burlingame, CA, USA), and fixed in acetone at room temperature for 30 minutes. The slides were air dried overnight and processed for X-GAL staining after fixing in 0.5% glutaraldehyde or stored at -35°C in airtight containers for later immunostaining.

In Phase 2 experiments, the right lung was dissected into three blocks – upper, middle, and lower. The left lung was divided into two sections – upper and lower. Each section of lung was then placed onto a small amount of OCT on a cork tile, snap frozen in liquid nitrogen and placed in a -80°C freezer. All samples were snap frozen within one hour of retrieval of the lungs. These larger sections enabled evaluation of both the larger airways (bronchi and bronchioles) in addition to the alveolar tissue. Cryosections were initially performed at a thickness of 10 and 20 microns (μm).

5.2.11 X-GAL staining of frozen sections

(P2, P4, P9, P11-13, P29, P30)

After thawing for one hour, the sections were washed twice in PBS and placed in 0.1% X-GAL staining solution (composition as described in 5.2.9) overnight at room temperature and protected from light. Sections were then washed twice in 3% DMSO in PBS to prevent hydrolysis of any residual X-GAL, and counterstained in 1% eosin for 10-15 seconds. The sections were dehydrated through alcohol (dipped

in 2% HCl ethanol in 70% IMS, washed in tap water, 70% IMS for 1 minute x 2, 100% IMS for 1 minute x 2, HistoClear for 1 minute x 2) prior to mounting with DPX mounting agent and coverslips. Microscopic examination was then performed to observe for the characteristic blue staining.

5.2.12 Antibodies for immunostaining

(a) Anti-molossin antibody: The mouse IgG1 monoclonal antibody, LC2-64, to the molossin peptide was used to assess distribution of the vector throughout the lung. This antibody was produced by fusing the NS-O mouse myeloma cell line with spleen cells from BALB/c mice immunised with molossin peptide conjugated to keyhole limpet hemocyanin (Collins 2000).

(b) Anti-Thy-1 antibody: The mouse IgG1 monoclonal antibody, F15-42-1, to human Thy-1 was used to assess human Thy-1 gene expression (Dalchau 1979).

(c) Rabbit anti-mouse antibody (RAM): Peroxidase-labelled rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) was prepared in 0.5% BSA/PBS and 20% normal rat serum to block antibodies cross-reacting with rat immunoglobulin.

(d) Anti-DA MHC class I antibody: MN4 was used as a positive control.

(e) Anti-human MHC class I antibody: W6/32 was used as a negative control.

5.2.13 Immunohistological staining

(a) Thy-1 gene expression

(P20, P22, P23, P24, P25, P26, P27, P28):

Sections thawed for one hour at room temperature and then quenched for five minutes in 0.6% H₂O₂ in methanol (to avoid non-specific immunoperoxidase staining). Two hundred µL of F15-42-1 (anti-Thy-1) monoclonal antibody were added to the sections at a saturating concentration in 0.5% BSA/PBS. For this protocol, 200µL of W6/32 antibody were used as the negative control, and 200µL of MN4 antibody as the positive control. After incubating for 30 minutes at room temperature in a humidified chamber, the slides were washed thrice in freshly prepared Tris buffered saline (0.05 M Tris, 0.15 M NaCl, pH 7.4). Two hundred µL of peroxidase-labelled rabbit anti-mouse immunoglobulin in 0.5% BSA/PBS and 20% normal rat serum were added and incubated for 30 minutes at room temperature. The slides were again washed and 200µL of freshly prepared diaminobenzidine (DAB, Sigma, Poole, Dorset, UK) were added for exactly six minutes. The slides were then washed in Tris buffered saline and lightly counterstained with Harris' haematoxylin (BDH, Merck Ltd, Poole, Dorset, UK) for 30 seconds, dehydrated and mounted with glass cover slips.

(b) Distribution of Poly-Mol vector

(P5, P6, P31, P32)

Sections thawed for one hour at room temperature and then quenched for five minutes in 0.6% H₂O₂ in methanol (to avoid non-specific immunoperoxidase staining). Two hundred µL of LC2-64 (anti-molossin) monoclonal antibody were added to the sections at a saturating concentration in 0.5% BSA/PBS. The subsequent methods were as described above.

For this protocol, F15-42-1 isotype antibody was used as a negative control, while for the positive control, preincubation of a section for 1 hour with 200 μ L of Poly-Mol peptide at 10 μ g/mL to establish maximal binding prior to staining with LC2-64, was included.

5.3 Results

5.3.1 Intratracheal instillation of the rat lung

This technique required certain refinements mainly related to the alteration of the paediatric laryngoscope to facilitate visualisation of the larynx and tracheal intubation. There was also operator dependent factors involved, with a definite learning curve experienced. In the first set of experiments, one rat was unable to be intubated and had to be terminated. Another became acutely cyanosed following intubation with signs of increased respiratory effort. This animal was also terminated and at autopsy was noted to have a ruptured trachea.

No other difficulties were experienced with the intubation procedure itself, although one of the rats, which received intraperitoneal chloroquine, became distressed, as evidenced by the production of porphyrins around its mouth and nose and ingestion of sawdust. This animal was also terminated. The rats, which received chloroquine as part of the tracheal instillate, demonstrated no ill effects.

Following intratracheal instillation of all solutions, there were “crackles” felt through the chest wall bilaterally, indicating intrapulmonary placement of the instillate. These were detected for a short period only and the rats returned to normal full activity soon after resolution of the anaesthetic effects. At 72 hours post-instillation, prior to the latest culling period, none of the rats showed aberrant behaviour or signs of respiratory embarrassment.

5.3.2 Phase 1 experimental results

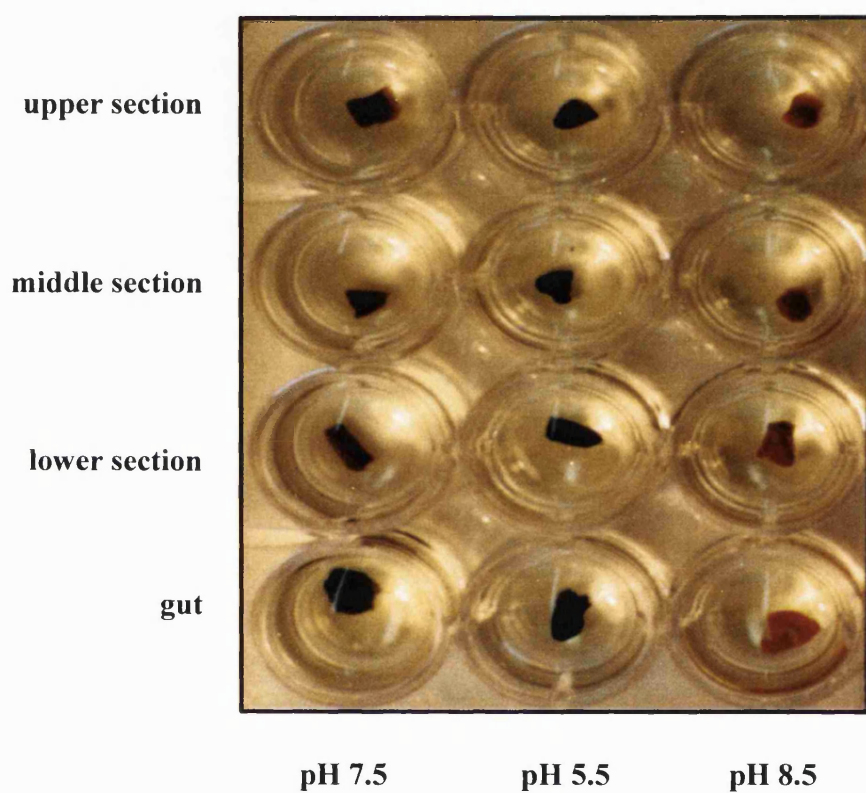
5.3.2.1 Studies with small cubes of lung tissue

There were marked differences in the macroscopic staining of the lung cubes. This was dependent upon the pH of the X-GAL staining solution used. At lower pH (5.5 and 7.5), intense blue colouration was observed throughout the cubes of control lung (P7 - instilled with normal saline) and in pieces of rat intestine. This relates to the high level of endogenous β -galactosidase present in the lung and gut. At pH 8.5, there was minimal staining in the control lung and gut pieces (Figure 5.2). Thus it followed that to determine true exogenous β -galactosidase activity, X-GAL staining only at pH 8.5 should be assessed.

There was no discernible difference in the intensity of X-GAL staining at pH 8.5 between the rat lung instilled with naked β -galactosidase DNA (P1) and the lung instilled with DNA and Poly-Mol vector (P3). When compared with the negative control lung, there was no evidence of increased X-GAL staining at the higher pH. However, as with the negative control samples, there were differences in staining observed at pH 7.5 and pH 8.5 (Figure 5.3). On repeat experiment, there was again no appreciable staining, compared to negative control, observed in the naked DNA or DNA with Poly-Mol treated lungs at pH 8.5 (P2 and P4, data not shown). As no attempts were made to assist with endocytic release, these results were not unpredictable.

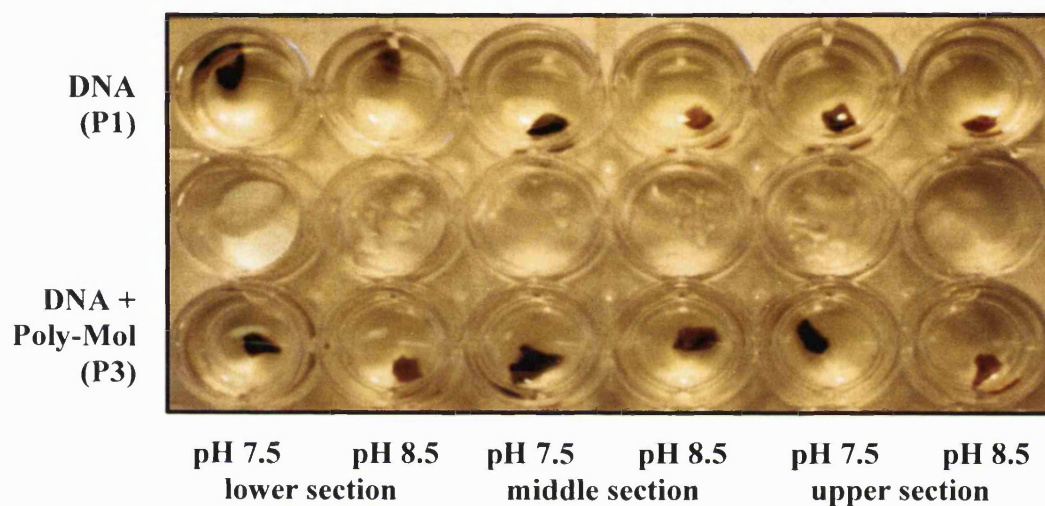
In lung cubes obtained from the rats instilled with DNA, Poly-Mol vector and chloroquine (P8), X-gal staining at pH 8.5 suggested slightly increased exogenous β -galactosidase activity compared with the negative control (Figure 5.4). This was also the case for cubes obtained from the lungs of rats instilled with DNA, Poly-Mol vector and fusogenic peptide, although the increase in staining was not as prominent (Figure 5.5).

Figure 5.2 X-GAL staining at varying pH of cubes of rat lung instilled with normal saline, and rat intestine



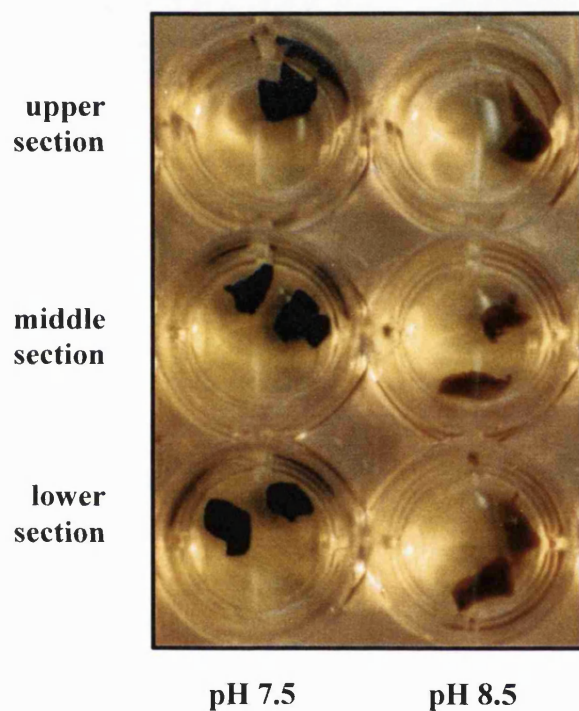
X-GAL staining method as described in text. Note positive (dark blue) staining for endogenous β -galactosidase in cubes of rat lung instilled with normal saline and rat intestine at lower pH (5.5 and 7.5). There is minimal staining at higher pH (8.5).

Figure 5.3 X-GAL staining at varying pH levels of cubes of rat lung instilled with naked DNA and DNA with Poly-Mol vector



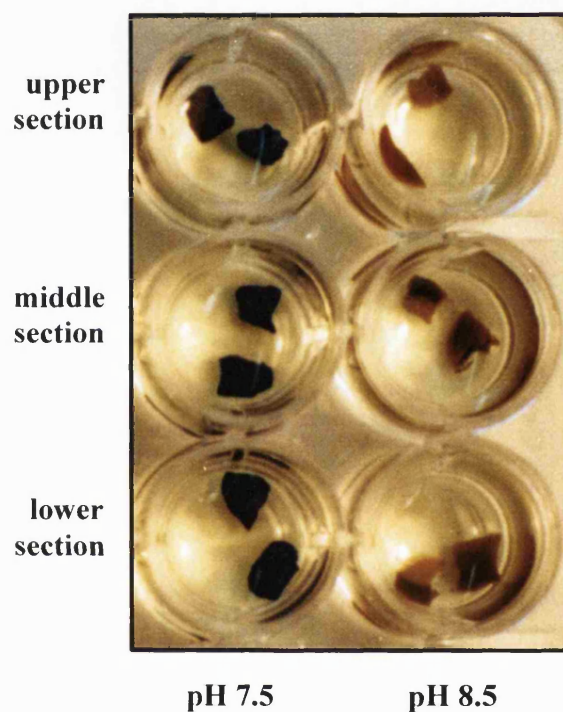
X-GAL staining method as described in text. At pH 7.5, positive X-GAL staining indicates endogenous β -galactosidase activity. There is no discernible staining at pH 8.5.

Figure 5.4 X-GAL staining at varying pH levels of cubes of rat lung instilled with DNA, Poly-Mol vector and chloroquine (P8)



X-GAL staining method as described in text. Note slight increase in staining in sections at pH 8.5, and increase in staining intensity in sections at pH 7.5 compared with negative controls (Figure 5.2).

Figure 5.5 X-GAL staining at varying pH levels of cubes of rat lung instilled with DNA, Poly-Mol vector and fusogenic peptide (P10)



X-GAL staining method as described in text. Note slight increase in staining in sections at pH 8.5, and increase in staining intensity in sections at pH 7.5 compared with negative controls (Figure 5.2).

5.3.2.2 X-GAL stained histological and frozen sections

(P2, P3, P4, P7, P8, P9, P10, P11, P12, P13)

The lung architecture was poorly preserved in those lungs not infused with glutaraldehyde or OCT, with difficulty in identifying alveolar structure in the cut sections. The lungs perfused with glutaraldehyde or OCT showed substantially better preservation of the parenchymal architecture.

Histological sections, either frozen or paraffin embedded, of X-GAL stained whole rat lungs showed no convincing evidence of β -galactosidase activity above background levels, when staining was performed at pH 7.5. When X-GAL staining was performed at pH 8.5, no β -galactosidase activity was observed in any of the sections. This again highlighted the difficulties in the interpretation of results of X-GAL staining.

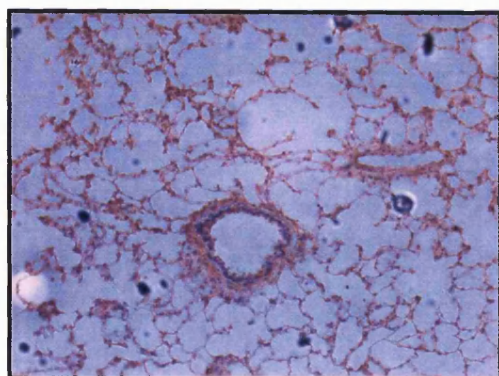
5.3.3 Phase 2 experimental results

All lung samples in Phase 2 experiments were processed as frozen sections, with no whole lung or block section samples obtained. Immunohistological staining was performed subsequently. Frozen sections were initially cut at a thickness of 10 μ m and 20 μ m in order to determine the optimum thickness. After processing, it was observed that the ideal thickness was 10 μ m. These sections demonstrated intact pulmonary architecture with minimal overlapping of alveolar cells as was seen in the 20 μ m sections. Thus, all subsequent frozen sections were cut at 10 μ m.

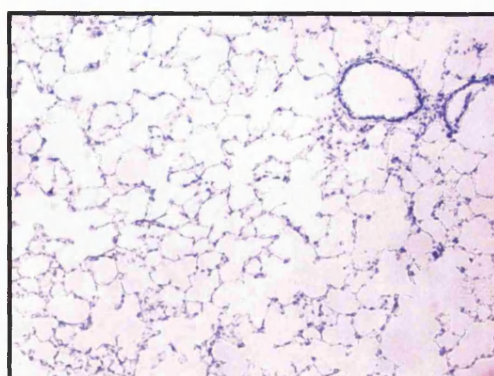
The initial immunoperoxidase staining frozen sections from rat lungs P20, P22, P25, and P26 showed marked background staining due to endogenous peroxidase activity and were difficult to interpret (Figure 5.6a). Therefore, all subsequent sections were first quenched with 0.6% hydrogen peroxide (H₂O₂) in methanol to remove endogenous peroxidase activity, prior to immunostaining (Figure 5.6b).

Figure 5.6 Micrographs (63x) of cryosections (10 μ m) of negative control rat lung (P26), (a) without, and (b) with, H₂O₂ quenching

Note good preservation of lung parenchyma and generalised positive immunoperoxidase (brown) staining throughout the unquenched section (a). This is removed after quenching with H₂O₂ (b).



(a)



(b)

5.3.3.1 Human Thy-1 studies

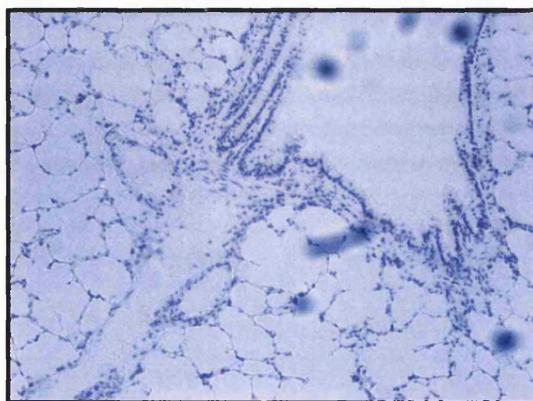
(P20, P22, P25, P27)

There was no evidence of human Thy-1 gene expression in any of the rat lungs instilled with the Thy-1 gene at a DNA concentration of 10µg/mL concentration. In view of the lack of reporter gene activity, the dose of DNA was increased to 100µg/mL per instillation together with the Poly-Mol vector, fusogenic peptide and lipofectamine. Despite the increased dose, there was again no Thy-1 gene expression observed in the sections of rat lungs. Figures 5.7 and 5.8 show sections from lung of rat P27 at different magnifications (63x and 126x) stained with antibodies for Thy-1 (test antibody), anti-DA class I (positive control) and anti-human class I (negative control), demonstrating this lack of Thy-1 expression in the test sections.

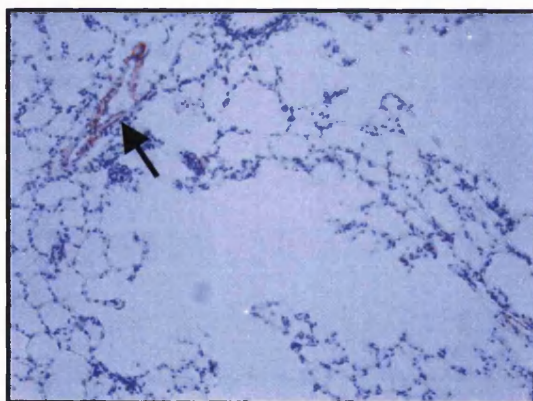
Figure 5.7 Micrographs (63x) of immunoperoxidase stained cryosections from rat lung instilled with Thy-1 reporter gene (100 μ g/mL), Poly-Mol vector, fusogenic peptide and Lipofectamine

(a) negative control antibody (W6/32); (b) positive control antibody (MN4); (c) Thy-1 antibody (F15-42-1). Note positive immunoperoxidase (brown) staining especially of airway (arrowed) in (b), with no evidence of staining in the test section (c).

(a)
negative
control



(b)
positive
control



(c)
test
section

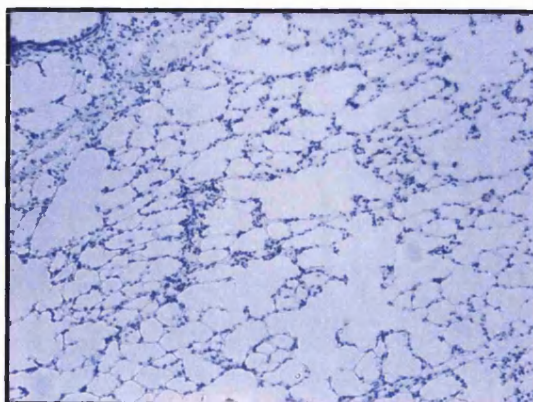
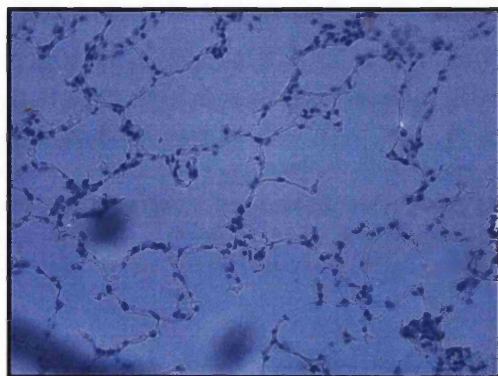


Figure 5.8 Micrographs as in Fig 5.7 at higher magnification (126x)

(a) negative control antibody (W6/32); (b) positive control antibody (MN4); (c) Thy-1 antibody (F15-42-1). Note positive immunoperoxidase (brown) staining (arrowed) in (b), with no evidence of staining in the test section (c).

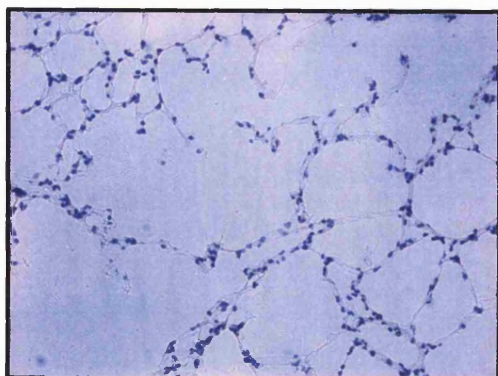
(a)
negative
control



(b)
positive
control



(c)
test
section



5.3.3.2 High dose β -GAL studies

(P29, P30)

A further attempt at β -GAL transfer was made using high dose DNA (100 μ g/mL), with Poly-Mol vector, fusogenic peptide and Lipofectamine (P29 and P30). Incubation for the X-GAL staining of cryosections was performed at pH 5.5, 7.0 and 8.5 respectively, for either four hours or overnight. The control sample was the Thy-1 construct instilled rat lung (P27).

At pH 5.5 all sections demonstrated markedly positive (blue) staining. There was no difference in the intensity of staining between the test sections and the negative control section. At pH 7.0 the intensity of the staining was less than at pH 5.5, but once again all sections, including the negative control, were positive. While at pH 8.5, all sections were negative, indicating the absence of exogenous β -galactosidase activity.

5.3.3.3 Vector distribution studies

(P5, P6, P31, P32)

Frozen sections of lungs retrieved from rats 1.5 to 2 hours after instillation with Poly-Mol vector and the β -galactosidase reporter gene (P5, P6), were stained with the Poly-Mol monoclonal antibody (LC2-64). For the negative control, the Thy-1 monoclonal antibody (F15-42-1) was used. The immunoperoxidase method was used after counterstaining with rabbit anti-mouse antibody.

A small amount of positive (brown) staining was noted around the airway epithelium in the negative control sample. However, positive staining was much more prominent in the test section, in both the airways and the alveoli (data not shown – see Phase 2 results below). The preliminary conclusion of this finding was that the vector appeared to bind to both epithelial and alveolar cells.

In the Phase 2 experiments, cryosections (10µm) were obtained from the lower part of the right lung in rats P31 and P32, 1.5-2 hours after instillation of the vector DNA complex. These sections were freeze dried overnight and fixed in acetone.

Immunostaining was as described above. The test antibody was the anti-Poly-Mol monoclonal antibody LC2-64. There were two negative control antibody stains, one of which was the human class I antibody W6/32. As there was insufficient time for expression of the Thy-1 reporter gene, the other negative control antibody was the anti-Thy-1 antibody, F15-42-1. For the positive control, sections were first treated with Poly-Mol vector at a concentration of 10µg/mL for approximately 60 minutes, then washed with Tris buffer. These were then stained with LC2-64.

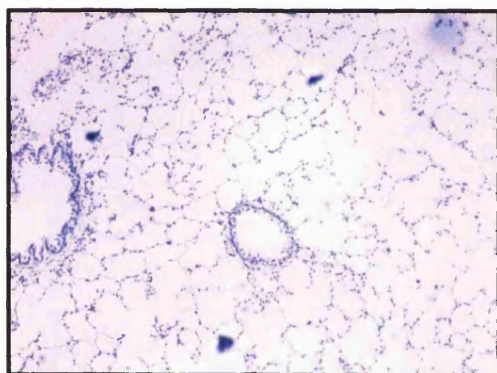
The positive control samples from both P31 and P32 lungs were heavily stained throughout, while both negative controls showed an absence of staining. At lower magnification (63x), there appeared to be minimal staining around the airway with more prominent staining of the alveoli (Figure 5.9). At higher magnification (126x), this appearance was confirmed with notable staining of alveolar macrophages and alveolar epithelium and a virtual absence of staining of the airway epithelium (Figure 5.10).

5.3.3.4 Inflammatory responses

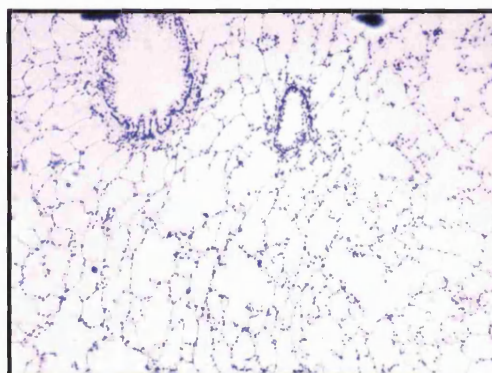
In all sections reviewed, both in the Phase 1 and the Phase 2 studies, there was no evidence of pulmonary inflammation observed.

Figure 5.9 Poly-Mol vector distribution study (63x)

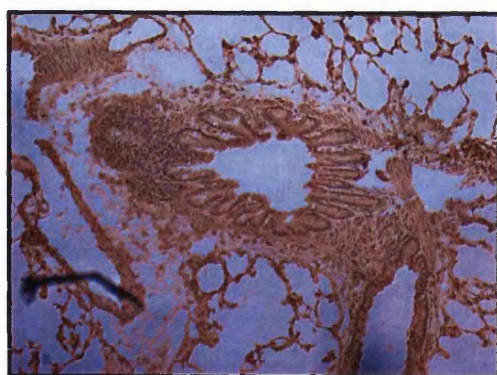
Micrographs (63x) of immunoperoxidase stained cryosections from rat lung (P31) instilled with Thy-1 reporter gene (10 μ g/mL), Poly-Mol vector, fusogenic peptide and Lipofectamine: (a) negative control antibody (W6/32); (b) negative control anti-Thy-1 antibody (F15-42-1); (c) positive control anti-Poly-Mol antibody (LC2-64); (d) test section anti-Poly-Mol antibody (LC2-64). Note positive immunoperoxidase (brown) staining throughout positive control section (c). The test section (d) shows positive staining of alveoli, but minimal staining of airway (arrowed).



