A thesis submitted to the University of London for the degree of Doctor of Philosophy

DEVELOPMENT OF HERPES VIRUS VECTORS FOR GENE DELIVERY TO DENDRITIC CELLS AND THE POTENTIAL IMMUNOTHERAPY OF CANCER

by

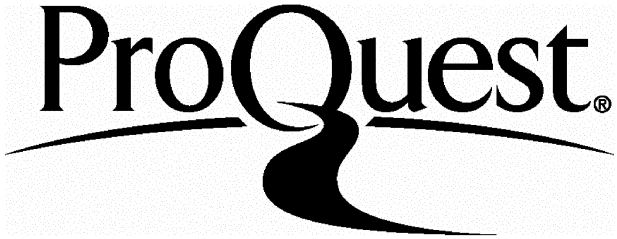
Laila Samady

May 2001

Department of Molecular Pathology and Immunology

Windeyer Institute of Medical Sciences

University College London



To my father and in memory of my beloved mother

Abstract

The greatest challenge in cancer immunotherapy is to overcome the fact that the immune system does not elicit an effective immune response to the tumor. This is partly due to the fact that tumors are deficient in presenting peptide antigens to T cells. One approach has been to use dendritic cells which are professional antigen presenting cells, to initiate an immune response. Dendritic cells may, therefore, provide the basis for generating a more effective anti-tumor response if a safe and efficient way is found to deliver genes encoding tumor antigens to them and to activate their T-cell stimulatory capacity. The development of a gene delivery system for dendritic cells is described in this thesis using herpes simplex virus (HSV-1) vectors for potential application in cancer immunotherapy.

Minimally disabled HSV-1 was found to infect dendritic cells efficiently even though dendritic cells were found not to be permissive for viral growth. This suggests that HSV-1 may naturally infect these cells However, slight viral growth was observed indicating that minimally disabled vectors may be toxic to the cells and affect their function. Thus, a panel of more disabled HSV-1 vectors were tested in dendritic cells. Two vectors were found to give optimal results in terms of balance of gene delivery and toxicity to cells. A partially disabled vector with deletions in the non-essential genes ICP34.5 and UL43, and inactivations in VP16 and vhs gave very high gene delivery (81% GFP expression at multiplicity of infection MOI=1) and was not toxic to cells. Furthermore, no viral growth was detected and only very low levels of immediate early (IE) proteins ICP0 and ICP22, but no ICP47 were expressed at MOI=1. A further disabled replication incompetent vector with deletions in essential genes ICP27 and ICP4, and deletions and/or inactivations in the non-essential genes ICP34.5, vmw65 and vhs, was also found to give high level transgene expression.

Selected HSV-1 based vectors were then used to study their effect on dendritic cell function. The inhibition of proteasomes in transduced cells showed that actual gene delivery was at higher efficiency than recorded by apparent protein expression levels. This suggested that cells infected with HSV process the antigen delivered to

them via the vector. The effect of HSV-1 was then assessed on several surface markers that are important for T-cell co-stimulation and activation. Minimally disabled HSV-1 prevents the up-regulation of these molecules in response to infection and this may be a possible route of escape of the virus from the immune system. However, a replication incompetent vector with vhs inactivated specifically up-regulated e.g. CD86 expression compared to an equivalent vector with vhs intact. This implicates the vhs protein of HSV-1 in immune escape. A replication incompetent vhs inactivated HSV-1 vector encoding hepatitis B surface antigen (HBsAg) was constructed in order to test the ability of transduced dendritic cells to specifically present a delivered antigen to T cells and thus elicit an immune response. Dendritic cells transduced with this vector stimulate a CD4+ T cell response specific to HBsAg *in vitro*. Finally a vector expressing the tumor-associated MUC-1 antigen was constructed which may be useful in the treatment of MUC-1 expressing cancers including breast and ovarian cancers.

Based on these results, replication incompetent vhs deleted HSV-1 vectors may provide an optimal means of delivering tumor antigens to dendritic cells for use in immunotherapy.

Acknowledgements

I wish to thank Dr. Robert Coffin, Professor David Latchman and Professor Benjamin Chain for all their guidance and support throughout this project. I wish also to thank Dr. Luci McCormack, Dr. Katherina Speidel and Dr. Steve Cleverley for their help and collaboration. I would like to express my appreciation to Dr. Caroline Lilley, Dr. James Palmer, Dr. Suzanne Thomas, Dr. Jill Smith, Dr. Barry Gibb and Dr. Michael Robinson for their help and contribution in constructing most of the viruses. I am also grateful to all the other members of the laboratory for their support and enthusiasm during the past three years.

Finally, I wish to thank my family for their encouragement and invaluable support.

Manuscripts in preparation

Samady, L., McCormack, L., Cleverley, S., Lilley, C., Smith, J., Latchman, D.S., Chain, B., Coffin, R.S. (2001) Deletion of the virion host shuttoff protein from herpes simplex virus (HSV) prevents HSV-mediated dendritic cell inactivation and allows the development of high efficiency vectors for dendritic cell-mediated antigen specific immune stimulation. Submitted.

Speidel, K., Pollara, G., **Samady, L.,** Ledderman, J., Katz, D.R., Coffin R., Chain, B. (2001) Function and phenotype in HSV-1 infected human peripheral blood dendritic cells. Submitted.

Abstracts

Samady, L., Cleverley, S., McCormack, L., Lilley, C., Robinson, M., Burton, S., McGrath, Y., Li, X., Latchman, D.S., Chain, B., Coffin, R.S. (2001) Herpes simplex virus vectors with the vhs protein deleted allow very high efficiency dendritic cell transduction together with DC activation: development of DC-mediated vaccines for cancer and infectious disease. Abstracts of the ASM conference on viral gene vectors.

Samady, L., Speidel, K., Chain, B., Ledderman, J., Latchman, D.S., Coffin, R.S. (2000) Development of a herpes virus vector-mediated approach to cancer immunotherapy using transduced human dendritic cells. *Molecular Therapy* May 2000 (Journal of the American Society of Gene Therapy)

Samady, L., Chain, B., Latchman, D.S., Coffin, R.S. (1999) Efficient transduction of human dendritic cells using herpes simplex virus-1 vectors: potential tools for cancer immunotherapy. *Cancer Gene Therapy* July 1999 (Journal of the International Society of Cancer Gene Therapy

Speidel., K., **Samady, L.,** Ledderman, J.A., Chain, B., Coffin, R.S., Katz, D. (1999) Development of transfected dendritic cells for immunotherapy of ovarian cancer. Abstracts of the British Cancer Research meeting.

Coffin, R.S., Lilley, C.E., Thomas, S.K., Gibb, B.J., Palmer, J.A., Robinson, M.J., Branston, R.H., Smith, J., **Samady, L.,** Chain, B., Latchman, D.S. (1998) Optimised herpes simplex virus vectors for the delivery of multiple genes to the nervous system and for immunotherapy. Abstracts of the 23rd International Herpesvirus workshop.

Abbreviations

A:	adenosine
AAV:	adenoassociated virus
Ad:	human adenovirus
APC:	antigen presenting cell
APS:	ammonium persulphate
ATP:	adenosine triphosphate
BHK:	baby hamster kidney
bis:	n,n'-methylene-bis-acrylamide
bp:	base pair
BSA:	bovine serum albumin
C:	cytosine
CAP-1:	carcinoembryonic antigen peptide
cDNA:	complementary DNA
CEA:	carcinoembryonic antigen
CD:	cluster determinant
CMC:	carboxymethylcellulose
CMV:	cytomegalovirus
CNS:	central nervous system
CPE:	cytopathic effect
cpm:	counts per minute
CTL:	cytotoxic T cell
CTP:	cytosine triphosphate
d:	deoxy
dd:	dideoxy
DC:	dendritic cell
ddH_20:	double distilled water
DMEM:	Dulbecco's modified Eagle's Medium
DMRIE:	1,2-dimyristyloxypropyl-3-dimethylhydroxy-ethylammonium bromide