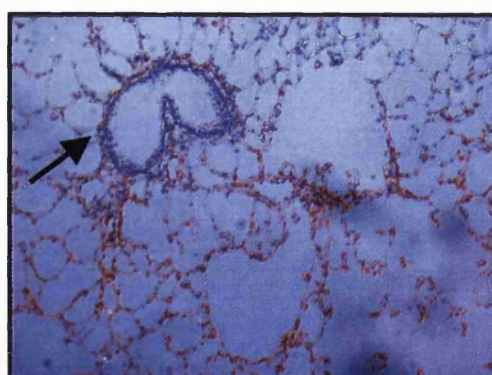
(a)
ne



(b)



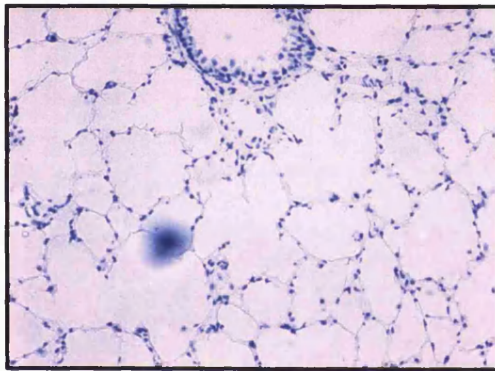
(c)



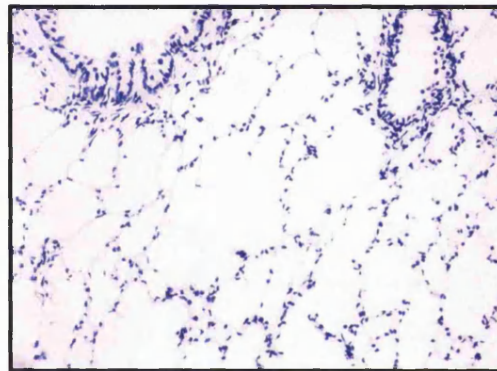
(d)

Figure 5.10 Poly-Mol vector distribution study (126x)

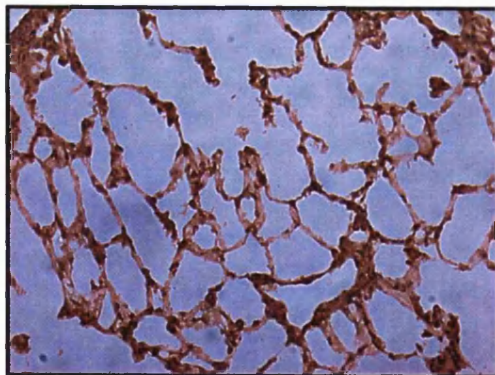
Micrographs (126x) of immunoperoxidase stained cryosections from rat lung (P31) instilled with Thy-1 reporter gene (10 μ g/mL), Poly-Mol vector, fusogenic peptide and Lipofectamine (as per figure 5.9): (a) negative control antibody (W6/32); (b) negative control anti-Thy-1 antibody (F15-42-1); (c) positive control anti-Poly-Mol antibody (LC2-64); (d) test section anti-Poly-Mol antibody (LC2-64). Note positive immunoperoxidase (brown) staining throughout positive control section (c). This confirms the findings in the test section (d) of minimal staining of the airway epithelium, but positive staining of alveolar macrophages (arrowed) and alveolar epithelium.



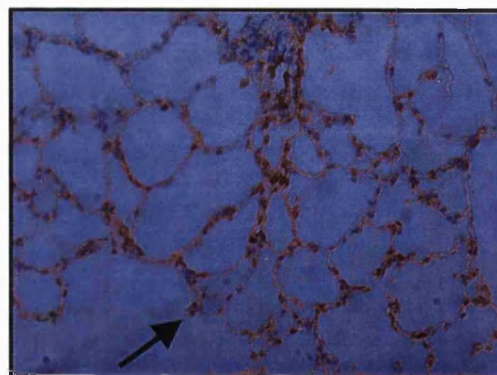
(a)



(b)



(c)



(d)

5.4 Discussion

Numerous problems need to be resolved before successful gene therapy can become a reality, especially with the use of non-viral gene vectors. A major challenge is posed by inefficient gene delivery. All of the currently available vectors seem to offer relatively poor entry into intact differentiated airway epithelium. Optimisation of existing adenoviral vectors may improve gene delivery. By creating less immunogenic adenoviral vectors, ie: those that express no endogenous viral genes, may represent one method of improving gene transfer (Schiedner 1998). Further development of liposomal preparations may also improve gene delivery.

However, the development of novel synthetic non-toxic, non-immunogenic and efficient gene vectors may constitute the way forward. One such novel vector system is the arginine-glycine-aspartate (RGD) containing peptide vector described in this study. The RGD motif is present in diverse natural ligands for several members of the integrin family and plays a crucial role in the ligand-integrin interaction (Hynes 1992). The specific RGDNP motif used was based on that found in the venom (molossin) of the American pit viper *Crotalus molossus molossus*. This venom binds preferentially to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, both of which are expressed on diverse cell types including respiratory epithelium. Binding of the vector (polylysine-molossin) to various tissues *in vitro*, including lung, has been well documented (Collins 2000).

Other factors important for the delivery of this integrin-targeted DNA vector *in vitro* have been explored previously (Collins 2000a). Some of these factors include the optimal concentration ratio of DNA to vector. Optimal gene transfection (*in vitro*) was obtained at a DNA concentration range of 2-8 μ g/mL, with a DNA:peptide ratio of 1:3. Another factor was the target cell exposure time. Although vector/DNA complexes saturated target binding sites within five minutes of incubation, lengthy exposure times (>2-3 hours) were essential for substantial gene transfer. In addition, exposure to chloroquine for 8-10 hours after uptake of vector/DNA complexes was essential for optimal gene transfer.

Utilising the optimal methodology determined in these *in vitro* studies, the same vector system was assessed *in vivo*. Firstly, a system to administer the DNA/vector complex to rat lung was developed. This was successfully achieved by direct instillation of the complex via a cannula into the trachea, after visualisation of the larynx with the aid of a modified paediatric laryngoscope. Apart from some early casualties associated with the initial learning curve, there were no problems associated with the technique of direct instillation of the vector-DNA complex. Unlike other methods of tracheal access that incorporate an open surgical procedure with exposure of the trachea, there was no obvious trauma to the airway associated with the intubation process. This lack of airway trauma may have had implications on the effectiveness of gene transfer observed in this study (see below).

The vector distribution experiments demonstrated that the administration of the DNA/vector complex was effective with widespread distribution of the complex throughout the lung. Sections from different areas of both lungs showed that the distribution was relatively uniform throughout. This indicated that the tracheal instillation used in this study was an efficient method of administering gene and vectors to rat lung. Interestingly, there appeared to be greater localisation of the vector in the alveolar epithelium than in the airway epithelium (Figure 5.9b). This suggests poor attachment of the vector to the airway epithelium, and may reflect inadequate exposure of the airway epithelium to the vector.

Disappointingly, there was no clear evidence of successful gene transfer demonstrated in either Phase 1 experiments using the β -galactosidase reporter gene, or in Phase 2 experiments when the Thy-1 reporter gene was used. The reasons for the change of gene marker have been discussed above, but despite this change there remained no histological evidence of reporter gene expression. Improved airway epithelial uptake of DNA/vector complex could possibly lead to better gene transfer and hence expression.

Using similar methodology, Jenkins *et al* demonstrated that gene transfer can be achieved with an integrin-targeted non-viral vector (Jenkins 2000). They used a

specific $\alpha_5\beta_1$ integrin binding peptide ([K]₁₆-GACRRETAWACG), in combination with a cationic liposome (Lipofectin) and lacZ (β -galactosidase) reporter gene. Chloroquine and fusogenic peptide were not used. They showed widespread β -galactosidase activity throughout airway epithelium and parenchymal cells, with no activity when using a control plasmid. A similar degree of transfection was found when using an adenoviral vector although this was associated with an inflammatory response, which was absent with the integrin-targeted vector.

It is unclear as to the discrepancy in the results achieved by Jenkins *et al* and those achieved in this study. Different integrin-targeted peptides were used and this may have influenced cellular uptake, although it was interesting that a non-specific (scrambled) peptide was also associated with gene transfection, albeit to a lesser extent. The authors did speculate that the cationic lipid, Lipofectin, might itself play a role in endocytic release, although this has not been proven in *in vitro* studies.

Another postulate for the enhanced gene transfection may be related to the method of DNA/vector administration. There are natural protective barriers to the airway epithelium including a layer of mucous and cilia. Partial or complete breakdown of these barriers may enhance uptake (Boucher 1999), and this mechanism may be one of the reasons that adenoviral vectors show reasonable gene transfer efficiency. Direct injury to the epithelium may enhance vector access, and this could explain why the open surgical tracheal administration as used by Jenkins *et al* was associated with gene transfection.

Another potential problem is the adequacy of the systemic chloroquine and the fusogenic peptide used for promoting escape of DNA/vector complexes from endocytic vesicles. Without effective endocytic escape mechanisms, DNA/vector complexes are degraded in lysosomes. These factors are currently being analysed in detail, and will be applied for gene delivery to the lung.

Despite the lack of gene transfer observed, the vector system used in this study appeared to be safe. Apart from one of the rats that received intraperitoneal

chloroquine and the early casualties associated with tracheal cannulation, none of the rats demonstrated untoward effects from the intratracheal administration of the DNA/vector complex. Furthermore, there was no evidence of inflammation noted in any of the lung sections examined following the administration of the complex.

In summary, the gene vector system used in these experiments appeared to be safe and showed widespread distribution throughout the lung. However, the successful *in vitro* gene transfer results could not be replicated in this *in vivo* model. Further work is being undertaken to extend these early studies, and it is hoped that this system will be of value for gene delivery to the lung.

Chapter 6 *In vitro* modulation of adenovirus infection by antisense oligodeoxynucleotides

6.1 Introduction

6.2 Methods

6.2.1 General Overview

6.2.2 Laboratory methods

6.2.2.1 293 cell culture

6.2.2.2 A549 cell culture

6.2.3 Virus culture and titring

6.2.3.1 Propagation of adenovirus 5-beta-galactosidase (AdV5- β gal)

6.2.3.2 Propagation of wild type adenovirus 5 (AdV5)

6.2.3.3 Titring of adenovirus 5-beta-galactosidase (AdV5- β gal)

6.2.3.4 Titring of wild type adenovirus 5 (AdV5)

6.2.4 Antisense oligodeoxynucleotides

6.2.4.1 Oligodeoxynucleotide design

6.2.4.2 Precipitation of oligodeoxynucleotides

6.2.5 Virus titring with oligodeoxynucleotides (ODNs)

6.2.5.1 Titring of adenovirus 5-beta-galactosidase (AdV5- β gal) and wild type adenovirus 5 (AdV5) with oligodeoxynucleotides (ODNs)

6.2.5.2 Titring with ODNs and Lipofectamine

6.2.5.3 End-protected versus fully-protected (phosphorothioated) ODNs

6.2.5.4 Wild type adenovirus 5 (AdV5) propagation for serial titring

6.2.5.5 Wild type adenovirus 5 (AdV5) propagation with ODNs for serial titring

6.2.5.6 Serial titring

6.2.6 Statistical methods

6.3 Results

6.3.1 Viral titring in 293 and A549 cell lines

6.3.2 Antisense ODNs in A549 cell line/wild type
adenovirus 5 system

6.3.2.1 E1AAS and PIXAS ODNs at 15 μ M concentration

6.3.2.2 E1AAS and PIXAS ODNs at varying concentrations

6.3.2.3 Antisense ODNs plus Lipofectamine

6.3.2.4 End-protected versus fully-protected antisense ODNs

6.3.2.5 Additional ODNs

6.3.2.6 Meta-analysis of E1AAS effect

6.3.2.7 Serial titring

6.4 Summary of results

6.5 Discussion

6.1 Introduction

Antisense oligodeoxynucleotides (ODNs) represent a novel approach to the treatment of viral infections, while the application of this methodology in the lung is of particular interest due to the lung's inherent accessibility. Although adenovirus pneumonitis is a relatively rare condition, there is no specific therapy currently available. As a consequence, adenovirus pneumonitis is potentially fatal in the lung transplant recipient. It seemed logical therefore, to evaluate antisense ODNs as a potential therapeutic option for this disease.

A biological assay was developed to evaluate the efficacy of antisense ODNs in modulating adenovirus infection *in vitro*. A system using cells derived from the lung together with a wild type strain of adenovirus was selected to reflect the *in vivo* situation as closely as possible. Various ODNs were assessed in this model under varying conditions. ODNs were designed with the intention to produce maximum reduction of adenovirus replication.

The main research questions were as follows:

- Can specific antisense oligodeoxynucleotides (ODNs) inhibit adenovirus replication and thus infection *in vitro*?
- If so, is this a specific antisense effect?
- Is the effectiveness of ODNs dependent upon their concentration?
- Does the addition of cationic lipids improve ODN effect?
- Does the manipulation of ODN structure produce any additional benefit?
- Is the ODN antisense effect related to time?

This chapter describes the development of the biological assay used in the study, together with how ODNs were designed and processed, while the results of the evaluation of a number of ODNs in modulating adenovirus infection are reported.

6.2 Methods

6.2.1 General Overview

The evaluation of antisense ODNs was performed using a biological assay. Fundamental to this assay was the method of virus titration by the standard plaque technique as previously described (Williams 1970). This involved the incubation of adenovirus with a cultured cell monolayer, followed by an overlay of agar-containing medium. After 7-14 days, plaques, representing areas of non-viable cells (ie: areas of viral cytopathic effect), were normally evident and visible macroscopically. The efficacy of antisense ODNs in modulating adenovirus infection can therefore be assessed by comparing the number of plaques in the ODN-treated with those in the untreated cell cultures.

In light of published experience (Quinlan 1986), local laboratory experience and reagent accessibility, initial pilot experiments incorporated the use of the 293 cell line with a modified (E1 region deficient) strain of adenovirus 5 (adenovirus 5-betagalactosidase, AdV5- β gal). After refinement of the methodology in this system and in order to simulate the clinical situation more closely, later experiments were performed using A549 cells, a human lung carcinoma cell line, with wild type adenovirus 5 (AdV5).

Oligodeoxynucleotides were designed to target the exon regions of the genes of interest (E1A, E1B and protein IX). They were constructed to bridge the initiation of translation (AUG) codon of the corresponding sense mRNA. As the half-life of unmodified phosphodiester ODNs is less than 15 minutes in the presence of serum, ODN backbones were chemically modified by the substitution of a non-bridging oxygen by a sulphur, resulting in a phosphorothioate linkage. This modification afforded more resistance to the breakdown of the ODNs by nucleases (Hoke 1991). These linkages were developed at either the terminal ends only (phosphorothioate end-protected) or throughout the entire ODN backbone (phosphorothioate fully-protected).

Various concentrations of the ODNs, ranging from 1 to 30 μM , were used in order to derive the optimum concentration. Lipofectamine was not routinely used, although its addition to the assay system was evaluated.

In order to determine the relationship between ODN antisense effect and time, ODNs were added to the culture medium of cell cultures infected with wild type adenovirus 5, with serial viral titring of the supernatant performed at subsequent time points (ranging from 24-96 hours). This set of experiments is thus described “serial titring”.

6.2.2 Laboratory methods

6.2.2.1 293 cell culture

293 cells are a human kidney cell line immortalised and transformed with the adenovirus type 5 (AdV5) E1A and E1B regions (Graham 1977). This cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK).

Propagation of 293 cells was as follows:

1. 293 culture medium: minimal essential medium (MEM, Gibco BRL, Paisley, UK); 2mM L-glutamine; and 10% heat-inactivated foetal calf serum (HIFCS – heated at 56⁰C for 30 minutes); penicillin (100U/mL) and streptomycin (100 $\mu\text{g/mL}$).
2. Cells suspended in 10mL of 293 culture medium, centrifuged at 200xg for 5 minutes, resuspended in 10mL of medium and viable cells counted by trypan blue exclusion.
3. Cells seeded at approximately 3.5×10^4 per cm^2 of culture flask, ie. 2.6×10^6 cells per 75cm^2 (T75) flask with 20mL of culture medium.
4. Cell cultures maintained in an humidified incubator at 37°C in 5% CO_2 /air.

5. Fresh culture medium exchanged after 3 days (cells 60-80% confluent).
6. Subculturing of cells performed on day 7 when 100% confluent: culture medium removed from T75 flask and cells incubated in 3mL warmed trypsin-EDTA solution for 5 minutes; 3mL culture medium added and cells dislodged by gentle shaking.
7. One mL aliquots of resulting suspension placed into six T75 flasks with 20mL of warmed medium and placed in humidified incubator at 37°C in 5% CO₂/air.
8. For viral titring experiments, 7mL of medium added to trypsin treated cells in T75 flask and 1mL aliquots of resulting suspension placed into 10 x 60mm culture dishes and incubated in 5mL culture medium at 37°C in 5% CO₂/air. Medium exchanged on day 3 and cells available for titring experiments when fully confluent on day 7.

6.2.2.2 A549 cell culture

This cell line is derived from lung epithelial cells (bronchial carcinoma). Cells were obtained from the European Collection of Animal Cell Cultures.

Propagation of A549 cells was as follows:

1. A549 culture medium: Dulbecco's modification of Eagle's medium (DMEM, Gibco BRL, Paisley, UK); 10% HIFCS; 2mM L-glutamine; penicillin (100U/mL) and streptomycin (100µg/mL).
2. As per 293 protocol.
3. As per 293 protocol.
4. As per 293 protocol.
5. As per 293 protocol.
6. As per 293 protocol with the exception that A549 cells, being more adherent to the culture flask, required longer incubation with trypsin-EDTA (20 minutes).
7. As per 293 protocol.

8. For initial viral titring experiments, 7mL of medium added to trypsin treated cells in T75 flask and 1mL aliquots of resulting suspension placed into 10 x 60mm culture dishes and incubated in 5mL of culture medium at 37° C in 5% CO₂/air. Medium exchanged on day 3 and cells ready for titring experiments when fully confluent on day 7. For later titring experiments, 6 well culture trays were used. These wells were of smaller diameter than the individual 60mm dishes and therefore 12mL of medium added to trypsin treated cells in T75 flask with 1mL aliquots distributed to 15 wells in the trays. These were incubated in 4mL of culture medium per well at 37°C in 5% CO₂/air until fully confluent on day 4.
9. A549 cells were not used after passage number 10.

6.2.3 Virus culture and titring

6.2.3.1 Propagation of adenovirus 5-betagalactosidase (AdV5-βgal)

This adenovirus has been rendered replication-incompetent by splicing out the E1A and E1B gene regions. Virus propagation is therefore only possible by culturing with 293 cells, which have these gene regions incorporated into their genome (see above).

The method of propagation was as follows:

1. T75 flasks of confluent 293 cells prepared.
2. AdV5-βgal supernatant of known plaque forming unit (PFU) activity diluted with phosphate buffered saline plus calcium and magnesium (PBS+ Ca⁺⁺/ Mg⁺⁺ see Appendix II for preparation details).
3. Following removal of culture medium, 293 cells were incubated with 2-3 PFUs/cell of AdV5-βgal supernatant at room temperature for 30 minutes. Fifteen mL of culture medium (MEM, 2mM L-glutamine, 2% HIFCS, penicillin [100U/mL] and streptomycin [100μg/mL]) were added to the cells and viral

supernatant and incubated at 37°C in 5% CO₂/air. (As there were approximately 1.6 x 10⁷ cells/T75 flask, 1mL (ie: 4 x 10⁷ PFUs) of virus supernatant diluted to 5mL with PBS + Ca⁺⁺/Mg⁺⁺ added per flask).

4. Cell lysis (viral cytopathic effect, CPE) was evident within 48-72 hours, at which stage supernatant collected and titring to assess PFU activity performed (see below).

6.2.3.2 Propagation of wild type adenovirus 5 (AdV5)

This adenovirus is capable of replication in cultured A549 cells. The methodology was similar to that for AdV5-βgal and was as follows:

1. T75 flasks of confluent A549 cells prepared.
2. 25 mL of AdV5 diluted supernatant (0.5 mL in 24.5 PBS+Ca⁺⁺/Mg⁺⁺) was added. Cells were incubated with virus at room temperature for 30 minutes, followed by the addition of 15 mL of medium (MEM, 2mM L-glutamine, 2% HIFCS, penicillin [100U/mL] and streptomycin [100μg/mL]) and incubated at 37°C in 5% CO₂/air for 4-5 days.
3. At this stage, the A549 cells appeared rounded up, ie: infected and not viable – demonstrating viral cytopathic effect.
4. The resulting supernatant was centrifuged at 200xg for 5 minutes.
5. Supernatant was then passed through a two micron filter and available for titring.

6.2.3.3 Titring of adenovirus 5-beta galactosidase (AdV5-βgal)

As discussed in the introduction of this methods section, viral titring by plaque assay was the fundamental technique utilised in this study. It was not only used for determining the relative “strength” of the viral supernatant, but also to enable assessment of the effect of various ODNs on cell cytopathic effect (CPE). The following describes the methods involved:

1. Preparation of medium/agarose overlay: equal volumes of medium (double concentrate MEM corrected to pH 6.8-7.2, 10% HIFCS, penicillin [100U/mL] and streptomycin [100µg/mL]), and 1% agarose warmed to 44°C.
2. AdV5-βgal supernatant diluted with PBS + Ca⁺⁺/Mg⁺⁺ to produce the desired test concentrations (normally ranging from 1:10²-1:10⁸ dilution).
3. Culture medium removed from prepared 60mm culture dishes of confluent 293 cells. For the negative control, 200 µL of PBS + Ca⁺⁺/Mg⁺⁺ were added per dish, while 200 µL of the various concentrations of AdV5-βgal supernatant were added to the 60 mm dishes in triplicate. These were then gently rocked to spread the virus and left at room temperature for 30 minutes.
5. Ten mL of the medium/agarose overlay poured over cells in each dish (promptly to avoid solidifying of the agarose) and placed in a 37°C incubator in 5% CO₂/air.
6. A plaque count was performed at day 7. The number of plaques per 60mm dish was counted and the absolute figure recorded. A further count was undertaken on day 10. A calculation of the plaque forming capacity of the supernatant was then determined (eg: 4 x 10⁷ PFUs/mL)

6.2.3.4 Titrating of wild type adenovirus 5 (AdV5)

This was performed initially using the 293 cell line (as above) and then repeated in the A549 cell line with a slightly modified technique.

The method used for the A549 cell line was essentially similar to the method described for titrating AdV5-βgal (6.2.3.3) apart from the following:

1. The medium used in the overlay was DMEM rather than MEM.
2. Additional medium/agarose overlay containing 0.002% neutral red (see Appendix III) was applied to the cells on day 10. This facilitated the identification of plaques when counted on day 14 (Williams 1970).

Titration of wild type AdV5 had also been attempted using HeLa cells and the A549 cell line without the neutral red overlay. These were unsuccessful, as plaques could not be determined in either assay. Therefore, for all subsequent wild type AdV5 titration procedures with A549 cells, with or without ODNs, the neutral red overlay method was used. Experiments were also performed to ascertain the best timing of the addition of neutral red overlay. This was determined to be day 7 with plaque counts performed on days 10 and 14.

6.2.4 Antisense oligodeoxynucleotides

6.2.4.1 Oligodeoxynucleotide design

The design and preparation of the antisense oligodeoxynucleotides (ODNs) used are described below under three phases. This relates to the chronological order that both the ODNs were produced and the experimental studies performed.

Phase 1. Antisense oligodeoxynucleotides (ODNs) to adenovirus E1A, E1B and protein IX genes.

As with the E1A antisense ODN described by Quinlan, ODNs were designed to interact with the relevant RNA at or around the initiation of translation (AUG) codon. Using the known sequence of adenovirus type 5 (Maat 1980), the following antisense oligomers of 20-21 bases in length were manufactured (the base numbers (n) corresponding to the published gene sequence are shown in parentheses). Initial ODNs were manufactured on an oligonucleotide synthesiser by Genosys, UK, but later (and predominantly) at the Rayne Institute, Kings College, London, UK.

E1A antisense (E1AAS) ODN (n 560 – 580) (Quinlan 1993):

5' - GTG GCA GAT AAT ATG TCT CAT - 3'
(molecular weight 6440)

E1B antisense (E1BAS) ODN (n 2019 – 2038)

5' – GA TGG GTT TCT TCG CTC CAT - 3'

(molecular weight 6071)

Protein IX antisense (PIXAS) ODN (n 3609 – 3628):

5' – TC AAA CGA GTT GGT GCT CAT - 3'

(molecular weight 6113)

In initial experiments, a sense ODN for E1A was also used for control purposes.

The sequence was as follows:

E1A sense (E1AS) ODN (n 560 – 580):

5' – ATG AGA CAT ATT ATC TGC CAC – 3'

(molecular weight 6369)

Nonsense ODNs for E1A and PIX were also synthesised to act as specific controls.

These ODNs were of the same composition as the antisense ODNs but of random sequence as follows:

E1A nonsense (E1ANS) ODN:

5' - TAC TTC GAT ATA TGA CAG GTG - 3'

(molecular weight 6440)

Protein IX nonsense (PIXNS) ODN:

5' - TC AAC AGG ATT GTG GCC TAT - 3'

(molecular weight 6113)

The above ODNs were phosphorothioate modified to provide protection from degradation by cellular nucleases. This strategy involved the substitution of a nuclease resistant linkage (phosphorothioate) for the more susceptible phosphodiester linkage at the 3' and 5' ends of the ODN.

Phase 2. Further ODNs were synthesised and assessed in the experimental model. These included ODNs designed from sequences further upstream of the initiation of translation (AUG) codon. Two further ODNs were produced for both the E1A and PIX genes labelled 2 and 3, their respective sequences were as follows:

E1A antisense 2 (E1AAS2) ODN (n 551 – 571):

5' – AAT ATG TCT CAT TTT CAG TCC – 3'

E1A antisense 3 (E1AAS3) ODN (n 542 – 562):

5' – CAT TTT CAG TCC CGG TGT CGG – 3'

PIX antisense 2 (PIXAS2) ODN (n 3600 – 3619):

5' – TTG GTG CTC ATG GCG GCG GC – 3'

PIX antisense 3 (PIXAS3) ODN (n 3592 – 3611):

5' – CAT GGC GGC GGC GGC TGC TG – 3'

These ODNs were also phosphorothioate end-protected.

Phase 3. Using computer software to predict RNA secondary structure, maps were developed for the RNA of both E1A and PIX (see Figures 6.1 and 6.2). From these, potential loop structures of unpaired bases within the RNA could be identified. These present as possible ideal targets for antisense ODNs. As identified in the figures, antisense ODNs were designed for both gene regions of interest. Two ODNs for each region were synthesised, and end-phosphorothioate modified. The sequences were as follows:

E1A antisense 4 (E1AAS4) ODN (n 721 – 740):

5' – CCT CCT CGT TGG GAT CTT CG – 3'

E1A antisense 5 (E1AAS5) ODN (n 891 – 910):

5' – CGG TAC AAG GTT TGG CAT AG – 3'

PIX antisense 4 (PIXAS4) ODN (n 3791 - 3810):

5' – CGG CTG AAG CGG CGG CGG AG – 3'

PIX antisense 5 (PIXAS5) ODN (n 4016 - 4035):

5' – TGT TTT AAA CCG CAT TGG GA – 3'

Assessment of fully-phosphorothioate modified (ie: fully-protected) ODNs was also undertaken. Only fully-phosphorothioated E1AAS and E1ANS ODNs were evaluated.

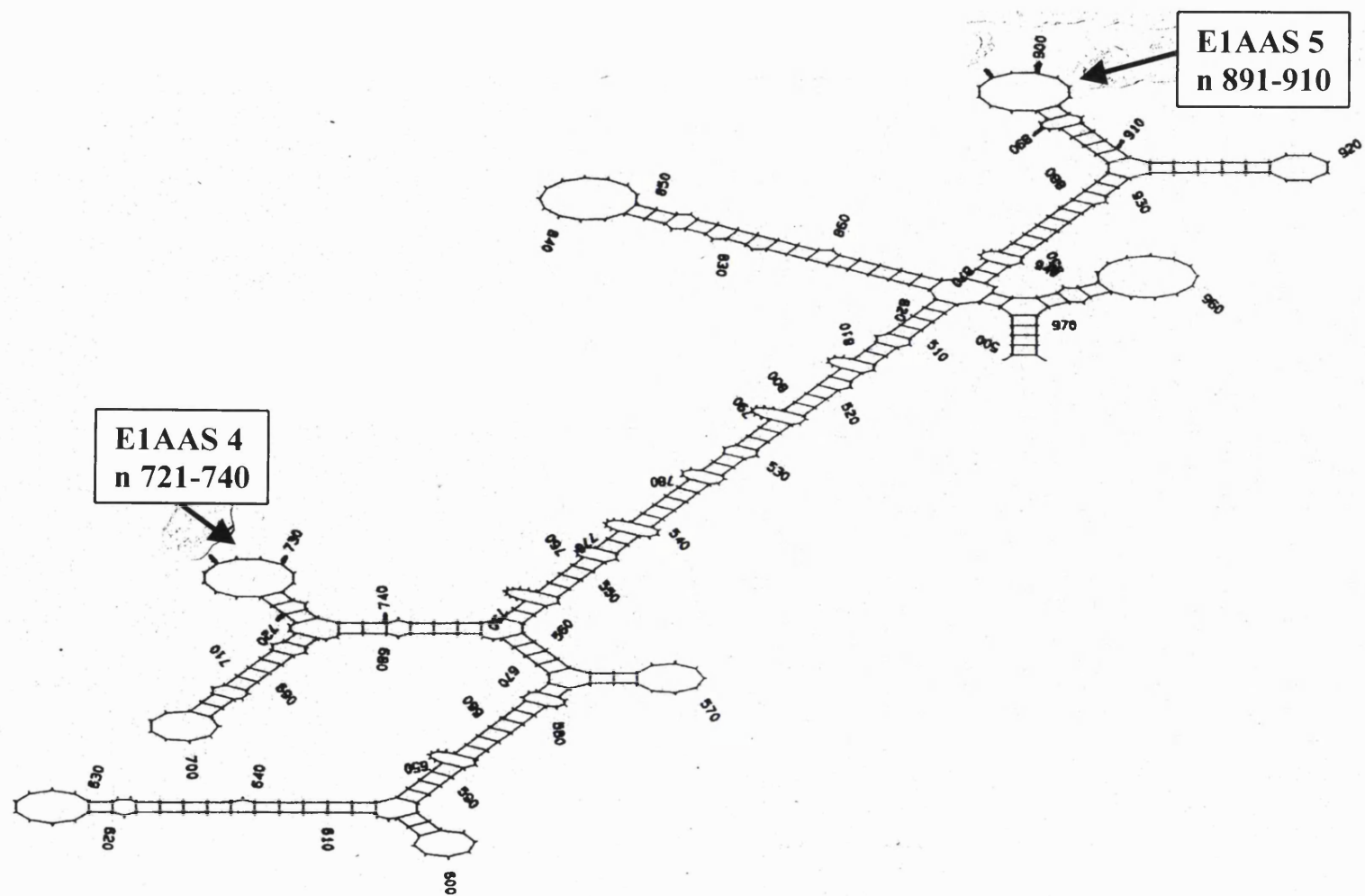


Figure 6.1 Predicted secondary RNA structure of E1A

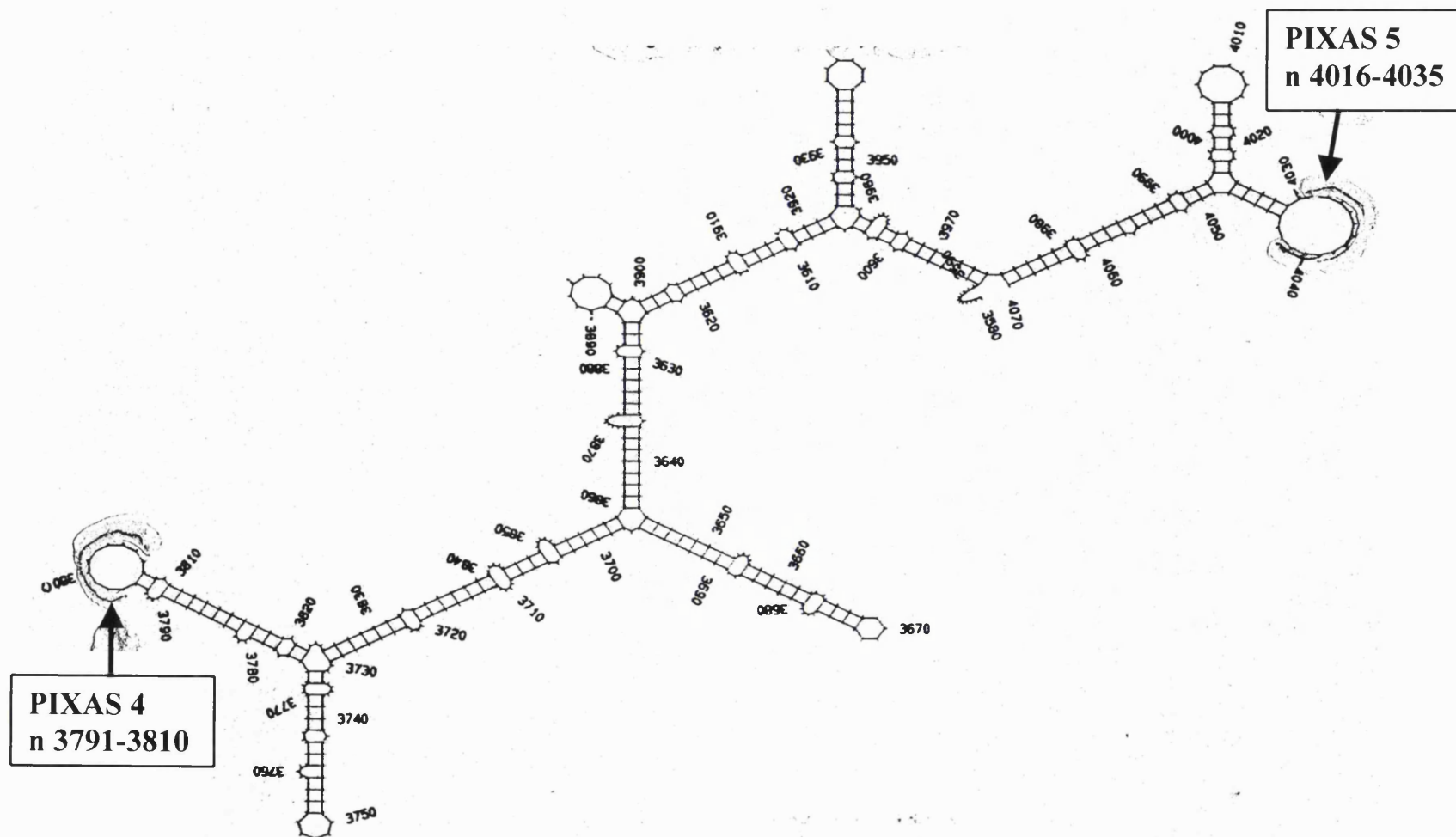


Figure 6.2 Predicted secondary RNA structure of PIX

6.2.4.2 Precipitation of oligodeoxynucleotides

1. Stock solution of oligonucleotide (approximately 0.5µg/µL). 300µL of suspended ODN plus 100µL of water aliquoted into 1.5mL eppendorf tube.
2. Forty µL of 3M Na acetate (pH4.5) added and vortexed.
3. 1.1mL of ethanol (i.e. 2.5 x vol) added and placed at -70°C for at least 30 minutes.
4. Centrifuged at 5000xg at 4°C for 10 minutes to form pellet and kept on ice.
5. One mL of 70% ethanol added as wash, then centrifuged for 5 minutes. Ethanol removed and vacuum dried for 10 minutes.
6. Pellet of ODN resuspended in 20µL of phosphate buffered saline. Optical density (OD) measured to derive concentration.

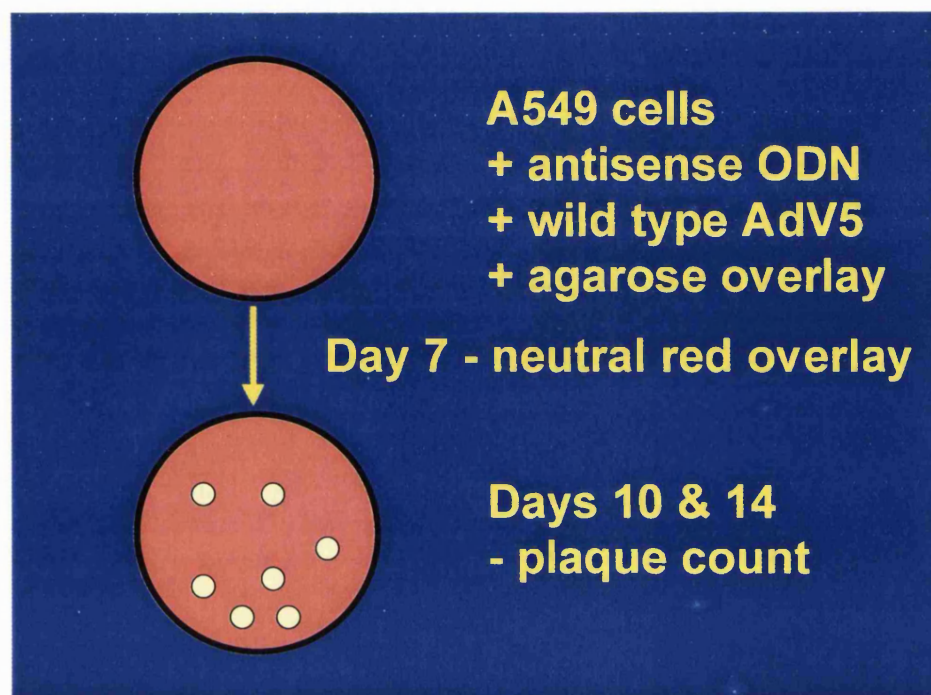
6.2.5 Virus titring with oligodeoxynucleotides (ODNs)

6.2.5.1. Titring of adenovirus 5-betagalactosidase (AdV5-βgal) and wild type adenovirus (AdV5) with oligodeoxynucleotides (ODNs)

For the initial pilot experiments, a small number of ODNs were evaluated in the AdV5-βgal/293 cell line system, while the majority of experiments were performed using the AdV5/A549 cell line protocol. ODNs were evaluated with and without the addition of Lipofectamine.

The titring protocol was similar to those described above, but only the methods for the AdV5/A549 system will be described here. This was the definitive technique used for the oligodeoxynucleotide (ODN) experiments. A simplified version of the method is shown in cartoon form in Figure 6.3.

Figure 6.3 Cartoon depicting method of viral plaque assay system with antisense oligodeoxynucleotides



There were two main alterations in the protocol during the course of the study. These were: 1) performing the experiments in sextuplicate rather than the initial triplicate; and, 2) conversion to 6 well culture trays rather than individual 60mL culture dishes. The introduction of 6 well trays made titring experiments technically easier and required small reductions in the volumes of overlay used – an initial overlay of 8mL, and the neutral red overlay of 4mL.

Titration of wild type adenovirus 5 (AdV5) in A549 cells treated with oligonucleotides (ODNs):

1. Desired concentration of ODN combined with medium (DMEM, 10% HIFCS, penicillin [100U/mL] and streptomycin [100µg/mL]) at 37°C.
2. Medium removed from 60mm culture dishes (or 6 well trays) of confluent 293 cells. Two mL plain medium added to positive and negative control plates/wells and 2mL of medium and ODN to others. Cells incubated at 37°C in 5% CO₂/air for 4 hours.
3. Medium +/- ODN removed from plates or wells and combined with appropriate volumes of medium/agarose overlay (equal volumes of double concentrate DMEM, 10% HIFCS, penicillin [200U/mL] and streptomycin [200µg/mL]), and 1% agarose) at 44°C.
4. 200 µL of PBS+Ca⁺⁺/Mg⁺⁺ added to the negative control cells, while to the positive control and ODN treated cells, 200µL of AdV5 supernatant diluted to yield approximately 20 plaques per plate/well (approximately 1:10⁶ dilution) were added.
5. After incubating at room temperature for 30 minutes, cells +/- virus were covered with 10mL (8mL for 6-well trays) of medium/agarose overlay (with or without ODN) and placed in incubator at 37°C in 5% CO₂/air.
6. Neutral red medium/agarose overlay added on day 7. Five mL (4mL for 6-well trays) of this final preparation were floated over the existing overlay in each of the dishes.
7. Plaque counts were performed on days 10 and 14 respectively.

6.2.5.2 Titration with ODNs and Lipofectamine

E1AAS, E1ANS, PIXAS and PIXNS ODNs were used at a concentration of 1µM. The ODN was suspended in medium without FCS and antibiotics. Lipofectamine was then added to a concentration of 10µg/mL (standard concentration used in ODN

experiments), mixed thoroughly with the ODN and medium, and allowed to bind for 45 minutes at 37°C.

Following the sequence as outlined in Section 6.2.5.2, medium was removed from 6 well dishes of confluent A549 cells and 1mL of the ODN/Lipofectamine/medium solution added to each of the 6 wells and incubated at 37°C in 5% CO₂/air for 5 hours. This solution was then removed and discarded. The cells were subsequently washed twice with 2mL DMEM without serum or antibiotics to remove any residual Lipofectamine. Adenovirus was then applied and titring performed as described above.

6.2.5.3 End-protected versus fully-protected (phosphorothioated) ODNs

Comparison was made between end-protected and fully-protected (phosphorothioated) ODNs (E1AAS and E1ANS). Plaque assay experiments were performed using 1µM concentrations of the respective ODNs as per above protocol.

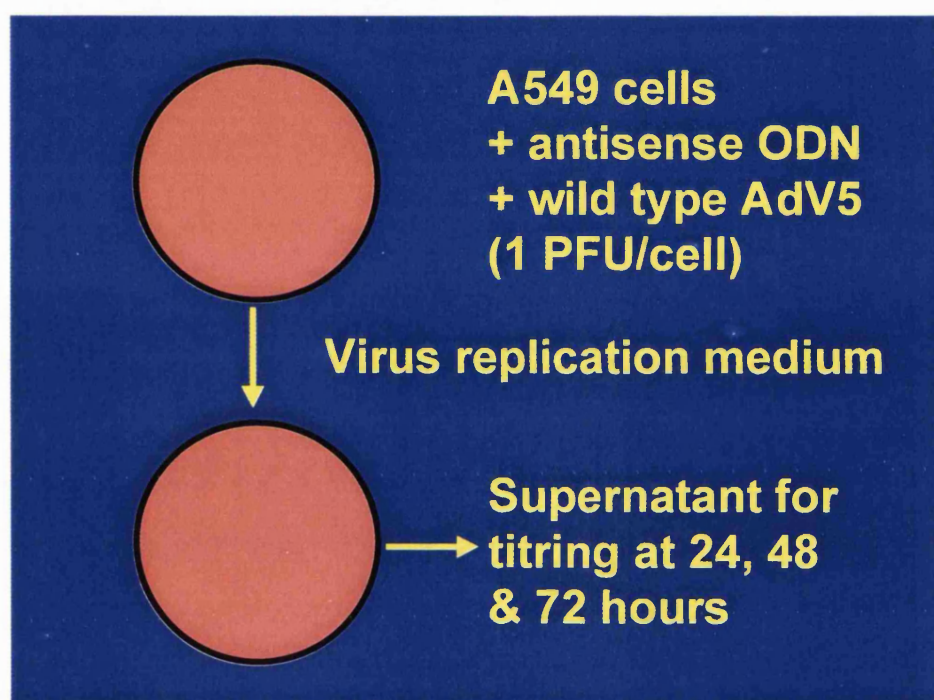
6.2.5.4 Wild type adenovirus 5 (AdV5) propagation for serial titring

1. 3 x 60mL dishes of 80% confluent A549 cells (viable cell count approximately 3.8×10^6 cells/plate).
2. Medium removed and wild type adenovirus 5 supernatant at 1 PFU/cell, (i.e. 3.8×10^6 PFUs/dish) suspended in PBS+Ca⁺⁺/Mg⁺⁺ added to the positive control dishes. PBS+ Ca⁺⁺/Mg⁺⁺ only to the negative control dish.
3. 8mL medium (DMEM, 2% HIFCS, 2mM L-glutamine, penicillin [100U/mL] and streptomycin [100µg/mL]) per plate.
4. One mL of supernatant removed from all three dishes at 24, 48, 72 and 96 hours and centrifuged at 200xg for 5 mins for later titring. Supernatant stored in 10% glycerol at -40°C.

6.2.5.5 Wild type adenovirus 5 (AdV5) propagation with ODNs for serial titring

Protocol as per 6.2.5.4, but with cells incubated with ODNs at a concentration of 1 μ M prior to the administration of virus. See Figure 6.4 for outline of technique.

Figure 6.4 Cartoon describing method of serial titring assay with antisense oligodeoxynucleotides



6.2.5.6 Serial titring

Controls were titred using standard methods for plaque counting as per 6.2.3.4. The supernatant collected at 24 hours was titred at dilutions of 1:1, 1:10² and 1:10⁴. At 48 hours 1:10⁴, 1:10⁶, 1:10⁸ dilutions were performed and at 72 hours 1:10⁶, 1:10⁸, 1:10¹⁰ were performed. Results of control titring indicated that at 24 hours 1:10²

dilution should be performed, at 48 hours $1:10^4$ concentration, and at 72 hours $1:10^6$ dilution.

After the first ODN experiment, it was realised that the dilutions of the supernatant at 24 and 48 hours required increasing, as the number of plaques formed was too great. Therefore, dilutions of $1:10^3$ and $1:10^5$ were used at 24 and 48 hours respectively for the subsequent experiment.

6.2.6 Statistical methods

Plaque counts were performed in a blinded fashion, predominantly in the presence of two observers. Data are presented as absolute plaque counts with median values. Comparison of median values of plaque counts was made between ODN treated samples and nonsense control samples, rather than positive controls, in order to eliminate any non-specific ODN effects. Non-parametric methods were employed for formal statistical analysis (Mann-Whitney analysis). All analyses were performed on Minitab (Release 11.21) for PCs. A p value of less than 0.05 was considered significant.

A meta-analysis of the results for E1AAS ODN (at $1\mu\text{M}$ concentration), the most commonly used ODN, was performed. The Fisher combined test was used for this analysis (Wolf 1986). This test analyses the combined results of a number of independent experiments planned to test a common hypothesis. The method is based on the product of probabilities from the different trials and will ultimately yield a p value for the combined results.

6.3 Results

This section has been subdivided into two major parts. The first describes how plaques were identified and titring defined, and in addition, briefly reviews the results of the initial pilot experiments. The second details the results of the definitive experiments involving ODNs under various conditions as outlined in the methods section.

6.3.1 Viral titring in 293 and A549 cell lines

The identification of plaques, indicating localised areas of cell death (viral cytopathic effect, CPE) was relatively straightforward in both the 293 and A549 cell lines, once the technique had been refined with the use of the neutral red overlay. This enabled easier detection of plaques (Figure 6.5). Whole plaques were counted and recorded on days 7 and 10 for the 293 cell system and on days 10 and 14 for the A549 cell system. Plaque counts were performed in a blinded fashion, and checked by a second observer in approximately 75% of experiments. There was no significant inter-observer error noted. Between 10-30 plaques per culture dish (or well) were considered an ideal number for the positive control sample. The amount of virus added to each culture dish was thus calibrated in an attempt to achieve this ideal number (ie: 10-30 plaque forming units per dish). Obviously, minor variations in experimental conditions occurred and so absolute plaque numbers could vary between experiments.

Figure 6.5

(a) Cell culture dish of A549 cells with no added virus (negative control)

Cells remain intact showing no evidence of plaque formation.



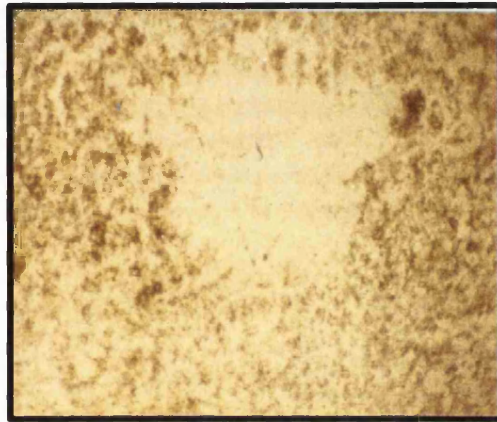
(b) Positive control cell culture dish of A549 cells with wild type adenovirus 5

Plaques identified by marker pen. Note the discrete pale area surrounding the mark.



(c) Plaques at higher magnification (x40)

These can be seen more clearly as areas of necrosed cells.



In the pilot antisense ODN experiments using the 293 cell line/adenovirus 5 β -gal system, antisense ODNs assessed included E1AAS, E1BAS, PIXAS. Control ODNs comprised E1AS and E1ANS. These experiments enabled refinement of the methodology, although results were inconclusive in demonstrating an antisense effect.

At this point, further assessment of antisense ODNs in modifying the cytopathic effect of adenovirus was explored in an *in vitro* system utilising wild type adenovirus and a lung-derived cell line. This was considered more reflective of the clinical situation. Thus, the A549 cell line and wild type adenovirus 5 system was developed.

6.3.2 Antisense ODNs in A549 cell line/wild type adenovirus 5 system

The viral plaque assay system was developed using A549 cells, a cell line derived from bronchial carcinoma cells, and wild type adenovirus 5. The methods were easily transferable from the 293 cell/adenovirus 5 β -gal system. Wild type adenovirus 5 was both propagated and titred in the A549 cell line (see methods section 6.2.3.2 and 6.2.3.4). Initially, E1AAS and PIXAS ODNs, and the corresponding control ODNs, were evaluated.

6.3.2.1 E1AAS and PIXAS ODNs at 15 μ M concentration

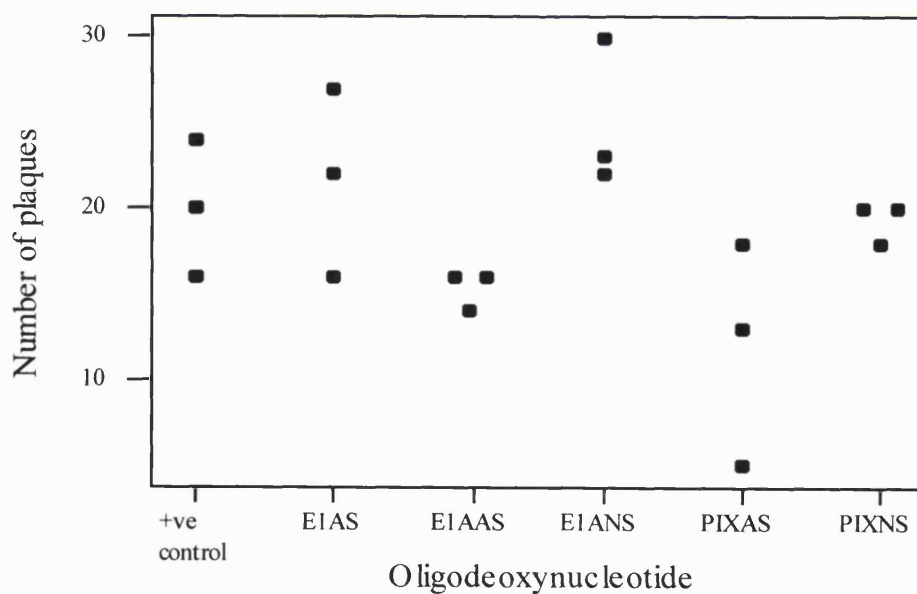
E1AAS, PIXAS, E1AS, E1ANS, PIXNS ODNs were added at a concentration of 15 μ M using the methods described above. The preparations were performed in triplicate. As there was only a small number of data points, formal statistical evaluation could not be performed. However, there was a strong trend for reduced plaque formation in the A549 cells treated with E1AAS and PIXAS ODNs compared with the control ODNs. There were reductions in plaque counts in the E1A antisense (E1AAS) ODN treated cells when compared with the sense (E1AS) and nonsense (E1ANS) control ODN treated cells. This was observed both on day 10 and day 14. Furthermore, PIX antisense (PIXAS) ODN treated cells had less plaque formation than the nonsense (PIXNS) control ODN cells at both days 10 and 14 (Table 6.1 and Figure 6.6).

Table 6.1 **Plaque counts in A549 cells using various ODNs, following wild type adenovirus 5 application**

A549 cell preparation	Plaque no. day 10	Plaque no. day 14
Negative control	0, 0, 0	0, 0, 0
Positive control	14, 18, 20	16, 20, 24
E1AAS (15 μ M)	11, 12, 12	14, 16, 16
E1AS (15 μ M)	15, 20, 27	16, 22, 27
E1ANS (15 μ M)	20, 21, 27	22, 23, 30
PIXAS (15 μ M)	4, 11, 12	5, 13, 18
PIXNS (15 μ M)	15, 17, 19	18, 20, 20

Each preparation performed in triplicate with ODNs added at 15 μ M concentration. Results are presented as absolute plaque counts.

Figure 6.6 **Plaque counts at day 14 in A549 cells treated with ODNs (15 μ M concentration) following wild type adenovirus 5 application (as per Table 6.1)**



6.3.2.2 ODNs at varying concentrations

To determine whether altering the concentration of ODN affects *in vitro* inhibition of adenovirus CPE, experiments were performed using different concentrations of E1AAS ODN. The initial pilot experiment compared 1 μ M, 5 μ M, 15 μ M, and 30 μ M concentrations of the targeted ODNs together with their respective nonsense control ODNs. However, there was no additional inhibitory effect from increasing the concentration of either antisense ODN to 30 μ M (data not shown).

Interestingly, it appeared that a lower concentration of ODN might still contribute substantially in reducing adenovirus CPE. Therefore, a definitive experiment was performed assessing the lower concentration (1 μ M) of E1AAS ODN and comparing this with the standard 15 μ M concentration, previously shown to have an inhibitory effect.

Plaque numbers from cells with E1ANS at 15 μ M were not significantly different to those in the positive control cells. When counts for the two negative control concentrations were pooled, there was no difference between this and the positive control count. As the plaque count for the E1ANS ODN at 1 μ M concentration was depleted, comparison was only made with the E1ANS 15 μ M nonsense control. Plaque formation at day 14 for both the 1 μ M and 15 μ M E1AAS ODN treated cells was reduced compared with the E1ANS 15 μ M nonsense control treated cells ($p=0.007$ and $p=0.02$, respectively, Table 6.2, Figure 6.7). There was no evidence of benefit in using the higher concentration of E1AAS ODN, with an improved reduction in plaque formation at the lower concentration. This between-concentration difference in plaque formation approached significance at the five percent level ($p=0.08$). It was therefore concluded that there was no enhancement of inhibition of plaque formation when using the higher concentrations of antisense ODN. Thus, subsequent experiments incorporated the use of antisense ODNs at 1 μ M concentration.

Table 6.2 Plaque counts in A549 cells using E1AAS ODN at 1 μ M and 15 μ M concentrations, following wild type adenovirus 5 application

A549 cell preparation	Plaque no. day 14	Median	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		
Positive control	19, 20, 20, 21, 28, 31	20.5	E1ANS 15 μ M p=0.47 E1ANS 1 & 15 μ M p=0.37
E1AAS (1 μ M)	13, 13, 17, 17, 18, 20	17.0	p=0.007*
E1ANS (1 μ M)	21, 23, 28, N/A, N/A, N/A	23.0	
E1AAS (15 μ M)	16, 19, 19, 20, 22, 22	19.5	p=0.02
E1ANS (15 μ M)	20, 22, 23, 23, 24, 29	23.0	
E1AAS (1 μ M) versus E1AAS (15 μ M)			p=0.08

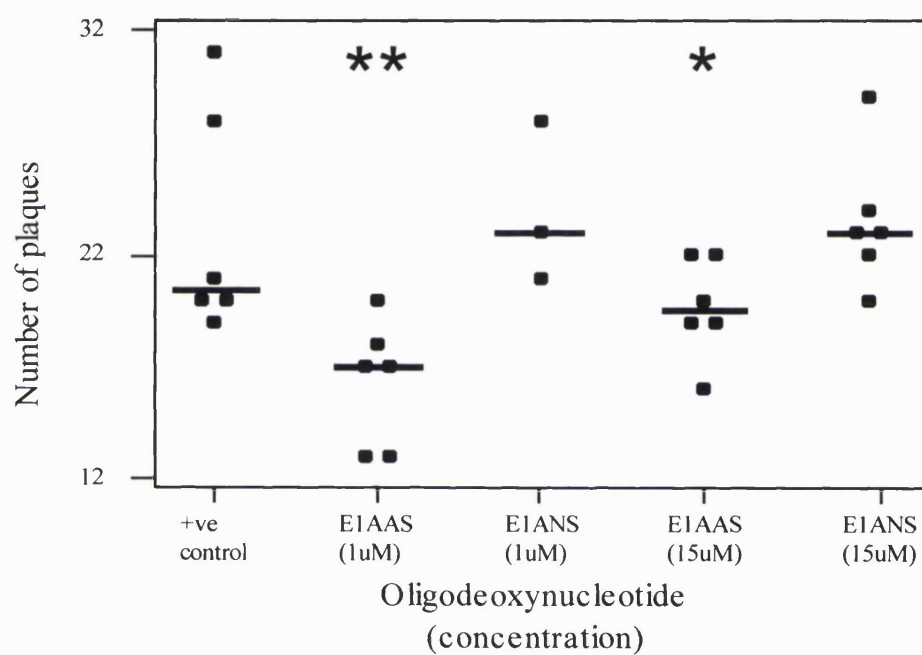
Results are presented as absolute plaque counts (in sextuplicate), with median values.

N/A, not available – plaque count void.

Comparison with nonsense controls by Mann-Whitney analysis.

*Comparison with E1ANS 15 μ M nonsense control, as only 3 data points for E1ANS control.

Figure 6.7 Plaque counts at day 14 in A549 cells using E1AAS ODNs at 1 μ M and 15 μ M concentrations, following wild type adenovirus 5 application (as per Table 6.2)



Median values are represented as horizontal bar.

* $p < 0.05$ compared with E1ANS (15 μ M)

** $p < 0.01$ compared with E1ANS (15 μ M)

6.3.2.3 Antisense ODNs plus Lipofectamine

Lipofectamine, being a cationic lipid preparation, facilitates the entry of DNA into cells. Its addition to the experimental protocol could be expected to aid the entry of antisense ODNs into target cells thereby increasing their antisense effect. Therefore, evaluation of the effect of Lipofectamine in enhancing the inhibition of adenovirus CPE was undertaken. The methods were described in section 6.2.3.8.

In this experiment, Lipofectamine was added at a concentration of 10µg/mL to all preparations together with the ODN at 1µM. The resultant plaque counts are shown in Table 6.3. Statistical analysis failed to demonstrate any difference in median plaque numbers between antisense ODN treated cells and nonsense control cells at day 10. However, day 14 median plaque numbers were significantly lower in the PIXAS ODN treated cells ($p=0.02$), but not the E1AAS ODN treated cells ($p=0.22$) when compared with the nonsense controls. There were no statistically significant differences in plaque counts between the positive and the nonsense controls, suggesting that Lipofectamine did not affect the plaque assay.

Table 6.3 **Plaque counts in A549 cells using E1AAS and PIXAS ODNs at 1 μ M concentration with Lipofectamine (10 μ g/mL), following wild type adenovirus 5 application**

A549 cell preparation	Plaque no. day 10 (median)	Comparison with nonsense control	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		0, 0, 0, 0, 0, 0	
Positive control	2, 4, 6, 6, 8, 9 (6.0)	E1ANS p=0.75 PIXNS p=0.69	11, 14, 15, 16, 16, 16 (15.5)	E1ANS p=0.51 PIXNS p=1.00
E1AAS (1 μ M)	4, 4, 6, 6, 7, 11 (6.0)	p=0.75	10, 10, 11, 13, 14, 15 (12.0)	p=0.22
E1ANS (1 μ M)	3, 5, 6, 7, 8, 12 (6.5)		10, 10, 16, 18, 18, 25 (17.0)	
PIXAS (1 μ M)	2, 3, 4, 4, 7, 8 (4.0)	p=0.63	8, 9, 10, 10, 13, 14 (10.0)	p=0.02
PIXNS (1 μ M)	2, 4, 5, 5, 6, 10 (5.0)		13, 14, 14, 15, 17, 19 (14.5)	

Results are presented as absolute plaque counts (in sextuplicate) together with median values. Comparison with nonsense control by Mann-Whitney analysis.

At this point, the experimental protocol was altered to ensure that the added Lipofectamine was not itself inhibited by other factors. Accordingly, Lipofectamine was combined with the ODN in Optimem medium without foetal calf serum and without antibiotics, while only the E1AAS ODN was evaluated together with its nonsense control.

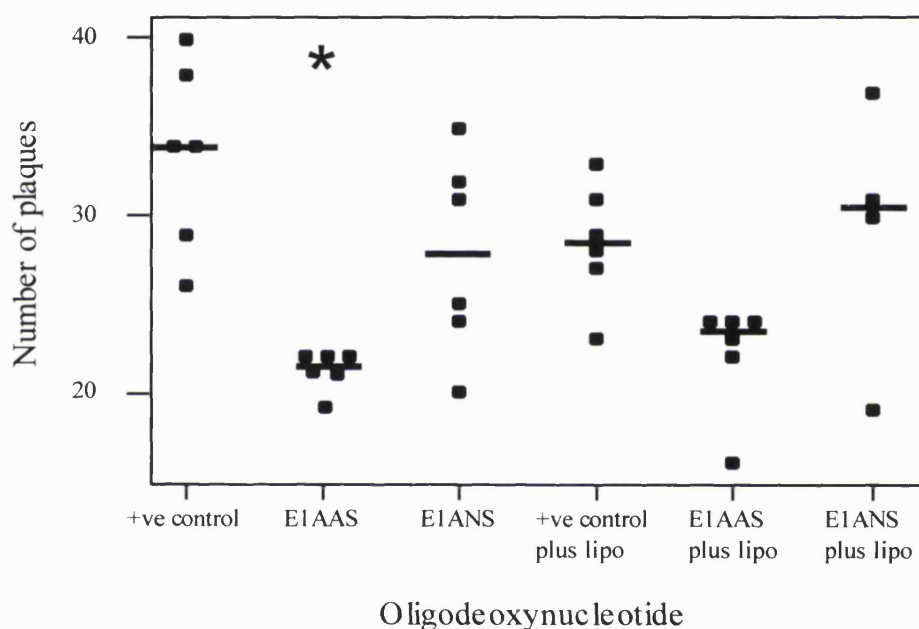
On this occasion plaque counts were performed on day 14 only. Plaque counts were significantly reduced in the E1AAS treated cells ($p=0.04$), only in the absence of Lipofectamine, as compared with nonsense controls. There was no difference in plaque counts in the nonsense ODN treated cells compared with the positive controls (Table 6.4). Thus, despite the modifications, this experiment failed to demonstrate that the addition of Lipofectamine produced any significant enhancement of plaque reduction.

Table 6.4 **Plaque counts in A549 cells using E1AAS ODNs at 1 μ M concentration in the absence and presence of Lipofectamine (10 μ g/mL, without FCS or antibiotics), following wild type adenovirus 5 application**

A549 cell preparation	Plaque no. day 14	Median	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		
Positive control (without lipofectamine)	26, 29, 34, 34, 38, 40	34.0	p=0.13
Positive control (with lipofectamine)	23, 27, 28, 29, 31, 33	28.5	p=0.67
E1AAS (1 μ M) (without lipofectamine)	19, 21, 21, 22, 22, 22	21.5	p=0.04
E1ANS (1 μ M) (without lipofectamine)	20, 24, 25, 31, 32, 35	28.0	
E1AAS (1 μ M) (with lipofectamine)	16, 22, 23, 24, 24, 24,	23.5	p=0.16
E1ANS (1 μ M) (with lipofectamine)	19, 30, 31, 37 N/A, N/A	30.5	

Results are presented as absolute plaque counts (in sextuplicate) together with median values.
Comparison with nonsense control by Mann-Whitney analysis.
N/A, not available – plaque count void.

Figure 6.8 Plaque counts at day 14 in A549 cells using E1AAS ODNs at 1 μ M concentration in the presence or absence of Lipofectamine (10 μ g/mL, without FCS or antibiotics), following wild type adenovirus 5 application (as per Table 6.4)



Median values are represented as horizontal bar.

lipo=Lipofectamine

* $p < 0.05$ compared with nonsense control.

6.3.2.4 End-protected versus fully-protected antisense ODNs

Using standard methodology (see 6.2.5.3), the first experiment showed no significant reduction in plaque numbers in either the end-protected or fully-protected antisense ODN treated cells when compared with nonsense controls (Table 6.5).

There was also no difference in the number of plaques in the nonsense compared with the positive controls. However, there were relatively small numbers of plaques observed in all preparations. Interestingly, there was a reduced number of plaques in the end-protected E1AAS ODN treated cells compared with the fully-protected E1AAS ODN treated cells at day 14 ($p=0.01$), suggesting that end-protected ODNs provided greater reduction of viral cytopathic effect.

On repeat experiment, there were statistically significant reductions in the number of plaques in the end-protected E1AAS ODN treated cells when compared with the nonsense controls at both day 10 and 14 ($p=0.005$ and $p=0.005$ respectively). This was true for the fully-protected E1AAS ODN treated cells only at day 14 ($p=0.05$, Table 6.6 and Figure 6.9). However, there was no difference between end-protected and fully-protected ODNs. The conclusion from these experiments was that there was no additional benefit of fully-phosphorothioated ODNs compared with end-phosphorothioated ODNs in reducing viral cytopathic effect.

Table 6.5 **Plaque counts in A549 cells using end-protected and fully-protected E1AAS ODNs at 1 μ M concentration, following wild type adenovirus 5 application**

A549 cell preparation	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0	
Positive control	4, 5, 5, 5, 6, 9 (5.0)	E1ANS p=0.46 E1ANS FP p=0.22
E1AAS (1 μ M)	2, 3, 3, 3, 4, 6 (3.0)	p=0.12
E1ANS (1 μ M)	3, 4, 4, 5, 6, 7 (4.5)	
E1AAS (1 μ M) FP	6, 6, 6, 8, 8, 9 (7.0)	p=0.93
E1ANS (1 μ M) FP	1, 6, 7, 7, 9, 10 (7.0)	
E1AAS versus E1AAS FP		p=0.01

Results are presented as absolute plaque counts (in sextuplicate) together with median values.

FP, fully-phosphorothioated bases, ie: fully-protected

Comparison with nonsense control and between end-protected and fully-protected by Mann-Whitney analysis.

Table 6.6 **Plaque counts in A549 cells using end-protected and fully-protected E1AAS ODNs at 1 μ M concentration, following wild type adenovirus 5 application**

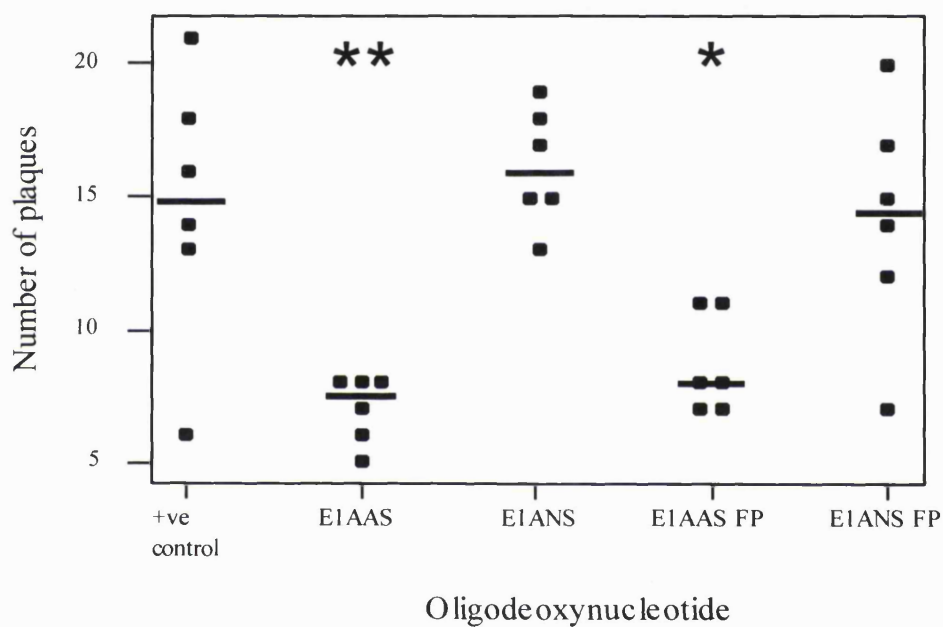
A549 cell preparation	Plaque no. day 10 (median)	Comparison with nonsense control	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		0, 0, 0, 0, 0, 0	
Positive control	4, 8, 10, 11, 18, 19 (10.5)	E1ANS p=0.57 E1ANS FP p=0.94	6, 13, 14, 16, 18, 21 (15.0)	E1ANS p=0.69 E1ANS FP p=0.87
E1AAS (1 μ M)	5, 5, 6, 7, 7, 8 (6.5)	p=0.005	5, 6, 7, 8, 8, 8 (7.5)	p=0.005
E1ANS (1 μ M)	10, 11, 12, 14, 14, 17 (13.0)		13, 15, 15, 17, 18, 19 (16.0)	
E1AAS (1 μ M) FP	5, 6, 6, 8, 8, 11 (7.0)	p=0.12	7, 7, 8, 8, 11, 11 (8.0)	p=0.05
E1ANS (1 μ M) FP	3, 10, 10, 12, 13, 15 (11.0)		7, 12, 14, 15, 17, 20 (14.5)	
E1AAS versus E1AAS FP		p=0.46		p=0.21

Results are presented as absolute plaque counts (in sextuplicate) together with median values.

FP, fully-phosphorothioated bases, ie fully-protected.

Comparison with nonsense control and between end-protected and fully-protected by Mann-Whitney analysis.

Figure 6.9 Plaque counts at day 14 in A549 cells using end-protected and fully-protected E1AAS ODNs at 1 μ M concentration, following wild type adenovirus 5 application (as per Table 6.6)



Median values are represented as horizontal bar.
 FP, fully-phosphorothioated or fully-protected.
 *p=0.05 when compared with nonsense control.
 **p<0.01 when compared with nonsense control.

6.3.2.5 Additional ODNs

As described in section 6.2.4.1, further ODNs were designed and evaluated for their inhibitory function on adenovirus cytopathic effect. The E1AAS ODN series 2 and 3 were designed to straddle the AUG initiation of translation codon, while the ODN series 4 and 5 were designed after computer assisted reconstruction of the relevant mRNAs was performed. The experimental methodology comprised the use of 1 μ M concentration ODN in the absence of Lipofectamine, as this had been shown previously not to be of value in the A549 cells.

Results from the first series (2 and 3) are shown in Table 6.7 and graphically in Figure 6.10. These indicate no reduction in plaque formation for all three E1AAS ODNs compared to the nonsense control ODN. Furthermore, the newly designed protein IX antisense ODNs, PIXAS2 and PIXAS3, showed no reduction in plaque formation when compared with the nonsense control (Figure 6.11).

Results from the second series of experiments using ODNs based on computer reconstructions of relevant mRNA (ODNs 4 and 5) are shown in Table 6.8. There was no difference in the number of plaques in the nonsense compared with the positive controls. On this occasion, the original E1AAS ODN showed a significant reduction in plaque formation at both day 10 ($p=0.01$) and day 14 ($p=0.006$) compared to the nonsense control. The E1AAS4 ODN treated cells also yielded a significant reduction in the number of plaques at day 10 ($p=0.02$), and at day 14 ($p=0.04$). However, the E1AAS5 ODN was not associated with a reduction in plaque formation at either day 10 or 14. In addition, there was no reduction in the number of plaques formed with the use of PIXAS4 or PIXAS5 ODNs when compared with the E1ANS nonsense control (Figure 6.13).

Table 6.7 **Plaque counts in A549 cells with E1AAS, E1AAS2, E1AAS3, PIXAS, PIXAS2, PIXAS3 ODNs at 1 μ M concentration, following wild type adenovirus 5 application**

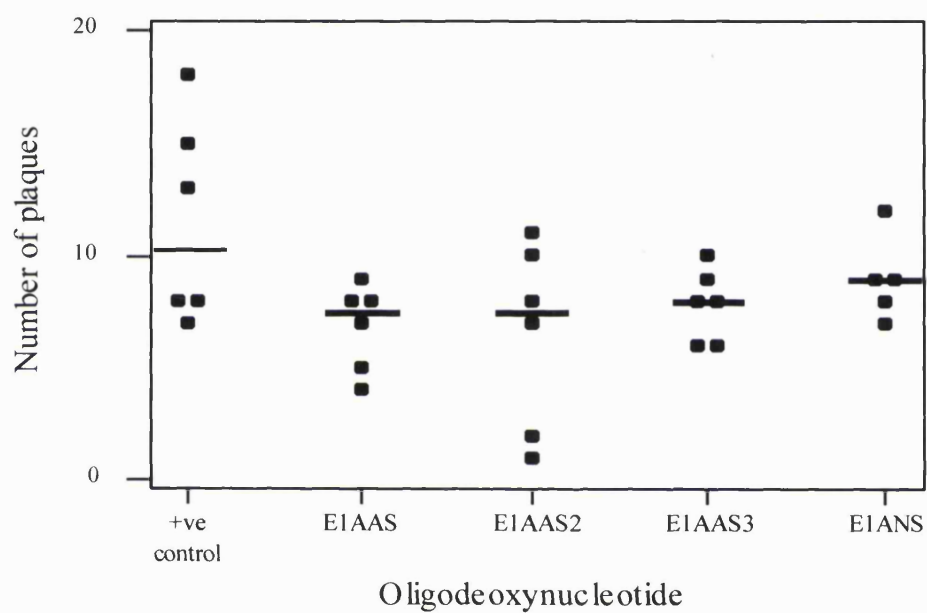
A549 cell preparation	Plaque no. day 10 (median)	Comparison with nonsense control	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		0, 0, 0, 0, 0, 0	
Positive control	5, 6, 6, 9, 13, 16 (7.5)	E1ANS p=0.36 PIXNS p=0.57	7, 8, 8, 13, 15, 18 (10.5)	E1ANS p=0.57 PIXNS p=0.57
E1AAS (1 μ M)	3, 5, 7, 7, 8, 8, (7.0)	p=0.85	4, 5, 7, 8, 8, 9 (7.5)	p=0.14
E1AAS2 (1 μ M)	0, 2, 3, 6, 7, 8 (4.5)	p=0.41	1, 2, 7, 8, 10, 11 (7.5)	p=0.41
E1AAS3 (1 μ M)	2, 5, 5, 5, 5, 6 (5.0)	p=0.25	6, 6, 8, 8, 9, 10 (8.0)	p=0.40
E1ANS (1 μ M)	4, 5, 6, 7, 11, N/A (6.0)		7, 8, 9, 9, 12, N/A (9.0)	
PIXAS (1 μ M)	3, 5, 7, 7, 9, 12 (7.0)	p=0.94	6, 7, 8, 9, 9, 13 (8.5)	p=0.63
PIXAS2 (1 μ M)	5, 6, 9, 12, 14, 16 (10.5)	p=0.23	6, 9, 10, 12, 14, 16 (11.0)	p=0.63
PIXAS3 (1 μ M)	5, 5, 6, 7, 9, 11 (6.5)	p=1.0	6, 9, 9, 11, 11, 14 (10.0)	p=0.81
PIXNS (1 μ M)	4, 5, 7, 8, 9, 11 (7.5)		5, 7, 9, 10, 11, 15 (9.5)	

Results are presented as absolute plaque counts (in sextuplicate) together with median values.

N/A, not available – plaque count void.

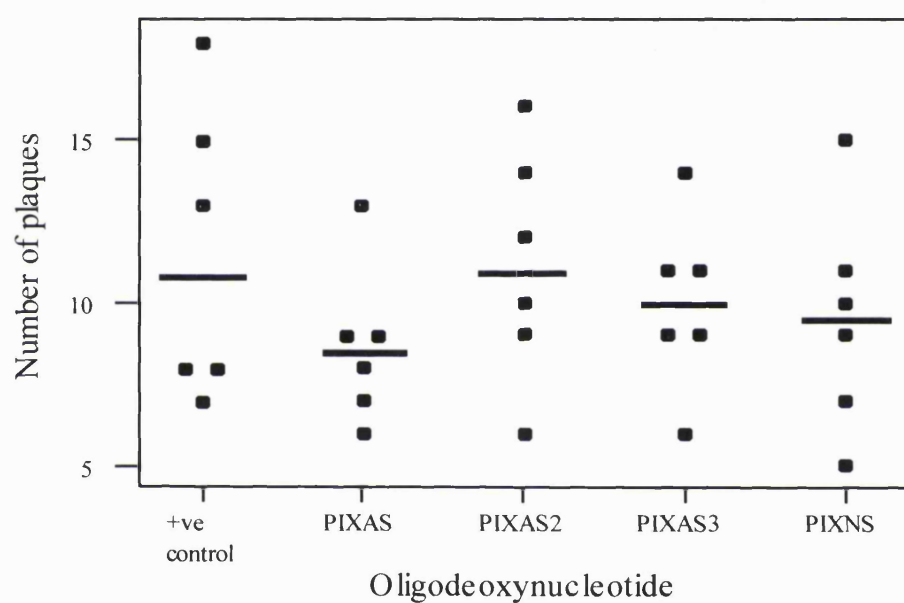
Comparison with nonsense control by Mann-Whitney analysis.

Figure 6.10 Plaque counts at day 14 in A549 cells with E1AAS, E1AAS2, E1AAS3 ODNs at 1 μ M concentration, following wild type adenovirus 5 application (as per Table 6.7)



Median values are represented as horizontal bar.

Figure 6.11 Plaque counts at day 14 in A549 cells with PIXAS, PIXAS2, PIXAS3 ODNs at 1 μ M concentration, following wild type adenovirus 5 application (as per Table 6.7)



Median values are represented as horizontal bar.

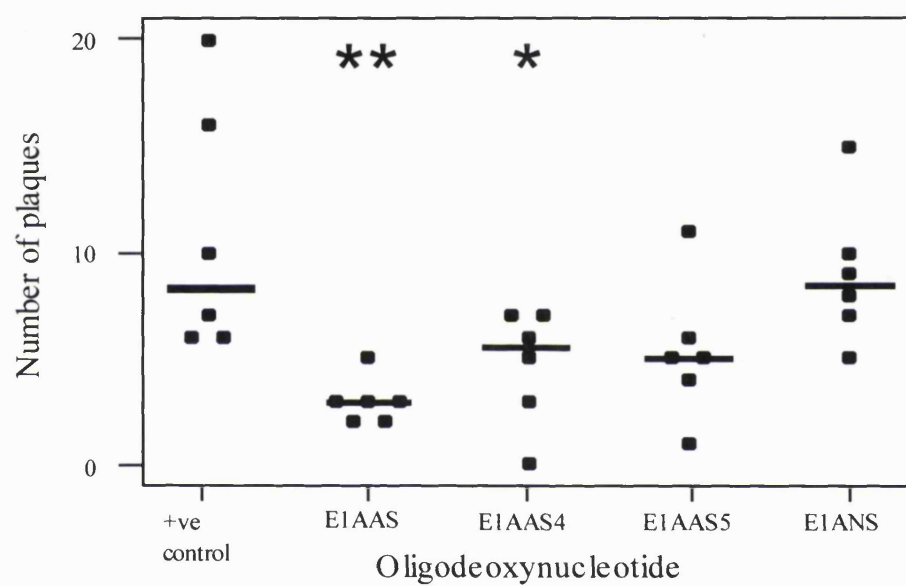
Table 6.8 **Plaque counts in A549 cells with E1AAS, E1AAS4, E1AAS5, PIXAS4, PIXAS5 ODNs at 1 μ M concentration, following wild type adenovirus 5 application**

A549 cell preparation	Plaque no. day 10 (median)	Comparison with nonsense control	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control	0, 0, 0, 0, 0, 0		0, 0, 0, 0, 0, 0	
Positive control	2, 4, 4, 6, 12, 16 (5.0)	E1ANS p=1.0	6, 6, 7, 10, 16, 20 (8.5)	E1ANS p=0.81
E1AAS (1 μ M)	0, 1, 1, 2, 2, 3 (1.5)	p=0.01	2, 2, 3, 3, 3, 5 (3.0)	p=0.006
E1AAS4 (1 μ M)	0, 1, 2, 3, 4, 4 (2.5)	p=0.02	0, 3, 5, 6, 7, 7 (5.5)	p=0.04
E1AAS5 (1 μ M)	1, 1, 2, 4, 4, 9 (3.0)	p=0.14	1, 4, 5, 5, 6, 11 (5.0)	p=0.09
E1ANS (1 μ M)	2, 5, 5, 6, 6, 8 (5.5)		5, 7, 8, 9, 10, 15 (8.5)	
PIXAS4 (1 μ M)	3, 4, 6, 6, 8, 9 (6.0)	p=0.63*	6, 6, 7, 10, 11, 12 (8.5)	p=1.0*
PIXAS5 (1 μ M)	3, 3, 5, 5, 9, 9 (5.0)	p=1.00 *	5, 9, 9, 10, 10, 12 (9.5)	p=0.63*

Results are presented as absolute plaque counts (in sextuplicate) together with median values.
Comparison with nonsense control by Mann-Whitney analysis.

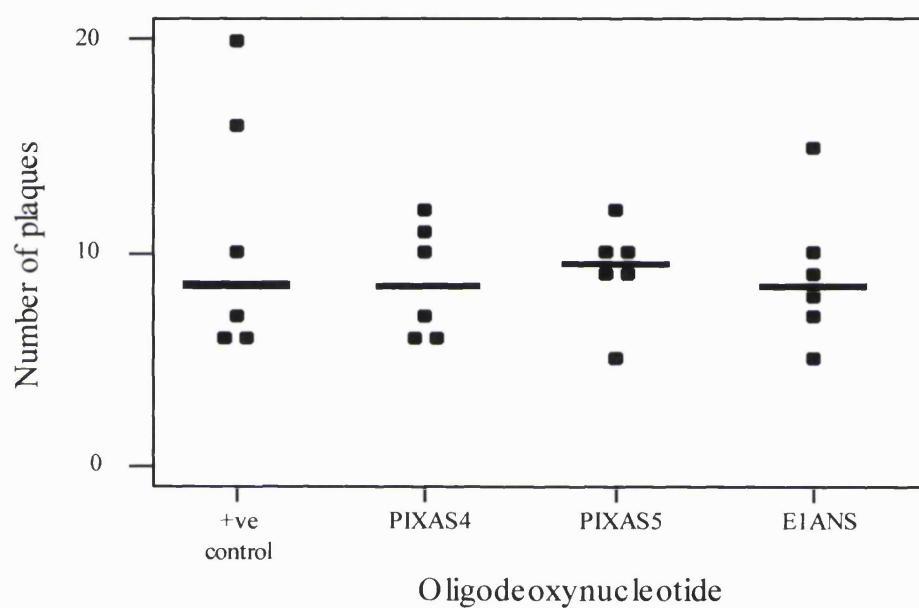
* Comparison with E1A nonsense control (E1ANS)

Figure 6.12 Plaque counts at day 14 in A549 cells with E1AAS, E1AAS4, E1AAS5 ODNs at 1 μ M concentration, following wild type adenovirus 5 application (as per Table 6.8)



Median values are represented as horizontal bar.
 * $p < 0.05$ when compared with nonsense control.
 ** $p < 0.01$ when compared with nonsense control.

Figure 6.13 Plaque counts at day 14 in A549 cells with PIXAS4 and PIXAS5 ODNs at 1 μ M concentration, following wild type adenovirus 5 application (as per Table 6.8)



Median values are represented as horizontal bar.

6.3.2.6 Meta-analysis of E1AAS effect

A meta-analysis (Fisher combined test) investigated the combined results of all the experiments in which the effects of 1 μ M E1AAS ODNs (end-phosphorothioated, without Lipofectamine) were compared with the effects of the appropriate nonsense controls. A second meta-analysis used similar combined experimental results to compare these ODNs with the positive controls.

These meta-analyses, with their greater statistical power, demonstrated highly significant differences between the E1AAS ODN treated cells and both the nonsense E1ANS ODN treated cells ($p < 0.005$) and the positive controls ($p < 0.005$). The results of these analyses strongly suggest that the E1AAS ODN reduces adenoviral plaque formation *in vitro*, and that this is a specific antisense effect.

Table 6.9 Meta-analysis of E1AAS inhibition of viral plaque formation

Analysis	p value of comparison with nonsense control
Table 6.3	p=0.22
Table 6.4	p=0.0435
Table 6.5	p=0.12
Table 6.6	p=0.0047
Table 6.7	p=0.14
Table 6.8	p=0.0059
Meta-analysis	p<0.005

6.3.2.7 Serial titring

The initial serial titring experiment was performed in triplicate, using E1AAS and PIXAS ODNs together with their respective nonsense controls. Titring was performed in triplicate on the supernatant samples collected at 24, 48 and 72 hours from each of the preparations. Plaque numbers were increased in all samples at 24 and 48 hours with no observed reduction in plaque numbers in the antisense ODN treated samples (Table 6.10). Serial titring at 72 hours did show a trend for a reduction in the number of plaques both in the E1AAS and PIXAS treated samples compared to nonsense controls.

In the subsequent experiment, the 24 and 48 hour supernatant samples were diluted further to $1:10^3$ and $1:10^5$ respectively in order to produce less unwieldy plaque counts. Titring was performed in sextuplicate. On this occasion only the E1AAS ODN was evaluated, but there was no observable reduction in plaque counts, as compared with the nonsense control, at any of the time points (Table 6.11, Figure 6.14). Interestingly, there was a statistically significant increase in the number of plaques formed in the ODN treated cells compared with the positive controls at 24 and 48 hours.

Table 6.10 Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS, E1ANS, PIXAS and PIXNS ODNs at 1µM concentration

A549 cell preparation	Plaque no. day 10	Plaque no. day 14
Negative control (24, 48 & 72 hr)	0, 0, 0	0, 0, 0
Positive control 24 hr (1:10 ²)	121, 123, 139	138, 158, 162
E1AAS (1µM) 24 hr (1:10 ²)	97, 114, 142	112, 126, 170
E1ANS (1µM) 24 hr (1:10 ²)	151, 164, 179	163, 174, 191
PIXAS (1µM) 24 hr (1:10 ²)	161, 172, 192	170, 198, N/A
PIXNS (1µM) 24 hr (1:10 ²)	88, 113, 114	106, 124, 135
Positive control 48 hr (1:10 ⁴)	80, 87, 119	93, 96, 134
E1AAS (1µM) 48 hr (1:10 ⁴)	123, 152, 177	150, 159, 184
E1ANS (1µM) 48 hr (1:10 ⁴)	N/A, 102, 113	N/A, 124, 128
PIXAS (1µM) 48 hr (1:10 ⁴)	N/A, 117, 140	N/A, 132, 158
PIXNS (1µM) 48 hr (1:10 ⁴)	174, 192, 200	191, 202, 209
Positive control 72 hr (1:10 ⁶)	16, 18, 21	20, 23, 27
E1AAS (1µM) 72 hr (1:10 ⁶)	9, 12, 16	9, 16, 20
E1ANS (1µM) 72 hr (1:10 ⁶)	20, 29, 43	23, 36, 63
PIXAS (1µM) 72 hr (1:10 ⁶)	6, 13, 17	14, 14, 22
PIXNS (1µM) 72 hr (1:10 ⁶)	17, 21, 24	18, 24, 31

Results are presented as absolute plaque counts (in triplicate).

N/A, not available – plaque count void.

Table 6.11 Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS and E1ANS ODNs at 1 μ M concentration

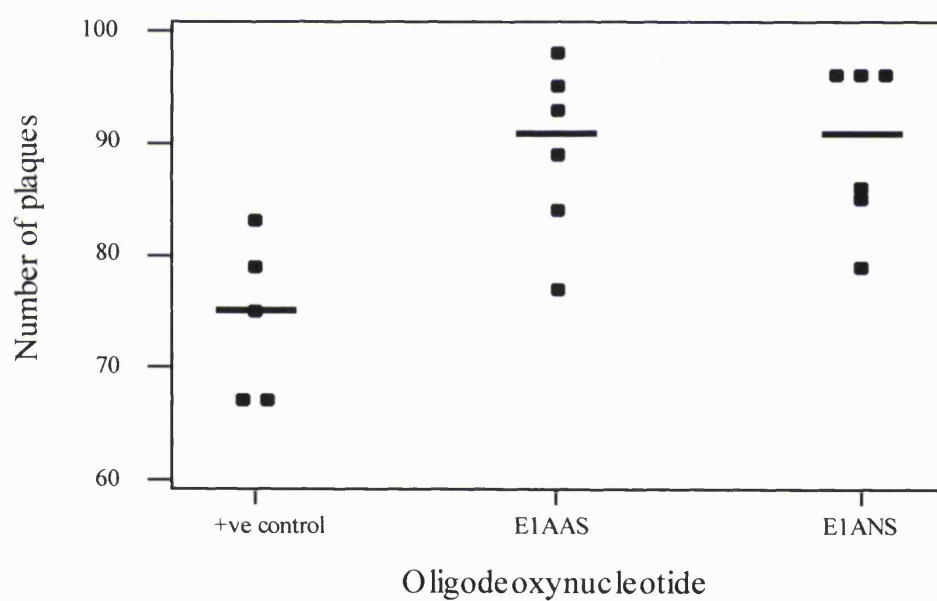
A549 cell preparation	Plaque no. day 10 (median)	Comparison with nonsense control	Plaque no. day 14 (median)	Comparison with nonsense control
Negative control (24, 48 & 72 hr)	0, 0, 0		0, 0, 0	
Positive control 24 hr (1:10 ³)	46, 50, 59, 64, 71, N/A (59)	p=0.06	67, 67, 75, 79, 83, N/A (75)	p=0.02
E1AAS (1 μ M) 24 hr (1:10 ³)	63, 72, 78, 78, 82, 82 (78)	p=0.9	77, 84, 89, 93, 95, 98 (91)	p=0.8
E1ANS (1 μ M) 24 hr (1:10 ³)	63, 69, 79, 80, 80, 89 (79.5)		79, 85, 86, 96, 96, 96 (91)	
Positive control 48 hr (1:10 ⁵)	37, 40, 48, 61, 71, 81 (54.5)	p=0.2	46, 59, 64, 70, 78, 90 (67)	p=0.2
E1AAS (1 μ M) 48 hr (1:10 ⁵)	45, 46, 54, 59, 67, 68 (56.5)	p=0.2	56, 62, 68, 77, 81, 83 (72.5)	p=0.5
E1ANS (1 μ M) 48 hr (1:10 ⁵)	52, 65, 66, 68, 70, 87 (67)		63, 71, 78, 78, 79, 98 (78)	
Positive control 72 hr (1:10 ⁶)	46, 51, 52, 61, 72, N/A (52)	p=0.008	64, 64, 71, 81, 88, N/A (71)	p=0.008
E1AAS (1 μ M) 72 hr (1:10 ⁶)	69, 74, 76, 88, 90, 95 (82)	p=0.09	81, 89, 101, 101, 103, 107 (101)	p=0.2
E1ANS (1 μ M) 72 hr (1:10 ⁶)	80, 87, 91, 103, 108, 111 (97)		91, 97, 103, 111, 115, 121 (107)	

Results are presented as absolute plaque counts (in sextuplicate) together with median values.
N/A, not available – plaque count void.

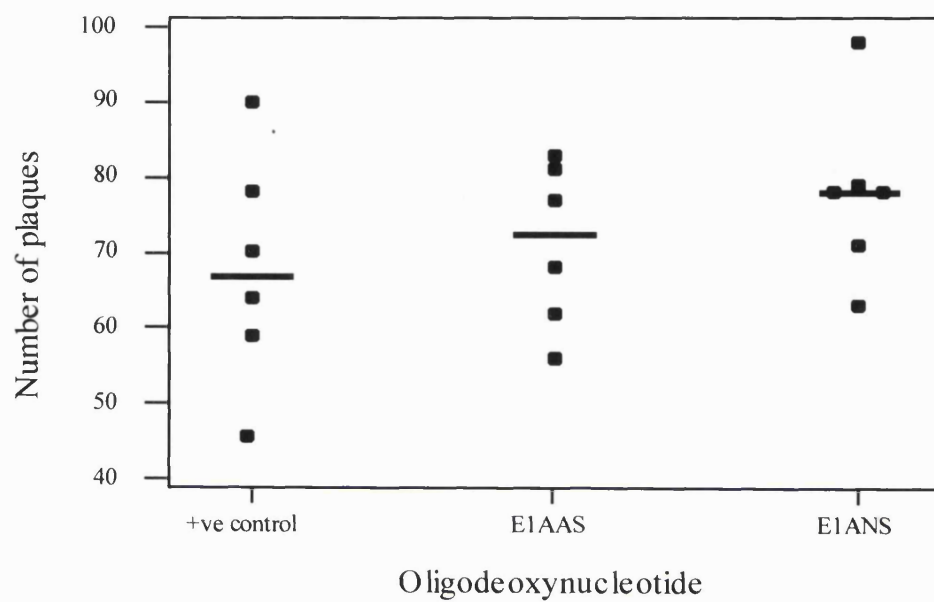
Figure 6.14

Serial titring of wild type adenovirus 5 on A549 cells from preparations with E1AAS and E1ANS ODNs at 1 μ M concentration. Plaque counts from day 14 (as per Table 6.11)

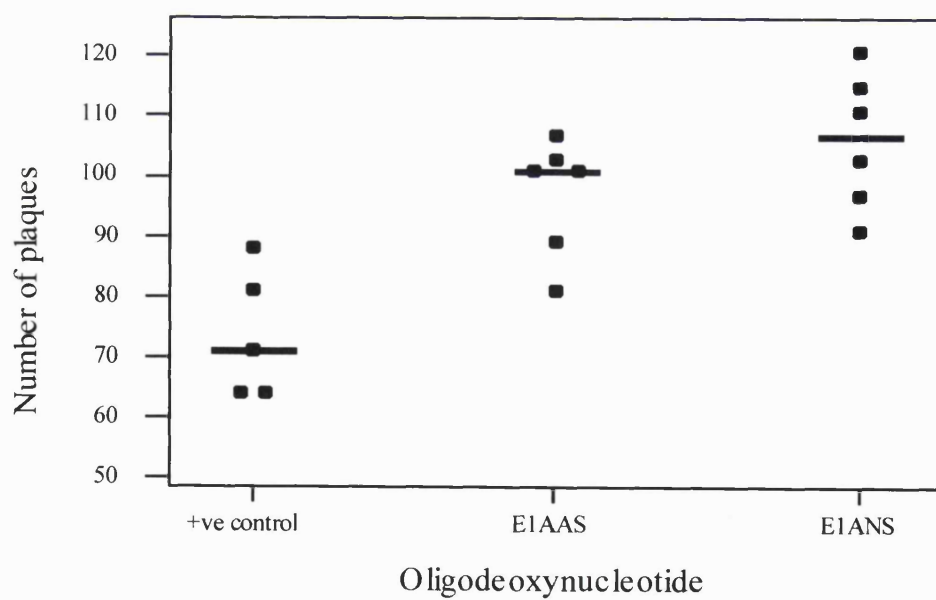
(a) 24 hours



(b) 48 hours



(c) 72 hours



6.4 Summary of results

- Antisense ODNs do inhibit adenovirus replication and thus infection *in vitro*. The most effective and consistent antisense ODN in this regard was that directed towards the E1A region of the adenoviral genome (E1AAS), which was associated with a small yet statistically significant reduction in viral plaque formation. Other ODNs assessed did show inhibition of plaque formation, but to a lesser extent and with less consistency.
- The reduction in plaque formation observed in this *in vitro* assay appears to be consequent upon a specific antisense effect. This is supported by the observation that nonsense (and sense) control ODNs were not generally associated with a reduction in adenovirus cytopathic effect.
- There was no demonstrable increased efficacy when using ODNs at higher concentrations. An ODN concentration of 1 μ M was as effective as 15 μ M concentration in reducing viral plaque formation and was the ultimate concentration used in the study.
- There was no evidence of enhanced antisense ODN effect resulting from the addition of Lipofectamine.
- There was no difference between end-protected and fully-protected E1AAS ODNs. Phosphorothioating each of the nucleotides (ie: fully-phosphorothioated or fully-protected ODN) did not produce enhancement of viral plaque reduction when compared with the end-phosphorothioated (end-protected) ODN.
- Only one additional ODN directed toward the E1A region of the adenoviral genome was shown to have an inhibitory effect on adenoviral plaque formation. This ODN (E1AAS4) was designed after computer assisted prediction of the relevant mRNA secondary structure. However, this ODN did not show inhibition of adenovirus infection superior to the original E1AAS ODN.

- Using the serial titring methodology, it was not possible to establish the time relationship of antisense ODN inhibition of adenoviral cytopathic effect.

6.5 Discussion

This study represents the first documented evidence that adenovirus 5 infection can be modulated by specific antisense oligonucleotides (ODNs) *in vitro*. As with their application in the modification of other viral infections (*in vitro*), including those related to papillomavirus (Cowser 1993), herpes simplex virus type 1 (Smith 1986) and human immunodeficiency virus (Zamecnik 1986), antisense ODNs show potential for the specific treatment of clinical adenoviral disease.

The most efficient and consistent antisense ODN in reducing adenovirus cytopathic effect was that targeted to the initiation of translation codon of the E1A gene. This ODN (E1AAS) was designed after that described previously (Quinlan 1993), and was one of the initial ODNs evaluated in this study. The addition of this E1AAS ODN reduced adenoviral plaque formation by up to 50%. Quinlan described the use of the E1A antisense ODN in an assay utilising the 293 cell line and replication deficient adenovirus 5. She showed that E1A activity was essential to maintain immortalisation of the cell line. However, there have been no previous reports of the use of this antisense ODN in modulating the replication of wild type human adenovirus 5.

The *in vitro* biological assay used in this study was developed to reflect more closely the clinical setting of adenoviral pneumonitis. A lung cell line (A549) was used in combination with “active” human adenovirus 5. At variance with the clinical situation was that the treatment, in the form of antisense ODN, was administered prior to the infective agent being introduced. However, in this protocol the antisense ODN remained in contact with the infected cells (in the agarose overlay) over the subsequent period. Interestingly, on occasions it was observed that fewer plaques developed in the antisense ODN treated cells as compared with the nonsense control cells between days 10 and 14. This observation suggests a prolonged antisense effect and that antisense ODN therapy may still be effective once viral infection becomes established.

In order to confirm that the *in vitro* ODN modulation of adenoviral infection was due to a specific antisense mechanism, nonsense control ODNs were used with the same base composition as the antisense ODN. A sense ODN was also used in the initial phase of the study. For the E1AAS ODN, the most effective ODN, there was consistently no inhibitory effect observed with either the nonsense or sense control ODNs used, thus supporting a specific antisense effect. Although there was some reduction in plaque formation with the PIXAS ODN, this was not as consistent or specific as for E1AAS.

Demonstration of a reduction in the target RNA or protein levels would further confirm a “true” antisense effect (Wagner 1994). In this study, attempts were made to evaluate reduction of the relevant targeted RNA by a reverse transcriptase-polymerase chain reaction (RT-PCR) method (data not shown). However, there were significant technical problems associated with this methodology, probably because of contamination by viral DNA, although there was no improvement following treatment with DNase.

There was no difference in antisense activity detected for the E1AAS ODN when used at concentrations varying between 1 μ M and 30 μ M. This was an interesting observation, as a higher concentration of the antisense ODN might be expected to produce a greater inhibitory effect. This phenomenon has been reported previously. Cowsert *et al*, in their evaluation of a number of antisense ODNs for the treatment of papillomavirus, demonstrated that the optimum concentration of ODN was dependent on the individual ODN. In dose-response experiments, the activity of one ODN was concentration dependent to 0.5 μ M, where it plateaued and exhibited no further activity to 5 μ M. The activity of another ODN remained dose dependent throughout the entire range tested, while one ODN showed a reduction in antisense activity with increasing concentration (Cowsert 1993).

The antisense ODN concentration used may be critical, not only for optimising antisense effect, but also for minimising side effects directly related to the ODN. These side effects may include cytotoxic effects. In a previous study assessing the

inhibition of human immunodeficiency virus type 1 replication *in vitro* by antisense ODNs, it was shown that cell cultures treated with a specific 28 oligomer antisense ODN at 1 μ M concentration showed evidence of toxicity (Liszewicz 1992). By reducing the ODN concentration to 0.1 μ M, the cells in culture remained viable and healthy.

In the current study, the optimum concentration of the most effective ODN (E1AAS) was 1 μ M. Concentrations below this were not evaluated, and it remains possible that an even lower concentration may be as, or even more, effective. In addition, there was no evidence of cytotoxicity, by visual examination of the cells, through the range of ODN concentrations assessed (ie: 1 μ M to 30 μ M). Naturally, both of these factors are important when considering *in vivo* therapy. Firstly, the treatment should be non-toxic, and secondly, the desired effect should be attainable at relatively low concentrations of ODN. The latter may be critical, as high tissue concentrations may be difficult to achieve as well as being cost prohibitive.

Cationic lipids have been reported to greatly enhance the uptake of DNA into cells. Of these cationic lipids, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) has been shown to be one of the most effective in this regard (Felgner 1987). Bennett and colleagues demonstrated a 6-15 fold increase in the association with cells of an antisense ODN in the presence of DOTMA compared to that obtained in the absence of DOTMA (Bennett 1992). Furthermore, antisense activity increased as a function of the concentration of DOTMA and with increasing time of incubation with the cationic lipid. It was expected, therefore, that an enhancement of antisense activity with the addition of cationic lipids might be demonstrated in the current study. However, there was no enhancement of antisense effect detected with the addition of cationic lipids (Lipofectamine), even following the removal of potentially inhibitory factors such as serum and antibiotics. The lack of effect may have been related to the concentration of Lipofectamine used. Higher concentrations may have produced the desired enhancement of ODN activity, but there were concerns that this would lead to cell toxicity. Bennett *et al* showed that

concentrations of DOTMA of 24 μ M or greater resulted in significant toxicity as analysed by loss of cells after overnight incubation (Bennett 1992).

As naturally occurring phosphodiester linkages in ODNs are susceptible to degradation by endogenous cellular nucleases, substitution of the 3'-end and/or 5'-end with nuclease resistant linkages, such as a phosphorothioate linkage, can protect the ODN from degradation (Hoke 1991). Substituting all the phosphodiester bonds with phosphorothioate bonds may induce further protection from nucleases (Stein 1993). Therefore, fully-phosphorothioated ODNs were compared with the standard end-protected ODNs for inhibition of adenoviral infection. There was no increased inhibition observed with the fully-phosphorothioated ODNs. On the contrary, the fully-phosphorothioated ODNs failed to demonstrate a statistically significant reduction in plaque numbers compared to the positive control, while the end-protected ODNs did.

It could be hypothesised that increasing the phosphorothioate content of the ODNs may have in some way reduced the affinity of the ODN for the targeted RNA, thereby reducing the antisense effect. Another explanation for the inferior results observed with the fully-phosphorothioated ODNs, is that, due to their increased binding capacity with proteins (Brown 1994), they may have bound with intranuclear proteins, thus interfering with their binding of the targeted RNA.

The number of possible antisense ODNs that can be developed is countless. ODNs that overlap the initiation of translation codon and extend either upstream or downstream appear to be the most effective inhibitors of translation, and thus major inhibitors of gene expression (Blake 1985). Accordingly, in this study, two further ODNs were designed to overlap the initiation of translation codon for both the E1A and protein IX (PIX) genes. None of these showed any enhanced effect over that achieved by the originally designed ODNs. After computer prediction of the secondary RNA structures for both the E1A and PIX genes, two additional ODNs were developed for both. These were targeted towards loop structures in the predicted mRNA. The ODNs designed for the E1A gene did demonstrate an effect,

but again this was not superior to that achieved by the original E1A antisense ODN (E1AAS). Overall, ODNs targeted to the E1A gene region demonstrated the greatest antisense effect.

This study was unable to elucidate the relationship between time and antisense effect. This was due in main part to the problems associated with the RT-PCR methodology. The antisense effect could be expected to occur early and therefore it was disappointing not to observe an early reduction in adenovirus titre in the serial titring experiments. This system may have been too insensitive to detect an inhibitory effect.

In conclusion, this study has shown that antisense ODNs can be effective in reducing adenoviral replication and thus infection *in vitro*. The most effective ODN was that targeted to the initiation of translation region of the E1A gene (E1AAS ODN). It showed consistent inhibition of adenoviral infection at the relatively low concentration of 1 μ M. Modifications, including fully-phosphorothioated linkages and the addition of cationic lipids produced no additional benefit. Given the lung's suitability for topical therapy, antisense ODNs offer potential as a specific topical treatment for adenoviral respiratory tract infections.

Chapter 7 Nuclear localisation of antisense oligodeoxynucleotides

7.1 Introduction

7.2 Methods

7.2.1 Fluorescent oligodeoxynucleotide

7.2.2 Cell culture and staining techniques

7.3 Results

7.4 Discussion

7.1 Introduction

Oligodeoxynucleotides (ODNs) are highly charged, hydrophilic molecules with molecular weights typically in the region of 6,000Da (for a 20mer). They are therefore incapable of penetrating directly through cellular membranes. Instead, like many viruses, ODN uptake proceeds by endocytosis. Previous studies have suggested that this process is saturable, temperature dependent, and can be at least partially competed by a large molar excess of unlabelled ODN. Unlike viruses, most naked ODNs remain trapped in vesicles, where they are either degraded in lysosomes or exported back into the extracellular medium (Vlassov 1994). This is a particular problem for ODNs, as a lower intracellular concentration limits their effect.

Encapsulation of ODNs into liposomes has been used to increase their delivery across membranes (Vlassov 1994). Moreover, this protects them from serum or lysosomal nucleases. Cationic lipids such as Lipofectamine have also been used as delivery vectors. This class of transfection agent contains positively charged amine groups that interact directly with the negatively charged phosphate residues of the ODN. These complexes have been shown to result in efficient delivery to the cytoplasm and nucleus in several cell lines (Bennett 1992).

Using confocal microscopy, Wagner and colleagues detailed the cellular uptake of fluorescein-labelled phosphorothioate ODNs in African green monkey cells. Relying on endocytosis alone, ODNs were localised in the cytoplasm. After complexing the ODNs with cationic liposome, 90% of the cells showed nuclear localisation of the fluorescent ODN. But after microinjection of ODNs into the cytoplasm of cells at a concentration of 25 μ M, all of the injected cells showed nuclear localisation of the ODN (Wagner 1993). Demonstrating that ODNs do enter the nucleus supports their expected mechanism of action.

Accordingly, experiments were designed to demonstrate cellular uptake and nuclear localisation of ODNs. Utilising similar methodology to that reported by Fisher *et al*,

attempts were undertaken to identify the intracellular presence of fluorescein-labelled ODNs with the use of a fluorescent microscope (Fisher 1993).

The main research questions were as follows:

- Do fluorescein-labelled ODNs enter the nucleus of A549 cells?
- If so, can the efficiency of ODN cell entry and nuclear localisation be enhanced by prolonged incubation or the addition of cationic lipids into the experimental protocol?
- Does the addition of adenovirus, an effective permeator of cell membranes, enhance nuclear localisation of ODNs?

7.2 Methods

7.2.1 Fluorescent oligodeoxynucleotide

The oligodeoxynucleotide used in these experiments was the E1AAS ODN as described in Chapter 6. The ODN was synthesised with fluorescein (X) labelled to the 5' end nucleotide. The sequence of the ODN was as follows:

5' – X G T G G C A G A T A A T A T G T C T C A T – 3'

The ODN (E1AAS-FL) was phosphorothioate end-protected and precipitated as previously described. It was used at a concentration of 1 μ M.

7.2.2 Cell culture and staining techniques

1. Monolayers of A549 cells were cultured in 2-chamber slides (Nunc, Naperville, IL, USA). 0.5×10^6 A549 cells were added to each chamber together with 1.5mL of medium - DMEM (Gibco BRL, Paisley, UK); 10% HIFCS; 2mM L-glutamine; penicillin (100U/mL) and streptomycin (100 μ g/mL). These were incubated at 37°C in 5% CO₂/air for 24 hours, at which stage, cells were approximately 60-70% confluent.
2. The culture medium was then removed from each of the chambers with 1mL of the respective ODN test medium added to each chamber, and incubated for four hours at 37°C in 5% CO₂/air.
3. In the initial experiment, 8 x 2-chamber slides were used. There were 2 negative control slides ie: with no added ODN, 2 slides with 1 μ M concentration of fluorosceinated E1AAS ODN; 2 slides with 1 μ M fluorosceinated ODN plus Lipofectamine at 10 μ g/mL; and two slides with ODN at 1 μ M concentration, together with approximately one plaque forming unit per cell of wild type adenovirus 5 (AdV5). In the Lipofectamine treated slide chambers, no foetal calf serum or antibiotics were added to the medium as these substances are known to interfere with the action of Lipofectamine. In addition, the cells had

been washed with DMEM without additives prior to the administration of Lipofectamine (and ODN). The resultant weight/weight ratio for ODN:Lipofectamine was 1:1.4. The following table summarises the components in each of the test conditions:

Table 7.1 Components of test medium for initial nuclear localisation experiments

Slide No.	ODN ^a	Lipofectamine	Adenovirus ^b	Medium ^c
1,2	-	-	-	1mL
3,4	1 μ M (7.3 μ g)	-	-	1mL
5,6	1 μ M	10 μ g	-	1mL ^d
7,8	1 μ M	-	1 PFU/cell	1mL

^a Fluoresceinated E1A antisense oligonucleotide (E1AAS-FL) was used in all nuclear localisation experiments.

^b Wild type adenovirus 5.

^c A549 culture medium: DMEM, 10% HIFCS, 2mM L-glutamine, Pen/Strep – volume per chamber.

^d Medium for lipofectamine treated cells did not include HIFCS or antibiotics

4. Preparation of slides for fluorescent microscopy: a small amount (approximately 1mL) of phosphate buffered saline (non sterile) was added to each of the chambers and the resulting PBS and medium mixture was removed leaving a small layer of liquid over the cells to prevent desiccation.
5. The cells were then washed thrice with PBS leaving a small amount of PBS covering the cells after the final wash.
6. Fixing solution, comprising analar methanol 3 parts to 1 part analar 1 molar glacial acetic acid was placed drop by drop into each of the chambers while gently rocked (the fixing solution was freshly made and used within 15 minutes). The solution was then discarded and further fixative applied on two occasions. After removal of the final fixative volume, the slides were allowed to air dry after inverting onto a paper towel.
7. The chambers and gaskets were then removed from the slides.

8. Counter-staining was performed with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), which stains nuclei blue. DAPI (2 mg/ml) was diluted 1:100 in glycerol. One to two drops of the DAPI/glycerol solution were applied to the fixed cells and covered with a double cover slip, which was pressed down gently. Further pressure was exerted onto the slides after covering with layers of paper towel.
9. The slides were then ready for fluorescent microscopy. Cells were viewed using a Zeiss Axioskop microscope with a charged-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) connected to a Macintosh Quadra computer. Image analysis was performed using SmartCapture software (Digital Scientific, Cambridge, UK).

The second phase of experiments included incubating the slides with the fluoresceinated ODN for 24 hours, as well as for 4 hours, in order to determine if longer incubation with the ODN improved nuclear uptake. In addition, high dose wild type adenovirus 5 (6×10^7 PFU/mL) was administered to assess its impact on ODN entry into the cytoplasm and nucleus. The conditions for this second phase of experiments are summarised in Table 7.2. Staining methods for fluoroscopic microscopy were as detailed above.

Table 7.2 Conditions for second phase nuclear localisation experiments

Slide No	ODN ^a	Lipofectamine	Adenovirus ^b	Medium ^c	Incubation (hours)
9, 10, 25, 26	-	-	-	1mL	4
11, 12	-	-	-	1mL	24
13, 14	1 μ M (7.3 μ g)	-	-	1mL	4
15, 16	1 μ M	-	-	1mL	24
17, 18, 27, 28	1 μ M	10 μ g	-	1mL ^d	4
19,20	1 μ M	10 μ g	-	1mL ^d	24
21, 22 29, 30	1 μ M	-	1PFU/cell	1mL	4
23, 24	1 μ M	-	1PFU/cell	1mL	24
31, 32	1 μ M	-	100 PFU/cell ^e	1mL	4

^a Fluoresceinated E1A antisense oligonucleotide (E1AAS-FL) was used in all nuclear localisation experiments.

^b Wild type adenovirus 5.

^c A549 culture medium: DMEM, 10% HIFCS, 2mM L-glutamine, Pen/Strep – volume per chamber

^d Medium for lipofectamine treated cells did not include HIFCS or antibiotics.

^e Neat wild type adenovirus 5 (approx 6x10⁷ PFU/mL) supernatant applied with ODN to cells.

In the final set of experiments (slide nos. 25-32, Table 7.2), counts of nuclei positive for the fluorescein marker under the various conditions described were performed on six randomly selected fields at 100x magnification. The number of nuclei containing the fluorescein-labelled ODN was compared with the total number of nuclei present in each field. Statistical evaluation of these results was by Chi square analysis.

7.3 Results

Fluoroscopic microscopy revealed generally good preparations of the processed slides with easy identification of A549 cell nuclei. The Lipofectamine treated slides showed increased background fluorescence. This may have been related to residual lipofectamine and fluorosceinated ODN that had bound to the slide.

The (negative) control slides showed clear definition of the cell nuclei with the observation of numerous mitotic figures, indicating active cell division. As expected, no fluorescence in any of the control slides was seen at either 20x or 100x magnification (Figure 7.1).

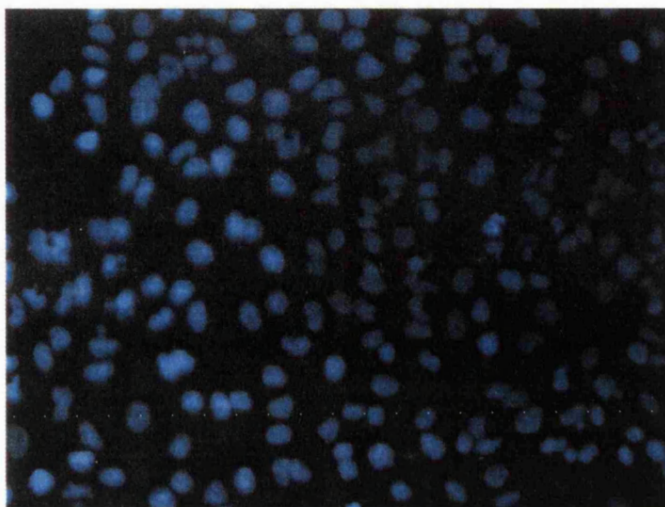
Figure 7.2 shows a representative example of A549 cells treated with fluorosceinated ODN alone. This micrograph at low magnification (20x) gives an overview of the distribution of the fluorosceinated ODN without the addition of agents to aid nuclear localisation. In the absence of confocal imaging, it is not possible to conclude that the fluorosceinated ODN resides in the nucleus. Three-dimensional reconstructions from multiple confocal images would be required to prove this unequivocally. However, the ODN is shown to be either adjacent to, or overlying, many of the nuclei in the field. At higher magnification (100x), the ODN was shown to completely overlie a number of nuclei, suggesting attachment to, or inclusion, in the nucleus (Figure 7.3).

The lipofectamine treated cells showed increased background fluorescence. This was most likely related to the inability to completely wash off excess lipofectamine bound to the ODN. However, alignment of the ODN to the nucleus could still be determined (Figure 7.4).

Figure 7.1 Fluoroscopic photomicrographs of control slides of A549 cells after nuclear staining with DAPI at: (a) 20x, and (b) 100x magnification

Note clear definition of cell nuclei and evidence of active cell division. No fluorescence was observed in any of the control slides.

(a)



(b)

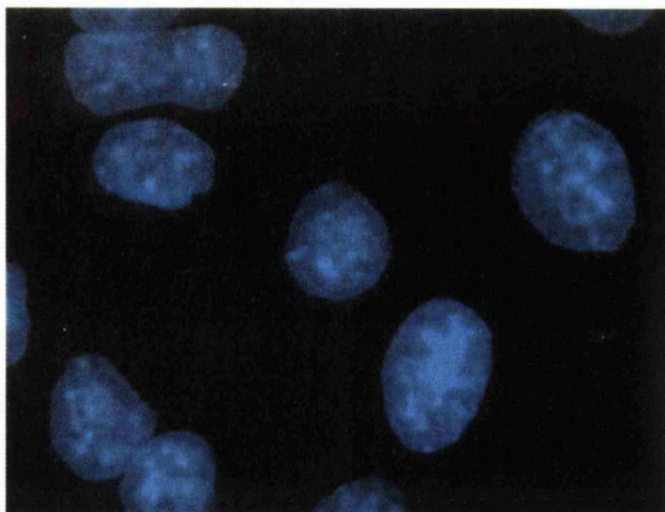
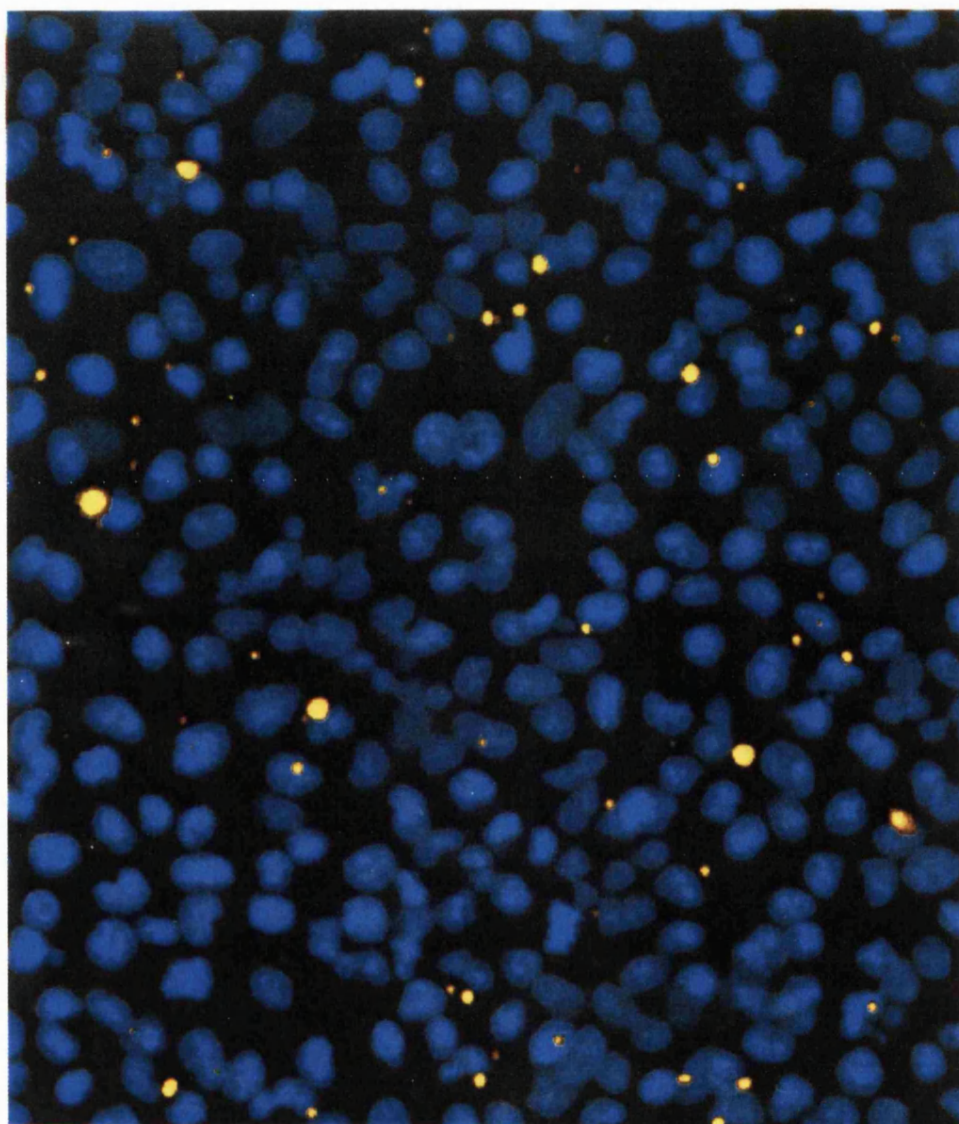


Figure 7.2 Fluoroscopic photomicrograph (20x magnification) of A549 cells treated with fluoresceinated ODN



The fluorescent ODN (yellow staining) appears to be situated mostly in the cytoplasm, with a smaller proportion adjacent to the nucleus.

Figure 7.3 Fluoroscopic photomicrographs (100x magnification) of A549 cells treated with fluoresceinated ODN

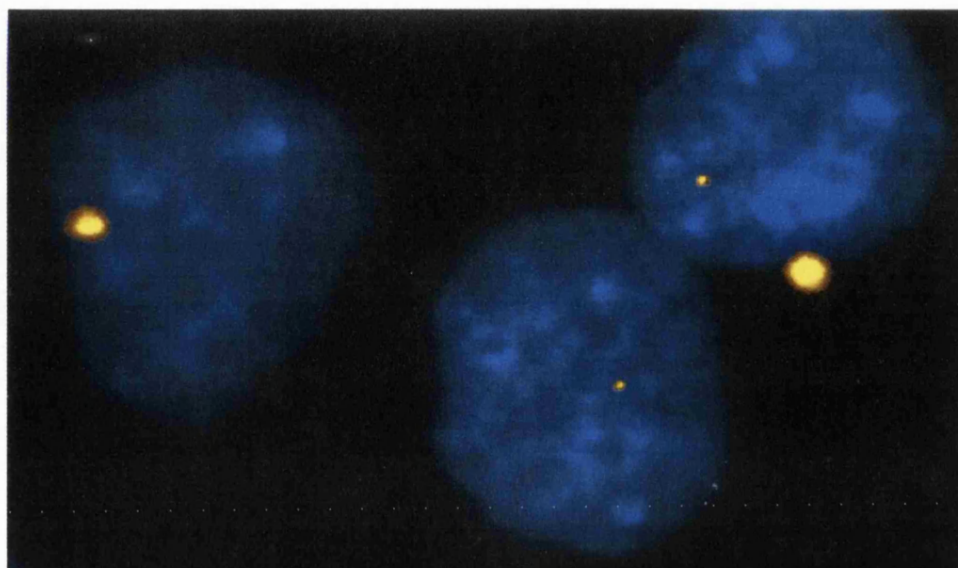
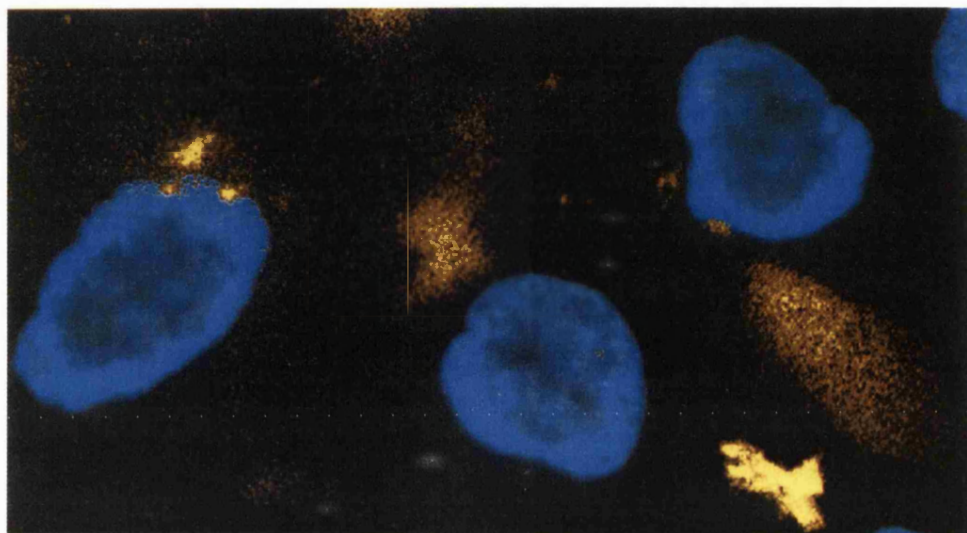


Figure 7.4 Fluoroscopic photomicrograph (100x magnification) of A549 cells treated with fluoresceinated ODN in the presence of Lipofectamine



Note the general increase in background fluorescence. This is likely to be related to excess lipofectamine bound to ODN remaining on the slide. Alignment of the ODN with the nuclei can still be determined.

A longer incubation period (24 hours) did not increase ODN localisation in cell nuclei (data not shown). In the final set of experiments (slides 25-32), the effect of high dose adenovirus on ODN nuclear uptake was compared with low dose adenovirus and Lipofectamine with a four-hour incubation. There was no discernible difference in the amount of ODN associated with nuclei between ODN alone, ODN with low dose or high dose adenovirus. In addition, there was no increase in the number of nuclei associated with ODN in the Lipofectamine treated samples (Table 7.3).

Table 7.3. Number of nuclei associated with fluorescent oligonucleotide per high power field (100x magnification) under various conditions

Field No.	ODN	ODN + AdV5 1PFU/mL	ODN + AdV5 100PFU/mL	ODN + Lipofectamine
1	1/9	0/10	0/20	2/15
2	1/6	1/13	1/25	0/22
3	2/11	1/32	2/22	3/26
4	1/11	12/16	1/21	2/17
5	1/16	0/10	3/17	8/16
6	0/15	1/15	3/18	1/14
Total (%)	6/68 (8.8%)	5/96 (5.2%)	10/123 (8.1%)	16/110 (14.5%)*

See legend of Table 7.2 for details of experimental conditions.

Fields were randomly selected.

Data are presented as number of nuclei positive for fluorescein-labelled ODN over total number of nuclei counted. Cumulative totals are shown together with overall percentage positive nuclei.

* Not significant by Chi square analysis.

7.4 Discussion

This study demonstrates no improvement in ODN localisation to the nucleus by Lipofectamine or after the administration of adenovirus at either low or high titre. Furthermore, given the limitations of the technique, it cannot be unequivocally concluded that a specific ODN directed toward the E1A gene of adenovirus 5 enters the nucleus of A549 cells.

Using similar techniques as described in this study, Bennett *et al* demonstrated that in the absence of cationic lipids, a FITC-labelled ODN appeared to be associated with cytoplasmic structures consistent with endosomal or lysosomal vesicles, based upon a punctate perinuclear cytoplasmic fluorescence. These findings were consistent with other reports examining the cellular distribution of ODNs (Loke 1989, Yakubov 1989). It was only in the presence of cationic lipids (DOTMA) that there was a greater association of ODNs with the nucleus (Bennett 1992). This observation was cited as confirmation of intranuclear deposition of ODN. The process was found to be both time and temperature dependent. Furthermore, the cytoplasmic distribution of ODNs in the presence of DOTMA was different, in that the structures that accumulated ODN were larger in size, suggesting a greater intracytoplasmic concentration of ODN.

It was disappointing that these findings could not be replicated in the current study in which there was no demonstrable enhancement of nuclear localisation in the presence of cationic lipids (Lipofectamine). This could be related to a variation in the cationic lipid preparation itself or to the relative ratio of ODN to cationic lipid. In the study of Bennett *et al*, 1 μ M concentration of ODN was used with 8 μ M DOTMA (Bennett 1992). In the reported experiments the ODN:Lipofectamine weight ratio was 1:1.4. Therefore, it's feasible that a greater concentration of Lipofectamine may have improved nuclear localisation.

Only one type and concentration of fluorescein-labelled antisense ODN was used in the study. This was the E1AAS ODN, which was shown to be the most effective

ODN tested in inhibiting adenoviral 5 replication *in vitro*. The concentration used was 1 μ M, which again was found to be effective (see Chapter 6). It is possible that a greater concentration of ODN, or even a different ODN, may have resulted in increased nuclear localisation.

It was hypothesised that the addition of adenovirus to the experimental protocol may enhance cellular uptake of ODN and thus lead to greater nuclear localisation.

Adenovirus is extremely efficient at entering cells. Attachment of the virus to the target cell is via a fibre protein and its receptor. Virus internalisation then occurs by receptor-mediated endocytosis (Chardonnet 1970, Fitzgerald 1983). Furthermore, adenovirus particles stimulate the internalisation of macromolecules into host cells (Defer 1990). This last characteristic was suggested as a possible mechanism to increase ODN uptake. In this study however, there was no enhancement of ODN cell uptake, as assessed by nuclear localisation, after the addition of low or high titre adenovirus 5. One reason for the observed lack of enhancement may relate to the timing of administration of ODN and adenovirus. The experimental protocol for this study involved adding the ODN and adenovirus simultaneously rather than four hours after the administration of the ODN as described in the protocol in Chapter 6. This may have induced some degree of inhibition of the adenovirus effect, for example, by interfering with adenovirus binding to the cell surface. There was no additional benefit in prolonging the incubation of the cells with the ODN, with no increase in the percentage of nuclei associated with the fluorescein-labelled ODN after 24 hours incubation compared with the four hour incubation.

In conclusion, this study demonstrates no increase in ODN nuclear localisation after prolonged exposure of cells to ODN, or in the presence of cationic lipids or adenovirus. As a consequence, it was not possible to prove unequivocally that the specific ODN studied enters the nuclei of A549 cells.

Chapter 8 Concluding remarks

Lung transplantation, both in adults and children, is an extremely challenging field of clinical medicine. The rate of complications, both in the short and long term, remains high despite improved surgical techniques and the development of more specific and less toxic immunosuppressive regimens. These complications often produce chronic graft dysfunction, otherwise known as obliterative bronchiolitis or bronchiolitis obliterans. Once established, this condition responds poorly to medical therapy, while retransplantation is more often palliative than curative. As a consequence, post transplant survival remains inferior to that for most other solid organ transplants.

Pre, peri and post operative factors have all been implicated in affecting the ultimate outcome of the pulmonary allograft. In essence, these factors produce varying degrees of lung injury, some of which may be reversible with prompt and appropriate treatment. Pre operative factors include those related to both the transplant recipient and the donor. The recipient's age and general clinical status may affect graft outcome, while the clinical status of the donor and the relative health of the donor lungs will be contributory factors. Peri operative factors include the duration of graft ischaemia, surgical events and the extent of lung reperfusion injury. Post operatively, rejection and infection are implicated in producing a significant degree of lung injury, while long term immunosuppressive therapy may affect allograft function either directly or indirectly. These injurious factors, plus others, were explored in detail in Chapter 1 of this thesis.

The clinical correlation of these injurious events in the transplanted lung is ultimately reflected by patient survival. The overall 5-year post transplant survival for both adults and children approximates 40-50%. However, this provides no indication of graft function or associated quality of life in those surviving. Further clinical correlates of these injurious events, including the effects on pulmonary function post transplant were described in Chapter 2.

In Chapter 4, a detailed assessment of pulmonary function over an extended post transplant period was presented for a group of paediatric lung transplant recipients with cystic fibrosis. Dynamic lung function demonstrated a statistically significant increase in all parameters for the group 12 months following transplantation. However, this improvement was relatively temporary, with a persistent reduction in lung flows and volumes over the succeeding post transplant course. By 36 months post transplant, only the forced vital capacity (FVC) was significantly different to pre transplant values.

Static lung volumes, total lung capacity and residual volume, were lower than pre transplant values to 12 months post transplant, thereafter demonstrating no significant difference to pre transplant values. Both of these observations were consistent with the development of obliterative bronchiolitis, which is ultimately associated with a mixed obstructive and restrictive defect in lung function, in the majority of survivors.

There were similarities noted between the post transplant pattern of pulmonary function in this paediatric cohort and that reported for adult transplant recipients. However, obstructive flow patterns were seen at an earlier stage in the paediatric group. This could be related simply to anatomical factors, with obstruction more evident in the smaller airways of the child and adolescent. An alternative hypothesis is that paediatric lung transplant recipients experience an increased number of injurious events to the lung graft, including rejection and infection, which in turn leads to an earlier onset of chronic graft dysfunction.

Also in Chapter 4, a unique method of measuring pulmonary function over a prolonged period has been presented. The area under the curve of FEV₁% (FEV₁% AUC) yielded an useful measure of pulmonary function for the individual lung transplant patient as well as for the entire cohort. This method enabled the relationship between pulmonary function and factors associated with injury in the lung allograft to be explored. Remarkably, there were statistically significant negative relationships between the duration of initial graft ischaemia and pulmonary

function to 36 months post transplant. These indicated that prolonged graft ischaemia negatively impacted upon post transplant lung function, and would suggest that limiting graft ischaemic time might improve the functional outcome of the allograft. Interestingly, there was no relationship demonstrated between the pre operative clinical status of the recipient (as assessed by FEV₁%) or the number of rejection and infection episodes in the first 12 months post transplant and FEV₁% AUC at any of the time points assessed.

Limiting the extent of lung injury was the philosophy behind the development of the novel therapeutic approaches, which constituted the latter sections of this thesis. The ability to introduce genes into the lung allograft, which may reduce the injurious effects of ischaemia and reperfusion or abrogate the rejection process, would obviously be highly advantageous. Numerous genes have been suggested in this regard, but one of the major problems currently is the lack of an efficient, non-toxic gene vector system. Both viral and non-viral vectors have been studied and the current status of each was reviewed in Chapter 3.

Currently, viral vectors appear to be the most efficient for gene transfer, but are associated with significant inflammatory reactions, which limit their use. Non-viral vectors are less toxic in this regard but possibly as a consequence, are less efficient for gene transfer. In Chapter 5 of this thesis, the evaluation of a non-viral, synthetic peptide gene vector in an *in vivo* rat lung model was described. This represented our unit's initial endeavours in administering the polylysine-molossin vector to the lungs, via the airway, of a live animal. Widespread distribution and attachment of the vector throughout the parenchyma of the lung was demonstrated, with relative sparing of the airway epithelium. In all the lung sections reviewed, at time points ranging from 1.5 to 72 hours post instillation, there was a complete absence of a cellular infiltrate indicating no evidence of an inflammatory reaction. However, despite adequate vector distribution and the lack of toxic effects, there was no discernible evidence of gene transfer to the airway epithelium or alveoli using two reporter genes. Since these experiments were performed, improved methods for

endocytic release have been developed (Zhang 2001), and these will soon be evaluated in this model.

The final section of this thesis was related to the development of specific anti-viral therapy. Viral infection of the transplanted lung represents another form of acute injury, and may be associated with significant allograft dysfunction in both the short and long term. Of particular concern is adenoviral infection of the lung, for which there is no specific treatment. This disease may produce an acute devastating pneumonitis or may predispose to the development of the more chronic disease, obliterative bronchiolitis.

In Chapter 3, the novel approach of antisense oligodeoxynucleotides (ODNs) for the treatment of viral infection was reviewed. The evaluation of specific antisense ODNs in the modification of adenoviral infection in an *in vitro* experimental model was detailed in Chapter 6. It was shown that antisense ODNs, especially one directed toward the E1A gene, can inhibit wild type adenovirus replication *in vitro* and this appeared to be a specific antisense effect. There was no enhancement of this effect with the addition of cationic lipids or altering the structure of the ODNs to increase their resistance to degradation by nucleases. As described in Chapter 7, efforts to maximise nuclear localisation of antisense ODNs with the use of cationic lipids and high titre adenovirus were shown to be not successful. It was concluded that antisense ODNs might offer a possible alternative treatment for adenoviral infection, although further *in vivo* assessment is essential.

Finally, lung transplantation offers many patients, adults and children, with end stage pulmonary disease the only chance of prolonged, good quality survival. To limit or even abolish injury to the lung allograft by reducing ischaemic damage and the incidence of rejection and infection is indeed a worthy goal. So much more so if this can be achieved with effective, non-toxic agents, similar to those described above.

References

A

Adesanya, M.R., Redman, R.S., Baum, B.J., O'Connell, B.C. (1996) Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. *Hum. Gene Ther.* **7**, 1085-1093.

Aeba, R., Stout, J.E., Francalancia, N.A., Keenan, R.J., Duncan, A.J., Yousem, S.A., Burckart, G.J., Yu, V.L., Griffith, B.P. (1993) Aspects of lung transplantation that contribute to increased severity of pneumonia. An experimental study. *J. Thorac. Cardiovasc. Surg.* **106**: 449-457.

Akhtar, S., Basu, S., Wickstrom, E., Juliano, R.L. (1991) Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic Acids Res.* **19**, 5551-5559.

Al-Dossari, G.A., Jessurun, J., Bolman, R.M. III., Kshetry, V.R., King, M.B., Murray, J.J., Hertz, M.I. (1995) Pathogenesis of obliterative bronchiolitis. Possible roles of platelet-derived growth factor and basic fibroblast growth factor. *Transplantation.* **59**, 143-145.

Al-Dossari, G.A., Kshetry, V.R., Jessurun, J., Bolman, R.M. III. (1994) Experimental large-animal model of obliterative bronchiolitis after lung transplantation. *Ann. Thorac. Surg.* **58**, 34-40.

Altman, D.G. (1991) In: *Practical statistics for medical research*. Chapman and Hall, London. 431-433.

Alton, E.W., Stern, M., Farley, R., Jaffe, A., Chadwick, S.L., Phillips, J., Davies, J., Smith, S.N., Browning, J., Davies, M.G., Hodson, M.E., Durham, S.R., Li, D., Jeffery, P.K., Scallan, M., Balfour, R., Eastman, S.J., Cheng, S.H., Smith, A.E., Meeker, D., Geddes, D.M. (1999) Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet*. **353**, 947-954.

Anderson, C.W., Baum, P.R., Gesteland, R.F. (1973) Processing of adenovirus 2 induced proteins. *J. Virol.* **12**, 241-252.

Anderson, W.F. (1984) Prospects for human gene therapy. *Science*. **226**, 401-409.

Andreu, G., Achkar, A., Couteil, J.P., Guillemain, R., Heshmati, F., Amrein, C., Chevalier, P., Guinvarch, A., Dore, M.F., Capron, F. (1995) Extracorporeal photochemotherapy treatment for acute lung rejection episode. *J. Heart Lung Transplant*. **14**, 793-796.

Aposhian, H.V. (1970) The use of DNA for gene therapy - the need, experimental approach, and implications. *Persp. Biol. Med.* **14**, 987-108.

Artemiou, O., Wieselthaler, G., Zuckermann, A., Wisser, W., Wekerle, T., Senbaklavaci, O., Birsan, T., Dekan, G., Kritzinger, M., Klepetko, W. (1997) Downsizing of the donor lung: peripheral segmental resections and lobar transplantation. *Transplant. Proc.* **29**, 2899-2900.

ATS statement. (1993) Guidelines for the initial management of adults with community-acquired pneumonia: diagnosis, assessment of severity and initial antimicrobial therapy. *Am. Rev. Respir. Dis.* **148**, 1418-1426.

August, C., Holzhausen, H-J., Schmoldt, A., Pompecki, R., Schroder, S. (1995) Histological and ultrastructural findings in chloroquine-induced cardiomyopathy. *J. Mol. Med.* **73**, 73-77.

Azad, R.F., Driver, V.B., Tanaka, K., Crooke, R.M., Anderson, K.P. (1993) Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. *Antimicrob. Agents. Chemother.* **37**, 1945-1954.

B

Balfour-Lynn, I.M., Martin, I., Whitehead, B.F., Rees, P.G., Elliott, M.J., de Leval, M.R. (1997) Heart-lung transplantation for patients under 10 with cystic fibrosis. *Arch. Dis. Child.* **76**, 38-40.

Bando, K.O., Paradis, I.L., Komatsu, K., Konishi, H., Matsushima, M., Keenan, R.J., Hardesty, R.L., Armitage, J.M., Griffith, B.P. (1995) Analysis of time-dependent risks for infection, rejection, and death after pulmonary transplantation. *J. Thorac. Cardiovasc. Surg.* **109**, 49-59.

Barnard, C.N. (1967) The operation. A human cardiac transplant: an interim report of a successful operation at Groote Schuur Hospital, Cape Town. *S. Africa J. Med.* **41**, 1271-1274.

Benham, A.M., Sawyer, G.J., Fabre, J.W. (1995) Indirect T cell allorecognition of donor antigens contributes to the rejection of vascularized kidney allografts. *Transplantation.* **59**, 1028-1032.

Bennett, F.C., Chiang, M.Y., Chan, H.C., Shoemaker, J.E.E., Mirabelli, C.K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharm.* **41**, 1023-1033.

Bennett, S.R.M., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F.A.P., Heath, W.R. (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature.* **393**, 478-480.

Bertling, W.M., Gareis, M., Paspaleeva, V., Zimmer, A., Kreuter, J., Nurnberg, E., Harrer, P. (1991) Use of liposomes, viral capsids, and nanoparticles as DNA carriers. *Biotechnol, Appl. Biochem.* **13**, 390-405.

Blake, K.R., Murakami, A., Miller, P.S. (1985) Inhibition of rabbit globin mRNA translation by sequence-specific oligodeoxyribonucleotides. *Biochemistry.* **24**, 6132-6138.

Borel, J.F., Kis, Z.L. (1991) The discovery and development of cyclosporine (Sandimmune). *Transplant. Proc.* **23**, 1867-1874.

Boucek, M.M., Faro, A., Novick, R.J., Bennett, L.E., Fiol, B., Keck, B.M., Hosenpud, J.D. (1999) The registry of the International Society for Heart and Lung Transplantation: third official pediatric report – 1999. *J. Heart Lung Transplant.* **18**, 1151-1172.

Boucher, R.C. (1999) Status of gene therapy for cystic fibrosis lung disease. *J. Clin. Invest.* **103**, 441-445.

Brandt, C.D., Kim, H.W., Vargosko, A.J., Jeffries, B.C., Arrobio, J.O., Rindge, B., Parrott, R.H., Chanock, R.M, (1969) Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. *Am. J. Epidemiol.* **90**, 484-500.

Braun, S.R., Birnbaum, M.L., Chopra, P.S. (1978) Pre- and postoperative pulmonary function abnormalities in coronary artery revascularization surgery. *Chest.* **73**, 316-320.

Bridges, N.D., Mallory, G.B., Huddleston, C., Canter, C.E., Spray, T.L. (1996) Lung transplantation in infancy and early childhood. *J. Heart Lung Transplant.* **15**, 895-902.

Bridges, N.D., Spray, T.L., Collins, M.H., Bowles, N.E., Towbin, J.A. (1998) Adenovirus infection in the lung results in graft failure after lung transplantation. *J. Thorac. Cardiovasc. Surg.* **116**, 617-623.

Brown, D.A., Kang, S.H., Gryaznov, S.M., DeDionisio, L., Heidenreich, O., Sullivan, S., Xu, X., Nerenberg, M.I. (1994) Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J. Biol. Chem.* **269**, 26801-26805.

Burke, C.M., Morris, A.J.R., Dawkins, K.D., McGregor, C.G.A., Yousem, S.A., Allen, M., Theodore, J., Harvey, J., Billingham, M.E., Oyer, P.E., Stinson, E.B., Baldwin, J.C., Shumway, N.E., Jamieson, S.W. (1985) Late airflow obstruction in heart-lung transplantation recipients. *Heart Transplant.* **IV**, 437-440.

Burke, C.M., Theodore, J., Baldwin, J.C., Tazelaar, H.D., Morris, A.J., McGregor, C., Shumway, N.E., Robin, E.D., Jamieson, S.W. (1986) Twenty-eight cases of human heart-lung transplantation. *Lancet.* **1**, 517-519.

Burke, C.M., Theodore, J., Dawkins, K.D., Yousem, S.A., Blank, N., Billingham, M.E., Van Kessel, A., Jamieson, S.W., Oyer, P.E., Baldwin, J.C., Stinson, E.B., Shumway, N.E., Robin, E.D. (1984) Post-transplant obliterative bronchiolitis and other late lung sequelae in human heart-lung transplantation. *Chest* **86**, 824-829.

C

Calne, R.Y., White, D.J.G., Thiru, S., Evans, D.B., McMaster, P., Dunn, D.C., Craddock, G.N., Pentlow, B.D., Rolles, K. (1978) Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* **2**, 1323-1327.

Cao, W., Mohacsi, P., Shorthouse, R., Pratt, R., Morris, R.E. (1995) Effects of rapamycin on growth factor-stimulated vascular smooth muscle cell DNA synthesis. Inhibition of basic fibroblast growth factor and platelet-derived growth factor action and antagonism of rapamycin by FK506. *Transplantation*. **59**, 390-395.

Capecchi, M.R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell*. **22**, 479-488.

Carrel, A., Guthrie, C.C. (1905) The transplantation of veins and organs. *Am. Med.* **10**, 1101.

Castaneda, A.R., Arnar, O., Schmidt-Habelman, P., Moller, J.H., Zamora, R. Cardiopulmonary autotransplantation in primates. (1972) *J. Cardiovasc. Surg.* **13**, 523-531.

Cavarocchi, N.C., Badellino, M. (1989) Heart/heart-lung transplantation. The domino procedure. *Ann. Thorac. Surg.* **48**, 130-133.

Chamberlain, D., Maurer, J., Chaparro, C., Idolor, L. (1994) Evaluation of transbronchial lung biopsy specimens in the diagnosis of bronchiolitis obliterans after lung transplantation. *J. Heart Lung Transplant.* **13**, 963-971.

Chandraker, A., Russell, M.E., Glysing-Jensen, T., Willett, T.A., Sayegh, M.H. (1997) T-cell costimulatory blockade in experimental chronic cardiac allograft rejection: effects of cyclosporine and donor antigen. *Transplantation*. **63**, 1053-1058.

Chardonnet, Y., Dales, S. (1970) Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virology*. **40**, 462-477.

Charpentier, B. (1993) In: Thompson, Catto (Eds). *GRD Immunol of renal transplant*. Edward Arnold, London. 112.

Chien, S., Zhang, F., Niu, W., Tseng, M.T., Gray, L. Jr. (2000) Comparison of University of Wisconsin, euro-collins, low-potassium dextran, and krebs-henseleit solutions for hypothermic lung preservation. *J. Thorac. Cardiovasc. Surg.* **119**, 921-930.

Chin, D.J., Green, G.A., Zon, G., Szoka, F.C., Straubinger, R.M. (1990) Rapid nuclear accumulation of injected oligodeoxyribonucleotides. *New Biologist.* **2**, 1091-1100.

Chu, G., Hayakawa, H., Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15**, 1311-1326.

Ciubotariu, R., Liu, Z., Colovai, A., Ho, E., Itescu, S., Ravalli, S., Hardy, M.A., Cortesini, R., Rose, E.A., Suciu-Foca, N. (1998) Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J. Clin. Invest.* **101**, 398-405.

Cohen, D.J., Loertscher, R., Rubin, M.F., Tilney, N.L., Carpenter, C.B., Strom, T.B. (1984) Cyclosporine: a new immunosuppressive agent for organ transplantation. *Ann. Intern. Med.* **101**, 667-682.

Collins, L., Gustafsson, K., Fabre, J.W. (2000) Tissue-binding properties of a synthetic peptide DNA vector targeted to cell membrane integrins. A possible universal nonviral vector for organ and tissue transplantation. *Transplantation.* **69**, 1041-1050.

Collins, L., Sawyer, G., Zhang, X.-H., Gustafsson, K., Fabre, J.W. (2000a) In vitro investigation of factors important for the delivery of an integrin-targeted nonviral vector in organ transplantation. *Transplantation.* **69**, 1168-1176.

Cooper, J., Stroka, D., Brostjan, C., Palmetshofer, A., Bach, F.H., Ferran, C. (1996) A20 blocks endothelial cell activation through an NF- κ B-dependent mechanism. *J. Biol. Chem.* **271**, 18068-18073.

Cooper, J.D., Billingham, M., Egan, T., Hertz, M.I., Higenbottam, T., Lynch, J., Maurer, J., Paradis, I., Patterson, G.A., Smith, C. (1993) A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. *J. Heart Lung Transplant.* **12**, 713-716.

Cooper, J.D., Patterson, G.A., Grossman, R., Maurer, J. (1989) Double-lung transplant for advanced chronic obstructive lung disease. *Am. Rev. Respir. Dis.* **139**, 303-307.

Copeland, J.G., Emery, R.W., Levinson, M.M., Icenogle, T.B., Riley, J.E., McAleer, M.J., Copeland, J.A., Dietz, R. (1986) Cyclosporine: an immunosuppressive panacea? *J. Thorac. Cardiovasc. Surg.* **91**, 26-39.

Costa, D.L., Lehmann, J.R., Harold, W.M., Drew, R.T. (1986) Transoral tracheal intubation of rodents using a fiberoptic laryngoscope. *Lab. Anim. Sci.* **36**, 256-261.

Cotten, M., Langle Rouault, F., Kirlappos, H., Wagner, E., Metchler, K., Zenke, M., Beug, H., Birnstiel, M.L. (1990) Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc. Natl. Acad. Sci. USA.* **87**, 4033-4037.

Couetil, J.P., Tolan, M.J., Loulmet, D.F., Guinvarch, A., Chevalier, P.G., Achkar, A., Birmbaum, P., Carpentier, A.F. (1997) Pulmonary bipartitioning and lobar transplantation: a new approach to donor organ shortage. *J Thorac. Cardiovasc. Surg.* **113**, 529-537.

Cowsert, L.M., Fox, M.C., Zon, G., Mirabelli, C.K. (1992) In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment for genital warts. *Antimicro. Agents. Chemother.* **37**, 171-177.

D

Dalchau, R., Fabre, J.W. (1979) Identification and unusual tissue distribution of the canine and human homologues of Thy-1 (theta). *J. Exp. Med.* **149**, 576-591.

Dalesandro, J., Akimoto, H., Gorman, C.M., McDonald, T.O., Thomas, R., Liggitt, H.D., Allen, M.D. (1996) Gene therapy for donor hearts: *ex vivo* liposome-mediated transfection. *J. Thorac. Cardiovasc. Surg.* **111**, 416-421.

Dauber, J.H., Paradis, I.L., Dummer, J.S. (1990) Infectious complications in pulmonary allograft recipients. *Clin. Chest Med.* **11**, 291-308.

De Blic, J., Peuchmaur, M., Carnot, F., Danel, C., Deruesne, M., Reynaud, P., Scheinmann, P., Brousse, N. (1992) Rejection in lung transplantation – an immunohistochemical study of transbronchial biopsies. *Transplantation.* **54**, 639-644.

Defer, C., Belin, M.-T., Caillet-Boudin, M.-L., Boulanger, P. (1990) Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. *J. Virol.* **64**, 3861-3873

Demikhov, V.P. (1960) Some essential points of the technique of transplantation of heart, lungs and other organs. In: *Experimental Transplantation of Vital Organs*. Moscow: Medgiz, State Press for Medical Literature.

Drazan, K.E., Olthoff, K.M., Wu, L., Shen, X-D, Gelman, A., Shaked, A. (1996) Adenovirus-mediated gene transfer in the transplant setting. *Transplantation*. **62**, 1080-1084.

Drazan, K.E., Wu, L., Olthoff, K.M., Jurim, O., Busuttil, R.W., Shaked, A. (1995) Transduction of hepatic allografts achieves local levels of viral IL-10 which suppress alloreactivity in vitro. *J. Surg. Res.* **59**, 219-223.

Duncan, S.R., Paradis, I.L., Yousem, S.A., Similo, S.L., Grgurich, W.F., Williams, P.A., Dauber, J.H., Griffith, B.P. (1992) Sequelae of cytomegalovirus pulmonary infections in lung allograft recipients. *Am. Rev. Respir. Dis.* **146**, 1419-1425.

E

Edwards, K.M., Thompson, J., Paolini, J., Wright, P.F. (1985) Adenovirus infections in young children. *Pediatrics*. **76**, 420-424.

Egan, T., Detterbeck, F.C., Mill, M., Paradowski, L.J., Lackner, R.P., Ogden, W.D., Yankaskas, J.R., Westerman, J.H., Thompson, J.T., Weiner, M.A. (1995) Improved results of lung transplantation for patients with cystic fibrosis. *J. Thorac. Cardiovasc. Surg.* **109**, 224-234.

Ellington, A.D., Szostak, J.W. (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature*. **346**, 818-822.

F

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M. (1987) Lipofectin: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA*. **84**, 7413-7417.

Fisher, K.J., Choi, H., Burda, J., Chen, S.J., Wilson, J.M. (1996) Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology*. **217**, 11-22.

Fisher, T.L., Terhorst, T., Cao, X., Wagner, R.W. (1993) Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acids Res.* **21**, 3857-3865.

Fitzgerald, D.J.P., Padmanabhan, R., Pastan, I., Willingham, M.C. (1983) Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell*. **32**, 607-617.

Flume, P., Egan, T., Paradowski, L., Detterbeck, F.C., Thompson, J.T., Yankaskas, J.R. (1994) Infectious complications of lung transplantation. Impact of cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **149**, 1601-1607.

Friedmann, T. (1971) In vitro reassembly of shell-like particle from disrupted polyoma virus. *Proc. Natl. Acad. Sci. USA.* **68**, 2574-2578.

Fujinami, R.S., Nelson, J.A., Walker, L., Oldstone, M.B. (1988) Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR β chain: a means for graft rejection and immunosuppression. *J. Virol.* **62**, 100-105.

G

Gao, S.Z., Schroeder, J.S., Alderman, E.L., Hunt, S.A., Silverman, J.F., Wiederhold, V., Stinson, E.B. (1987) Clinical and laboratory correlates of accelerated coronary artery disease in the cardiac transplant patient. *Circulation*. **76** (suppl 5), 56-61.

Gao, W.Y., Han, F.S., Storm, C., Egan, W., Cheng, Y.C. (1992) Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology. *Molec. Pharmac.* **41**, 223-229.

Garovoy, M.R., Reddish, M.A., Busch, G.J., Tilney N.L. (1982) Immunoglobulin-secreting cells recovered from rejected human renal allografts. *Transplantation.* **33**, 109-111.

Gassas, A., Ehtisham, S., Aurora, P, Whitehead, B., Whitmore, P., Rees, P.G., Tsang, V.T., Elliott, M.J., de Leval, M.R. (1999) The effect of pre lung transplant clinical status on post transplant outcome for children with cystic fibrosis: a ten year experience. (Abstract) *J. Heart Lung Transplant.* **18**, 83.

Gibson, I. (1994) Antisense DNA and RNA strategies: new approaches to therapy. *J. R. Coll. Physicians Lond.* **28**, 507-511.

Ginsberg, H.S. (1984) The adenoviruses. Plenum, New York.

Glanville, A.R., Burke, C.M., Theodore, J., Baldwin, J.C., Harvey, J., Vankessel, A., Robin, E.D. (1987a) Bronchial hyper-responsiveness after human cardiopulmonary transplantation. *Clin. Sci.* **73**, 299-303.

Glanville, A.R., Baldwin, J.C., Burke, C.M., Theodore, J., Robin, E.D. (1987) Obliterative bronchiolitis after heart-lung transplantation: apparent arrest by augmented immunosuppression. *Ann. Intern. Med.* **107**, 300-304.

Ghosh-Choudhury, G., Haj-Ahmed, Y., Graham, F.L. (1987) Protein IX component of the human adenovirus capsid is essential for the packaging of full length genomes. *EMBO. J.* **6**, 1733-1739.

- Gould, F.K., Freeman, R., Taylor, C.E., Ashcroft, T., Dark, J.H., Corris, P.A. (1993) Prophylaxis and management of cytomegalovirus pneumonitis after lung transplantation: a review of experience in one center. *J. Heart Lung Transplant.* **12**, 695-699.
- Graham, F.L., Prevec, L. (1991) Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in molecular biology.* **7**, 109.
- Graham, F.L., Smiley, J.R., Russell, W.C., Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59-74.
- Graham, F.L., Van Der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* **52**, 456-460.
- Graham, F.L., Van Der Eb, A.J., Heijneker, H.L. (1974) Size and location of the transforming region in human adenovirus type 5 DNA. *Nature.* **251**, 687-690.
- Greber, U.F., Webster, P., Weber, J., Helenius, A. (1996) The role of the adenovirus protease on virus entry into cells. *EMBO. J.* **15**, 1766-1777.
- Griep, R.B., Stinson, E.B., Dong, E.J., Phillips, R.C., Morrell, R.M., Shumway, N.E. (1972) Use of anti-thymocyte globulin in human heart transplantation. *Circulation.* **45**, 147-153.
- Gryzan, S., Paradis, I.L., Zeevi, A., Duquesnoy, R.J., Dummer, J.S., Griffith, B.P., Hardesty, R.L., Trento, A., Nalesnik, M.A., Dauber, J.H. (1988) Unexpectedly high incidence of *Pneumocystis carinii* infection after lung-heart transplantation: implication for lung defense and allograft survival. *Am. Rev. Respir. Dis.* **137**, 1268-1274.

Gudmundsdottir, H., Turka, L.A. (1999) T cell costimulatory blockade: new therapies for transplant rejection. *J. Am. Soc. Nephrol.* **10**, 1356-1365.

H

Halloran, P., Mathew, T., Tomlanovich, S., Groth, C., Hooftman, L., Barker, C. (1997) Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *N. Engl. J. Med.* **338**, 161-165.

Halloran, P.F., Broski, A.P., Batiuk, T.D., Madrenas, J. (1993) The molecular immunology of acute rejection: an overview. *Transplant Immunol.* **1**: 3-27.

Halloran, P.F., Homik, J., Goes, N., Lui, S.L., Urmson, J., Ramassar, V., Cockfield, S.M. (1997) The "injury response": a concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. *Transplant. Proc.* **29**, 79-81.

Hardy, J.D., Webb, W.R., Dalton, M.L., Walker, G.R. (1963) Lung homotransplantation in man: report of the initial case. *JAMA.* **186**, 1065.

Hardy, K.A., Schidlow, D.V., Zaeri, N. (1988) Obliterative bronchiolitis in children. *Chest.* **93**, 460-466.

Hart, S.L., Arancibia-Carcamo, C.V., Wolfert, M.A., Mailhos, C., O'Reilly, N.J., Ali, R.R., Coutelle, C., George, A.J.T., Harbottle, R.P., Knight, A.M., Larkin, F.P., Levinsky, R.J., Seymour, L.W., Thrasher, A.J., Kinnon, C. (1998) Lipid-mediated enhancement of transfection by a nonviral integrin-targeting vector. *Hum. Gene Ther.* **9**, 575-585.

Hart, S.L., Harbottle, R.P., Cooper, R., Miller, A., Williamson, R., Coutelle, C. (1995) Gene delivery and expression mediated by an integrin-binding peptide. *Gene Ther.* **2**, 552-554.

Hart, S.L., Knight, A.M., Harbottle, R.P., Mistry, A., Hunger, H.D., Cutler, D.F., Williamson, R. (1994) Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *J. Biol. Chem.* **269**, 12468-12474.

Hasegawa, T., Iacono, A., Yousem, S.A. (1999) The significance of bronchus-associated lymphoid tissue in human lung transplantation: is there an association with acute and chronic rejection? *Transplantation.* **67**, 381-385.

Heidenreich, O., Kang, S-H., Xu, X., Nerenberg, M. (1995) Application of antisense technology to therapeutics. *Mol. Med. Today.* **1**, 128-133.

Helene, C., Toulme, J. (1990) Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim. Biophys. Acta.* **1049**, 99-125.

Hertz, M.I., Jessurun, J., King, M.B., Sarik, S.K., Murray, J.J. (1993) Reproduction of the obliterative bronchiolitis lesion after heterotopic transplantation of mouse airway. *Am. J. Pathol.* **142**, 1945-1951.

Hierholzer, J.C. (1992) Adenoviruses in the immunocompromised host. *Clin. Microbiol. Rev.* **5**, 262-274.

Higenbottam, T., Jackson, M., Woolman, P., Lowry, R., Wallwork, J. (1989) The cough response to ultrasonically nebulized distilled water in heart-lung transplantation patients. *Am. Rev. Respir. Dis.* **140**, 58-61.

Higenbottam, T.W., Stewart, S., Penketh, A., Wallwork, J. (1987) The diagnosis of lung rejection and opportunistic infection by transbronchial lung biopsy.

Transplant. Proc. **19**, 3777-3778.

Hoke, G.D., Draper, K., Freier, S.M., Gonzalez, C., Driver, V.B., Zounes, M.C., Ecker, D.J. (1991) Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. *Nucleic Acids Res.* **19**, 5743-5748.

Hosenpud, J.D., Bennett, L.E., Keck, B.M., Fiol, B., Boucek, M.M., Novick, R.J. (1999) The registry of the International Society for Heart and Lung Transplantation: sixteenth official report – 1999. *J. Heart Lung Transplant.* **18**, 611-626.

Hynes, R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* **69**, 11-25.

I

Isberg, R.R. (1991) Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. *Science.* **252**, 934-938.

Isberg, R.R., Tran Van Nhieu, G. (1994) Binding and internalization of microorganisms by integrin receptors. *Trends. Microbiol.* **2**, 10-14.

Ishikawa, Y., Homcy, C.J. (1992) High efficiency gene transfer into mammalian cells by a double transfection protocol. *Nucleic Acids Res.* **20**, 4367.

Iverson, P.L., Zhu, S., Meter, A., Zon, G. (1992) Cellular uptake and subcellular distribution of phosphorothioate oligonucleotides into cultured cells. *Antisense Res. Dev.* **2**, 211-222.

J

Jackson, D.A., Symon, R.H., Berg, P. (1972) Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose peron of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **69**, 2904-2909.

Jenkins, R.G., Herrick, S.E., Meng, Q-H., Kinnon, C., Laurent, G.J., McAnulty, R.J., Hart, S.L. (2000) An integrin-targeted non-viral vector for pulmonary gene therapy. *Gene Therapy*. **7**, 393-400.

Jurado Chacon, M., Hernandez Mohedo, F., Navarro Mari, J.M., Ferrer Chaves, C., Escobar Vedia, J.L., de Pablos Gallego, J.M. (1998) Adenovirus pneumonitis successfully treated with intravenous ribavirin. *Haematologica*. **83**, 1128-1129.

K

Kantrowitz, A., Haller, J.D., Joos, H., Cerruti, M.M., Carstensen, H.E. (1968) Transplantation of the heart in an infant and an adult. *Am. J. Cardiol*. **22**, 782-790.

Karlsson, S., Van Doren, K., Schweiger, S.G., Nienhuis, A.W., Gluzman, Y. (1986) Stable gene transfer and tissue-specific expression of a human globin gene using adenoviral vectors. *EMBO. J*. **5**, 2377-2385.

Kato, K., Kaneda, Y., Sakurai, M., Nakanishi, M., Okada, Y. (1991) Direct injection of hepatitis virus DNA into liver induced hepatitis in adult rats. *J. Biol. Chem*. **266**, 22071-22074.

Keenan, R.J., Iacono, A., Dauber, J.H., Zeevi, A., Yousem, S.A., Ohori, N.P., Burckart, G.J., Kawai, A., Smaldone, G.C., Griffith, B.P. (1997) Treatment of refractory acute allograft rejection with aerosolized cyclosporine in lung transplant recipients. *J. Thorac. Cardiovasc. Surg.* **113**, 335-340.

Keenan, R.J., Lega, M.E., Dummer, J.S., Paradis, I.L., Dauber, J.H., Rabinowich, H., Yousem, S.A., Hardesty, R.L., Griffith, B.P., Duquesnoy, R.J. (1991) Cytomegalovirus serologic status and postoperative infection correlated with risk of developing chronic rejection after pulmonary transplantation. *Transplantation.* **51**, 433-438.

Kemeny, D.M., Noble, A., Holmes, B.J., Diaz-Sanchez, D. (1994) Immune regulation: a new role for the CD8+ T cell. *Immunol. Today.* **15**, 107-110.

Kesten, S., Chaparro, C. (1999) Mycobacterial infections in lung transplant recipients. *Chest.* **115**, 741-745.

Klein, J., Juretic, A., Baxevanis, C.N., Nagy, Z.A. (1981) The traditional and a new version of the mouse H-2 complex. *Nature.* **291**, 455-460.

Kornberg, A. (1968) The synthesis of DNA. *Scientific American.* **219**, 64-78.

Kramer, M.R. (1993a) Bronchiolitis obliterans following heart-lung transplantation. *Respir. Med.* **88**, 9-15.

Kramer, M.R., Denning, D.W., Marshall, S.E., Ross, D.J., Berry, G., Lewiston, N.J., Stevens, D.A., Theodore, J. (1991) Ulcerative tracheobronchitis after lung transplantation: a new form of invasive aspergillosis. *Am. Rev. Respir. Dis.* **144**, 552-556.

Kramer, M.R., Marshall, S.E., Starnes, V.A., Gamberg, P., Amitai, Z., Theodore, J. (1993) Infectious complications in heart-lung transplantation. Analysis of 200 episodes. *Arch. Intern. Med.* **153**, 2010-2016.

Kramer, M.R., Stoeck, C., Lewiston, N.J., Starnes, V.A., Theodore, J. (1992) Trimethoprim-sulphamethoxazole prophylaxis for *Pneumocystis carinii* infections in heart-lung and lung transplantation – how effective and for how long? *Transplantation*. **53**, 586-589.

Kramer, M.R., Stoeck, C., Whang, J.L., Berry, G.J., Sibley, R., Marshall, S.E., Patterson, G.M., Starnes, V.A., Theodore, J. (1993b) The diagnosis of obliterative bronchiolitis after heart-lung transplantation: low yield of transbronchial lung biopsy. *J. Heart Lung Transplant.* **12**, 675-681.

Krensky, A.M., Clayberger, C. (1994) Transplant immunology. *Pediatr. Clin. North Am.* **41**, 819-839.

L

Latchman, D.S. (1994) Herpes simplex virus vectors for gene therapy. *Mol. Biotechnol.* **2**, 179-195.

Lawrence, E.C. (1990) Diagnosis and management of lung allograft rejection. *Clin. Chest Med.* **11**, 269-278.

Lawrence, E.C., Holland, V.A., Young, J.B., Windsor, N.T., Brousseau, K.P., Noon, G.P., Whisnand, H.H., Debaek, M.E., Nelson, D.L. (1989) Dynamic changes in soluble interleukin-2 receptor levels after lung or heart-lung transplantation. *Am. Rev. Respir. Dis.* **140**, 789-796.

Lechler, R.I., Lombardi, G., Batchelor, J.R., Reinsmoen, N., Bach, F.H. (1990) The molecular basis of alloreactivity. *Immunol. Today*. **11**, 83-88.

Ledley, F.D. (1994a) Development in somatic gene therapy. *Exp. Op. Invest. Drugs*. **3**, 913-921.

Ledley, F.D. (1994b) Non-viral gene therapies. *Curr. Opinion Biotechnol*. **5**, 626-636.

Lenschow, D.J., Zeng, Y., Thistlewaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S., Bluestone, J.A. (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4-Ig. *Science*. **257**, 789-792.

Lentz, D., Bergin, C.J., Berry, G.J., Stoehr, C., Theodore, J. (1992) Diagnosis of bronchiolitis obliterans in heart-lung transplantation patients: importance of bronchial dilatation on CT. *AJR*. **159**, 463-467.

Leonetti, J.P., Mechti, N., Degols, G., Gagnor, G., Lebleu, B. (1991) Intracellular distribution of microinjected antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA*. **88**, 2702-2706.

Lewis, J.B., Mathews, M. (1980) Control of adenovirus early gene expression: a class of immediate early products. *Cell*. **21**, 303-313.

Liszewicz, J., Sun, D., Weichold, F.F., Thierry, A.R., Lusso, P., Tang, J., Gallo, R.C., Agrawal, S. (1994) Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. *Proc. Natl. Acad. Sci. USA*. **91**, 7942-7946.

Liu, C., Deng, S., Yang, Z., Kucher, T., Guo, F., Gelman, A., Chen, H., Naji, A., Shaked, A., Brayman, K.L. (1999) Local production of CTLA4-Ig by adenoviral-mediated gene transfer to the pancreas induces permanent allograft survival and donor-specific tolerance. *Transplant. Proc.* **31**, 625-626.

Lloyd, K.S., Barnard, P., Holland, V.A., Noon, G.P., Lawrence E.C. (1990) Pulmonary function after heart-lung transplantation using larger donor organs. *Am. Rev. Respir. Dis.* **142**, 1026-1029.

Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S., Neckers, L.M. (1989) Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA.* **86**, 3474-3478.

Low, D.E., Kaiser, L.R., Haydock, D.A., Trulock, E., Cooper, J.D. (1993) The donor lung: infectious and pathologic factors affecting outcome in lung transplantation. *J. Thorac. Cardiovasc. Surg.* **106**, 614-621.

Lower, R.R., Shumway, N.E. (1960) Studies of orthotopic homotransplantation of the canine heart. *Surg. Forum.* **11**, 18.

M

McElvaney, N.G. (1996) Is gene therapy in cystic fibrosis a realistic expectation? *Curr. Opin. Pulm. Med.* **2**, 466-471.

McGregor, C.G.A., Baldwin, J.C., Jamieson, S.W., Billingham, M.E., Yousem, S.A., Burke, C.M., Oyer, P.E., Stinson, E.B., Shumway, N.E. (1985) Isolated pulmonary rejection after combined heart-lung transplantation. *J. Thorac. Cardiovasc. Surg.* **90**, 623-626.

McPhaull, J.J. Jr., Stastny, P., Freeman, R.B. (1981) Specificities of antibodies eluted from human cadaveric renal allografts. Multiple mechanisms of renal allograft injury. *J. Clin. Invest.* **67**, 1405-1414.

Macdonald, P., Mundy, J., Rogers, P., Harrison, G., Branch, J., Glanville, A., Keogh, A., Spratt, P. (1995) Successful treatment of life-threatening acute reperfusion injury after lung transplantation with inhaled nitric oxide. *J. Thorac. Cardiovasc. Surg.* **110**, 861-863.

Maat, J., Van Bevern, C.P., Van Ormondt, H. (1980) The nucleotide sequence of adenovirus type 5 early region E1: the region between map positions 8.0 (hindiii site) and 11.8 (smaI site). *Gene.* **10**, 27-38.

Macek, V., Sorli, J., Kopriva, S., Marin, J. (1994) Persistent adenoviral infection and chronic airway obstruction in children. *Am. J. Respir. Crit. Care. Med.* **150**, 7-10.

Mankad, P.S., de Leval, M.R. (1997) Heart-lung transplantation. In: Franco, K.L., (Ed). *Pediatric cardiopulmonary transplantation*. Futura, New York. 167-184.

Mann, F.C., Priestley, J.T., Markowitz, J. (1933) Transplantation of the intact mammalian heart. *Arch. Surg.* **26**, 219.

Marshall, S.E., Lewiston, N.J., Kramer, M.R., Sibley, R.K., Berry, G., Rich, J.B., Theodore, J., Starnes, V.A. (1991) Prospective analysis of serial pulmonary function studies and transbronchial biopsies in single-lung transplant recipients. *Transplant. Proc.* **23**, 1217-1219.

Maslo, C., Girard, P.M., Urban, T., Guessant, S., Rozenbaum, W. (1997) Ribavirin therapy for adenovirus pneumonia in an AIDS patient. *Am. J. Respir. Crit. Care. Med.* **156**, 1263-1264.

Mastrangeli, A., Danel, C., Rosenfeld, M.A., Stratford-Perricaudet, L., Perricaudet, M., Pavirani, A., Lecocq, J.P., Crystal, R.G. (1993) Diversity of airway epithelial cell targets for *in vivo* recombinant adenovirus-mediated gene transfer. *J. Clin. Invest.* **91**, 225-234.

Maurer, J.R., Tullis, E., Grossman, R.F., Vellend, H., Winton, T.L., Patterson, G.A. (1992) Infectious complications following isolated lung transplantation. *Chest* **101**, 1056-1059.

Merrick, A.F., Shewring, L.D., Sawyer, G.J., Gustafsson, K.T., Fabre, J.W. (1996) Comparison of adenovirus gene transfer to vascular endothelial cells in cell culture, organ culture, and *in vivo*. *Transplantation*. **62**, 1085-1089.

Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., Roche, A.C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res.* **21**, 871-878.

Miller, D.G., Adam, M.A., Miller, A.D. (1990) Gene transfer by retrovirus vector occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* **10**, 4139-4142.

Milligan, J.F., Matteucci, M.D., Martin, J.C. (1993) Current concepts in antisense drug design. *J. Med. Chem.* **36**, 1923-1937.

Miroshnichenko, O.I., Ponomareva, T.I., Tikchonenko, T.I. (1989) Inhibition of adenovirus 5 replication in COS-1 cells by antisense RNAs against the viral E1a region. *Gene*. **84**, 83-89.

Moretta, L., Ciccone, E., Moretta, A., Hoglund, P., Ohlen, C., Karre, K. (1992) . Allorecognition by NK cells: nonself or no self? *Immunol. Today*. **13**, 300-306.

Murtagh, P., Cerqueiro, C., Halac, A., Avila, M., Kajon, A. (1993) Adenovirus type 7 respiratory infections: a report of 29 cases of acute lower respiratory disease. *Acta Paediatr.* **82**, 557-561.

N

Nakae, S., Webb, W.R. Theorides, T., Sugg, W.L. (1967) Respiratory function following cardiopulmonary denervation in dog, cat and monkey. *Surg. Gynaecol. Obstet.* **125**, 1285-1292.

Naldini, L., Blomer, U., Gallat, P. Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D. (1996) *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science.* **272**, 263-267.

Nathan, C.F., Murray, H.W., Cohn, Z.A. (1980) The macrophage as an effector cell. *N. Engl. J. Med.* **303**, 622-626.

Neptune, W.B., Cookson, B.A., Bailey, C.P. (1953) Complete homologous heart transplantation. *AMA. Arch. Surg.* **66**, 174.

Newham, P., Humphries, M.J. (1996) Integrin adhesion receptors: structure, function and implications for biomedicine. *Mol. Med. Today.* **2**, 304-313.

Novick, R.J., Andreassian, B., Schafers, H.J., Haverich, A., Patterson, G.A., Kaye, M, P., Menkis, A.H., McKenzie, F.N. (1994) Pulmonary retransplantation for obliterative bronchiolitis: intermediate-term results of a North American-European series. *J. Thorac Cardiovasc. Surg.* **107**, 755-763.

Novick, R.J., Menkis, A.H., McKenzie, F.N. (1992) New trends in lung preservation: a collective review. *J. Heart Lung Transplant.* **11**, 377-392.

Noyes, B.E., Kurland, G., Orenstein, D.M., Fricker, F.J., Armitage, J.M. (1994)
Experience with pediatric lung transplantation. *J. Pediatr.* **124**, 261-268.

O

Ohori, N.P., Michaels, M.G., Jaffe, R., Williams, P., Yousem, S.A. (1995)
Adenovirus pneumonia in lung transplant recipients. *Hum. Pathol.* **26**, 1073-1079.

Otulana, B.A., Higenbottam, T., Scott, J., Clelland, C., Igboaka, G., Wallwork, J.
(1990) Lung function associated with histologically diagnosed acute lung rejection
and pulmonary infection in heart-lung transplant patients. *Am. Rev. Respir. Dis.*
142, 329-332.

Otulana, B.A., Mist, B.A., Scott, J.P., Wallwork, J., Higenbottam, T. (1989) The
effect of recipient lung size on lung physiology after heart-lung transplantation.
Transplantation. **48**, 625-629.

P

Parham, P., Ohta, T. (1996) Population biology of antigen presentation by MHC
class I molecules. *Science.* **272**, 67-74.

Patterson, G.M., Wilson, S., Whang, J.L., Harvey, J., Agacki, K., Patel, H.,
Theodore, J. (1996) Physiologic definitions of obliterative bronchiolitis in heart-
lung and double lung transplantation: a comparison of the forced expiratory flow
between 25% and 75% of the forced vital capacity and forced expiratory volume in
one second. *J. Heart Lung Transplant.* **15**, 175-181.

Penketh, A.R.L., Higenbottam, T.W., Hutter, J., Coutts, C., Stewart, S., Wallwork, J. (1988) Clinical experience in the management of pulmonary opportunistic infection and rejection in heart-lung transplant recipients. *Thorax*. **43**, 762-769.

Pierschbacher, M.D., Ruoslahti, E. (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*. **309**, 30-33.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem.* **269**, 12918-12924.

Platt, J.L., Fischel, R.J., Matas, A.J., Reif, S.A., Bolman, R.M., Bach, F.H. (1991) Immunopathology of hyperacute rejection in a swine-to-primate model. *Transplantation*. **52**, 214-220.

Q

Qin, L., Chavin, K.D., Ding, Y., Favaro, J.P., Woodward, J.E., Lin, J., Tahara, H., Robbins, P., Shaked, A., Ho, D.Y. (1995) Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model. Immunosuppression with TGF-beta 1 or vIL-10. *Transplantation*. **59**, 809-816.

Qin, L., Chavin, K., Ding, Y., Tahara, H., Favaro, J., Woodward, J., Susuki, T., Robbins, P., Lotze, M., Bromberg, J. (1996) Retrovirus mediated gene transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J. Immunol.* **156**, 2316-2323.

Quinlan, M.P. (1993) Expression of antisense E1A in 293 cells results in altered cell morphologies and cessation of proliferation. *Oncogene* **8**, 257-265.

R

Rabinowich, H., Zeevi, A., Paradis, I.L., Yousem, S.A., Dauber, J.H., Kormos, R., Hardesty, R.L., Griffith, B.P., Duquesnoy, R.J. (1990) Proliferative responses of bronchoalveolar lavage lymphocytes from heart-lung transplant patients.

Transplantation. **49**, 115-121.

Radley-Smith, R.C., Burke, M., Pomerance, A., Yacoub, M.H. (1995) Graft vessel disease and obliterative bronchiolitis after heart-lung transplantation in children.

Transplant. Proc. **27**, 2017-2018.

Randhawa, P.S., Yousem, S.A., Paradis, I.L., Dauber, J.A., Griffith, B.P., Locker, J. (1989) The clinical spectrum, pathology and clonal analysis of Epstein-Barr virus-associated lymphoproliferative disorders in heart-lung transplant recipients. *Am. J. Clin. Pathol.* **92**, 177-185.

Reid, K.R., McKenzie, F.N., Menkis, A.H., Novick, R.J., Pflugfelder, P.W., Kostuk, W.J., Ahmad, D. (1990) Importance of chronic aspiration in recipients of heart-lung transplants. *Lancet.* **336**, 206-208.

Reitz, B.A., Burton, N.A., Jamieson, S.W., Bieber, C.P., Pennock, J.L., Stinson, E.B., Shumway, N.E. (1980) Heart and lung transplantation. Autotransplantation and allotransplantation in primates with extended survival. *J. Thorac. Cardiovasc. Surg.* **80**, 360-372.

Reitz, B.A., Stinson, E.B. (1982) Cardiac transplantation--1982. *JAMA.* **248**, 1225-1227.

Reitz, B.A., Wallwork, J.L., Hunt, S.A., Pennock, J.L., Billingham, M.E., Oyer, P.E., Stinson, E.B., Shumway, N.E. (1982) Heart-lung transplantation: successful therapy for patients with pulmonary vascular disease. *N. Engl. J. Med.* **306**, 557-564.

Report working party. (1993) Standardization of lung function tests. *Eur. Respir. J.* **6**, Suppl. 16.

Ridge, J.P., Di Rosa, F., Matzinger, P. (1998) A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and T-killer cell. *Nature*. **393**, 474-478.

Rodighiero, V. (1989) Therapeutic drug monitoring of cyclosporin. Practical applications and limitations. *Clin. Pharmacokinet.* **16**, 27-37.

Rogers, S., Pfuderer, P. (1968) Use of viruses as carriers of added genetic information. *Nature*. **219**, 749-751.

Rosenkrantz, A.A., Yachmenev, S.V., Jans, D.A., Serebryakova, N.V., Murav'ev, V.I., Peters, R., Sobolev, A.S. (1992) Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Experimental Cell Res.* **199**, 323-329.

Rosenthal, M., Bain, S.H., Cramer, D., Helms, P., Denison, D., Bush, A., Warner, J.O. (1993a) Lung function in white children aged 4 to 19 years: I-spirometry. *Thorax*. **48**, 794-802.

Rosenthal, M., Cramer, D., Bain, S.H., Denison, D., Bush, A., Warner, J.O. (1993b) Lung function in white children aged 4 to 19 years: II-- Single breath analysis and plethysmography. *Thorax*. **48**, 803-808

Roth, G., Curiel, T., Lacy, J. (1994) Epstein-Barr viral nuclear antigen 1 antisense oligodeoxynucleotide inhibits proliferation of Epstein-Barr virus-immortalized B cells. *Blood*. **84**, 582-587.

Ruuskanen, O., Meurman, O., Sarkkinen, H. (1985) Adenoviral diseases in children: a study of 105 hospital cases. *Pediatrics* **76**, 79-83.

S

Salmons, B., Gunzburg, W.H. (1993) Targeting of retroviral vectors for gene therapy. *Hum. Gen. Ther.* **4**, 129-141.

Sambrook, J., Botchan, M., Gallimore, P., Ozanne, B., Pettersson, U., Williams, J., Sharp, P.A. (1974) Viral DNA sequences in cells transformed by simian virus 40, adenovirus type 2 and adenovirus type 5. *Cold Spring Harbor Sym. Quant. Biol.* **39**, 615-632.

Sanders, M.H., Owens, G.R., Sciruba, F.C., Rogers, R.M., Paradis, I.L., Griffith, B.P., Hardesty, R.L. (1989) Ventilation and breathing pattern during progressive hypercapnia and hypoxia after human heart-lung transplantation. *Am. Rev. Respir. Dis.* **140**, 38-44.

Sayegh, M.H., Turka, L.A. (1998) The role of T-cell costimulatory activation pathways in transplant rejection. *N. Engl. J. Med.* **338**, 1813-1821.

Scarborough, R.M., Rose, J.W., Naughton, M.A., Phillips, D.R., Nannizzi, L., Arfsten, A., Campbell, A.M., Charo, I.F. (1993) Characterization of the integrin specificities of disintegrins isolated from American pit viper venoms. *J. Biol. Chem.* **268**, 1058-1065.

Schiedner, G., Morral, N., Parks, R.J., Wu, Y., Koopmans, S.C., Lagston, C., Graham, F.L., Beaudet, A.L., Kochanek, S. (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* **18**, 180-183.

Scott, J.P., Higenbottam, T.W., Sharples, L., Clelland, C.A., Smyth, R.L., Stewart, S., Wallwork, J. (1991) Risk factors for obliterative bronchiolitis in heart-lung transplant recipients. *Transplantation.* **51**, 813-817.

- Sedmark, D.D., Orosz, C.G. (1991) The role of vascular endothelial cells in transplantation. *Arch. Pathol. Lab. Med.* **115**, 260-265.
- Serrano-Ikkos, E., Lask, B., Whitehead, B., Eisler, I. (1998) Incomplete adherence after paediatric heart and heart-lung transplantation. *J. Heart Lung Transplant.* **17**, 1177-1183.
- Shaw, J.P., Kent, K., Bird, J., Fishback, J., Froehler, B.F. (1991) Modified deoxyoligo-nucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res.* **19**, 747-750.
- Shewring, L., Collins, L., Lightman, S.L., Hart, S., Gustaffsson, K., Fabre, J.W. (1997) A nonviral vector system for efficient gene transfer to corneal endothelial cells via membrane integrins. *Transplantation.* **64**, 763-769.
- Shimotohno, K., Temin, H.M. (1981) Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell.* **26**, 67-77.
- Simil, S., Linna, O., Lanning, P., Heikkinen, E., Ala-Houhala, M. (1981) Chronic lung damage caused by adenovirus 7: a ten-year follow-up study. *Chest* **80**, 127-131.
- Simsir, A., Greenebaum, E., Nuovo, G., Schulman, L.L. (1998) Late adenovirus pneumonitis in a lung transplant recipient. *Transplantation.* **65**, 592-594.
- Skeens, J.L., Fuhrman, C.R., Yousem, S.A. (1989) Bronchiolitis obliterans in heart-lung transplantation patients: radiologic findings in eleven patients. *AJR.* **153**, 253-256.

Sly, P.D., Soto-Quiros, M.E., Landau, L.I., Hudson, I., Newton-John, H. (1984) Factors predisposing to abnormal pulmonary function after adenovirus type 7 pneumonia. *Arch. Dis. Child.* **59**, 935-939.

Smith, C.C., Aurelian, L., Reddy, M., Miller, P.S., Ts'o, P.O.P. (1986) Antiviral effect of an oligo (nucleotide methylphosphonate) complementary to the splice-junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. *Biochemistry.* **83**, 2787-2791.

Smyth, R.L., Higenbottam, T.W., Scott, J.P., McGoldrick, J.P., Whitehead, B.F., Helms, P., de Leval, M., Wallwork, J. (1989) Early experience of heart-lung transplantation. *Arch. Dis. Child.* **64**, 1225-1230.

Smyth, R.L., Higenbottam, T.W., Scott, J.P., Wreghitt, T.C., Stewart, S., Clelland, C.A., McGoldrick, J.P., Wallwork, J. (1990) Herpes simplex virus infection in heart-lung transplant recipients. *Transplantation.* **49**, 735-739.

Snell, G.I., de Hoyos, A., Krajden, M., Winton, T., Maurer, J.R. (1993) *Pseudomonas cepacia* in lung transplant recipients with cystic fibrosis. *Chest.* **103**, 466-471.

Spray, T.L., Mallory, G.B., Canter, C.B., Huddleston, C.B. (1994) Pediatric lung transplantation: indication, techniques, and early results. *J. Thorac. Cardiovasc. Surg.* **107**, 990-1000.

Starnes, V.A., Marshall, S.E., Lewiston, N.J., Theodore, J., Stinson, E.B., Shumway, N.E. (1991) Heart-lung transplantation in infants, children and adolescents. *J. Pediatr. Surg.* **26**, 434-438.

Stein, C.A., Cheng, Y.C. (1993) Antisense oligonucleotides as therapeutic agents – Is the bullet really magical? *Science.* **261**, 1004-1012.

Sundaresan, S., Alevy, Y.G., Steward, N., Tucker, J., Trulock, E.P., Cooper, J.D., Patterson, G.A., Mohanakumar, T. (1995) Cytokine gene transcripts for tumor necrosis factor- α , interleukin-2, and interferon- γ , in human pulmonary allografts. *J. Heart Lung Transplant.* **14**, 512-518.

Sykes. M., Sachs, D.H., Nienhuis, A.W., Pearson, D.A., Moulton, A.D., Bodine, D.M. (1993) Specific prolongation of skin graft survival following retroviral transduction of bone marrow with an allogeneic major histocompatibility complex gene. *Transplantation.* **55**, 197-202.

T

Tabin, C.J., Hoffmann, J.W., Goff, S.P., Weinberg, R.A. (1982) Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. *Mol. Cell. Biol.* **2**, 426-436.

Takahashi, S. (1990) Conformation of membrane fusion-active 20-residue peptides with or without lipid bilayers. Implication of alpha-helix formation for membrane fusion. *Biochemistry.* **29**, 6257-6264.

Tatum, E.L. (1966) Molecular biology, nucleic acids and the future of medicine. *Perspect. Biol. Med.* **10**, 19-32.

Theodore, J. (1990a) Physiology of the lung in human heart-lung transplantation. *Cardiovasc. Clin.* **20**, 57-69.

Theodore, J., Jamieson, S.W., Burke, C.M., Reitz, B.A., Stinson, E.B., Van Kessel, A., Dawkins, K.D., Herran, J.J., Oyer, P.E., Hunt, S.A. (1984) Physiologic aspects of human heart-lung transplantation: pulmonary function status of the post-transplanted lung. *Chest.* **89**, 349-357.

Theodore, J., Starnes, V.A., Lewiston, N.J. (1990) Obliterative bronchiolitis. *Clin. Chest Med.* **II**, 309-321.

Thet, L.A. (1983) A simple method of intubating rats under direct vision. *Lab. Anim. Sci.* **33**, 368.

Tidd, D.M., Warenus, H.M. (1989) Partial protection of oncogene, antisense oligodeoxynucleotides against serum nuclease degradation using terminal methylphosphonate groups. *Br. J. Cancer.* **60**, 343-350.

Tilney, N.L., Schmid, C., Azuma, H., Heemann, U.W. (1994) Transplant immunology: an introduction. *Transplant Immunol.* **2**, 99-102.

Tilney, N.L., Whitley, W.D., Diamond, J.R., Kupiec-Weglinski, J.W., Adams, D.H. (1991) Chronic rejection – an undefined conundrum. *Transplantation.* **52**, 389-398.

Toronto Lung Transplant Group. (1986) Unilateral lung transplantation for pulmonary fibrosis. *N. Engl. J. Med.* **14**, 1140.

Trulock, E.P., Bolman, R.M., Genton, R. (1989) Pulmonary disease caused by *Mycobacterium chelonae* in a heart-lung transplant recipient with obliterative bronchiolitis. *Am. Rev. Respir. Dis.* **140**, 802-805.

Tullius, S.G., Tilney, N.L. (1995) Both alloantigen-dependent and -independent factors influence chronic allograft rejection. *Transplantation.* **59**, 313-318.

V

Valentine, V.G., Robbins, R.C., Wehner, J.H., Patel, H.R., Berry, G.J., Theodore, J. (1996) Total lymphoid irradiation for refractory acute rejection in heart-lung and lung allografts. *Chest*. **109**, 1184-1189.

Vella, J.P., Sayegh, M.H. (1997) Current and future immunosuppressive therapies: impact on chronic allograft dysfunction. *J. Nephrol.* **10**, 229-231.

Vella, J.P., Sayegh, M.H., Turka, L.A. (1998) Developments in the clinical science of transplantation during the 20th century. *Pediatr. Transplantation*. **2**, 257-262.

Vincenti, F., Kirkman, R., Light, S., Bumgardner, G., Pescovitz, M., Halloran, P., Neylan, J., Wilkinson, A., Ekberg, H., Gaston, R., Backman, L., Burdick, J. (1998) Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N. Engl. J. Med.* **338**, 161-165.

Vinogradov, S.V., Suzdaltseva, Y., Alakhov, V.Yu., Kabanov, A.V. (1994) Inhibition of herpes simplex virus 1 reproduction with hydrophobized antisense oligonucleotides. *Biochem. Biophys. Res. Commun.* **203**, 959-966.

Viola, A., Schroeder, S., Sakakibara, Y., Lanzavecchia, A. (1999) T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science*. **283**, 680-682.

Vlassov, V.V., Balakireva, L.A., Yakubov, L.A. (1994) Transport of oligonucleotides across natural and model membranes. *Biochem. Biophys. Acta*. **1197**, 95-108.

W

Waaga, A.M., Rocha, A. M., Tilney, N.L. (1997) Early risk factors contributing to the evolution of long-term allograft dysfunction. *Transplant. Rev.* **11**, 208-216.

Wagner, E., Cotten, M., Foisner, R., Birnstiel, M.L. (1991) Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. USA.* **88**, 4255-4259.

Wagner, E., Zenke, M., Cotton, M., Beug, H., Birnstiel, M.L. (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. USA.* **87**, 3410-3414.

Wagner, J.A., Reynolds, T., Moran, M.L., Moss, R.B., Wine, J.J., Flotte, T.R., Gardner, P. (1998) Efficient and persistent gene transfer of AAV-CFTR in maxillary sinus. *Lancet.* **351**, 1702-1703.

Wagner, R.W. (1994) Gene inhibition using antisense oligodeoxynucleotides. *Nature.* **372**, 333-335.

Wagner, R.W., Matteucci, M.D., Lewis, J.G., Gutierrez, A.J., Moulds, C., Froehler, B.C. (1993) Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science.* **260**, 1510-1513.

Walker, R.C., Paya, C.V., Marshall, W.F., Strickler, J.G., Wiesner, R.H., Velosa, J.A., Habermann, T.M., Daly, R.C., McGregor, C.G. (1995) Pretransplantation sero-negative Epstein-Barr virus status is the primary risk factor for posttransplantation lymphoproliferative disease in adult heart, lung and other solid organ transplantations. *J. Heart Lung Transplant.* **14**, 214-221.

Walker, S., Sofia, M.J., Kakarla, R., Kogan, N.A., Wierichs, L., Longley, C.B., Bruker, K., Axelrod, H.R., Midha, S., Babu, S., Kahne, D. (1996) Cationic facial amphiphiles: a promising class of transfection agents. *Proc. Natl. Acad. Sci. USA*. **93**, 1585-1590.

Walther, W., Stein, U. (2000) Viral vectors for gene transfer. A review of their use in the treatment of human diseases. *Drugs*. **60**, 249-271.

Wakita, T., Wands, J.R. (1994) Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. In vitro model for selection of target sequence. *J. Biol. Chem*. **269**, 14205-14210.

Wang, J., Jiao, S., Wolff, J.A., Knechtle, S.J. (1992) Gene transfer and expression in rat cardiac transplants. *Transplantation*. **53**, 703-705.

Wang, J., Ma, Y., Knechtle, S.J. (1996) Adenovirus-mediated gene transfer into rat cardiac allografts. Comparison of direct injection and perfusion. *Transplantation*. **61**, 1726-1729.

Watson, D.C., Reitz, B.A., Baumgartner, W.A., Raney, A.A., Oyer, P.E., Stinson, E.B., Shumway, N.E. (1979) Distant heart procurement for transplantation. *Surgery*. **86**, 56-59.

Webb, W.R., Howard, H.S. (1957) Restoration of function of the refrigerated heart. *Surg. Forum*. **8**, 302.

Wei, C-M., Gibson, M., Spear, P.G., Scolnick, E.M. (1981) Construction and isolation of a transmissible retrovirus containing the src gene of harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *J. Virol*. **39**, 935-944.

Weisner, R.H., Ludwig, J., Van Hoek, B., Krom, R.A. (1991) Current concepts in cell-mediated hepatic allograft rejection leading to ductopenia and liver failure.

Hepatology. **14**, 721-729.

Weiss, D.J., Liggitt, D., Clark, J.G. (1997) *In situ* histochemical detection of beta-galactosidase activity in lung: assessment of X-gal reagent in distinguishing *lacZ* gene expression and endogenous beta-galactosidase activity. *Hum. Gene Ther.* **8**, 1545-1554.

Wharton, S.A., Martin, S.R., Ruigrok, R.W., Skehel, J.J., Wiley, D.C. (1988) Membrane fusion by peptide analogues of influenza virus haemagglutinin. *J. Gen. Virol.* **69**, 1847-1857.

Whitehead, B., Dinwiddie, R., Adwani, S., Fabre, J., Rees, P., Elliott, M., de Leval, M. (1994) Pulmonary function in children with cystic fibrosis following heart-lung transplantation. (Abstract) *Pediatr. Pulmonol.* Suppl **10**, 272.

Whitehead, B., Helms, P., Goodwin, M., Martin, I., Lask, B., Serrano, E., Scott, J.P., Smyth, R.L., Higenbottam, T.W., Wallwork, J., Elliott, M., de Leval, M. (1991) Heart-lung transplantation for cystic fibrosis. 1: Assessment. *Arch. Dis. Child.* **66**, 1018-1021.

Whitehead, B., Rees, P., Sorensen, K., Bull, C., Higenbottam, T.W., Wallwork, J., Fabre, J., Elliott, M., de Leval, M. (1993) Incidence of obliterative bronchiolitis after heart-lung transplantation in children. *J. Heart Lung Transplant.* **12**, 903-908.

Whitehead, B., Scott, J.P., Helms, P., Malone, M., Macrae, D., Higenbottam, T.W., Smyth, R.L., Wallwork, J., Elliott, M., de Leval, M. (1992) Technique and use of transbronchial biopsy in children and adolescents. *Pediatr. Pulmonol.* **12**, 240-246.

Whitehead, B.F., Rees, P.G., Sorensen, K., Bull, C., Fabre, J., de Leval, M.R., Elliott M.J. (1995) Results of heart-lung transplantation in children with cystic fibrosis. *Eur. J. Cardiothorac. Surg.* **9**, 1-6.

Whitehead, B.F., Stoehr, C., Wu, C.J., Patterson, G., Burchard, E.G., Theodore, J., Clayberger, C., Starnes, V.A. (1993A) Cytokine gene expression in human lung transplant recipients. *Transplantation.* **56**, 956-961.

Wickham, T.J., Mathias, P., Cheresch, D.A., Nemerow, G.R. (1993) Integrins $\alpha\beta 3$ and $\alpha\beta 5$ promote adenovirus internalization but not virus attachment. *Cell.* **73**, 309-319.

Wildevuur, C.R.H., Benfield, J.R. (1970) A review of 23 human lung transplantations by 20 surgeons. *Ann. Thorac. Surg.* **9**, 489-515.

Williams, J.F. (1970) Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. *J. Gen. Virol.* **9**, 251-255.

Wolf, F.M. (1986) Meta-analysis. Quantitative methods for research synthesis. *Sage University Paper series on Quantitative Applications in the Social Sciences.* Sage publications. Beverly Hills. 07-059.

Wolff, J.A., Lederberg, J. (1994) An early history of gene transfer and therapy. *Hum. Gene Ther.* **5**, 469-480.

Wreghitt, T.G., Hakim, M., Cory, P.R., English, T.H.A., Wallwork, J. (1986) The impact of donor transmitted CMV and *Toxoplasma gondii* disease in cardiac transplantation. *Transplant. Proc.* **18**, 1375-1376.

Wu, G.Y., Wu, C.H. (1987) Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* **262**, 4429-4432.

Wu, G.Y., Wu, C.H. (1988) Evidence for targeted gene delivery to hepG2 hepatoma cells in vitro. *Biochemistry*. **27**, 887-892.

Wulffraat, N.M., Geelen, S.P., van Dijken, P.J., de Graeff-Meeder, B., Kuis, W., Boven, K. (1995) Recovery from adenovirus pneumonia in a severe combined immunodeficiency patient treated with intravenous ribavirin. *Transplantation*. **59**, 927.

Y

Yacoub, M., Al-Kattan, K.M., Tadjkarimi, S., Eren, T., Khaghani, A. (1997) Medium term results of direct bronchial arterial revascularisation using IMA for single lung transplantation (SLT with direct revascularisation). *Eur. J. Cardio-Thorac. Surg.* **11**, 1030-1036.

Yakubov, L., Khaled, Z., Zhang, L.M., Truneh, A., Vlassov, V., Stein, C.A. (1993) Oligodeoxynucleotides interact with recombinant CD4 at multiple sites. *J. Biol. Chem.* **268**, 18818-18823.

Yakubov, L.A., Deeva, E.A., Zarytova, V.F., Ivanova, E.M., Ryte, A.S., Yurchenko, L.V., Vlassov, V.V. (1989) Mechanism of oligonucleotide uptake by cells: involvement of specific receptors. *Proc. Natl. Acad. Sci. USA*. **86**, 6454-6458.

Yamabe, K., Kamiike, W., Shimizu, S., Waguri, S., Hasegawa, J., Okuno, S., Yoshioka, Y., Sawa, Y., Uchiyama, Y., Matsuda, H. (1997) Prevention of hypoxic liver injury by *in vivo* transfection of the human bcl-2 gene. *Transplant. Proc.* **29**, 384-385

Yang, N.S., Burlholder, J., Roberts, B., Martinell, B., McCabe, D. (1990) *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. *Proc. Natl. Acad. Sci. USA*. **87**, 9568-9572.

Yang, Y., Su, Q., Wilson, J.M. (1996) Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* **70**, 7209-7212.

Yao, G.Q., Grill, S., Egan, W., Cheng, Y.C. (1993) Potent inhibition of Epstein-Barr virus by phosphorothioate oligodeoxynucleotides without sequence specification. *Antimicrob. Agents. Chemother.* **37**, 1420-1425.

Yousem, S.A., Berry, G.J., Cagle, P.T., Chamberlain, D., Husain, A.N., Hruban, R.H., Marchevsky, A., Otori, N.P., Ritter, J., Stewart, S., Tazelaar, H.D. (1996) Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: lung rejection study group. *J. Heart Lung Transplant.* **15**, 1-15.

Yousem, S.A., Burke, C.M., Billingham, M.E. (1985) Pathologic pulmonary alterations in long-term human heart-lung transplantation. *Hum. Pathol.* **16**, 911-923.

Yousem, S.A., Dauber, J., Keenan, R., Paradis, I.L., Zeevi, A., Griffith, B.P. (1991) Does histologic acute rejection in lung allografts predict the development of bronchiolitis obliterans? *Transplantation.* **52**, 306-309.

Z

Zahradnik, J.M., Spencer, M.J., Porter, D.D. (1980) Adenovirus infection in the immunocompromised patient. *Am. J. Med.* **68**, 725-732.

Zamecnik, P.C., Goodchild, J., Taguchi, Y., Sarin, P.S. (1986) Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA. *Proc. Natl. Acad. Sci. USA.* **83**, 4143-4146.