DMSO:	dimethylsulphoxide
DNA:	deoxyribonucleic acid
DOTAP:	1,2-doleoyloxy-3-(trimethylammonio)propane
ds:	double stranded
E:	early (class of genes)
ECL:	enhanced chemiluminescence
EDTA:	ethylenediaminetetra-acetic acid
ER:	endoplasmic reticulum
FCS:	fetal calf serum
μg:	microgram
g:	gram
G:	guanosine
gB:	glycoprotein B (similarly for other glycoproteins)
GFP:	green fluorescent protein
GM-CSF:	granulocyte-macrophage colony-stimulating factor
β-gal:	beta-galactosidase
GTP:	guanosine triphosphate
HBsAg:	hepatitis B surface antigen
HBSS:	Hank'a balanced salt solution
HBV:	hepatitis B virus
HEPES:	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HLA:	human leukocyte antigen
HIV:	human immunodefiency virus
HMBA:	hexamethylene bisacetamide
HRP:	horseradish peroxidase
HPV:	human papilloma virus
HSV-1:	herpes simplex virus type 1
IAA:	isoamyl alcohol
ICAM:	intercellular adhesion molecule
ICP:	infected cell polypeptide
IE:	immediate early genes

IFN:	interferon
Ig:	immunoglobulin
IL:	interleukin
IR:	inverted repeat
kb:	kilobase
kDa:	kilo Dalton
µl:	nicro litre
l:	litre
L:	late (class of genes)
LAP:	latency active promoter
LAT:	latency associated transcript
LB:	Luria Bertani medium
LFA:	lymphocyte function-associated antigen
LTR:	long terminal repeat
M:	molar
mA:	milliamps
MAGE:	melanoma antigen E
Mart:	melanoma antigen recognised by T cells
mg:	milligram
MHC:	major histocompatibility complex
MIP-3α:	macrophage inflammatory protein 3 alpha
ml:	millilitre
mM:	millimolar
MMLV:	Moloney murine leukaemia virus
MMTV:	mouse mammary tumor virus
MOI:	multiplicity of infection
mRNA:	messenger RNA
MSV:	murine simian virus
MUC-1:	mucin gene
MW:	molecular weight
ng:	nanogram

nm:	nanometre
nt:	nucleotide
NGF:	nerve growth factor
NK:	natural killer cells
Oct:	octamer binding protein
OD:	optical density
ORF:	open reading frame
p:	plasmid
PAGE:	polyacrylamide gel electrophoresis
PAP:	prostate antigen peptide
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEM:	polymorphic epithelial mucin
pfu:	plaque forming unit
PMSA:	prostate-specific membrane antigen
PNS:	peripheral nervous system
RNA:	ribonucleic acid
rpm:	revolutions per minute
RSV:	rous sarcoma virus
RT:	room temperature
SDS:	sodium dodecyl sulphate
ss:	single stranded
SSC:	standard sodium citrate
T:	thymidine
TAE:	Tris-acetate EDTA buffer
TBE:	Tris-borate EDTA buffer
TCR:	T cell receptor
TEMED:	N,N,N'N'-tetramethylethylenediamine
TGF-β:	transforming growth factor-β
TH:	tyrosine hydroxylase
TIL:	tumor-infiltrating lymphocytes

TK:	thymidine kinase
TNF:	tumor necrosis factor
Tris:	thy(hydroxyl)aminomethane
TTP:	thymidine triphosphate
Tween-20	polyoxyethylene-sorbtan monolaurate
UL:	unique long (region)
US:	unique short (region)
UV:	ultraviolet
V:	volt
VV:	vaccine virus
vhs:	virion host shutoff protein
VP:	virion polypeptide
VSV-G:	vesicular stomatitis virus G protein
v/v:	volume for volume
wt:	wild-type
w/v:	weight for volume
X-Gal:	4-chloro, 5-bromo, 3-indolyl-β-galactosidase

Contents

Index of figures

Chapter IV

Chapter V

Chapter VI

Chapter VII

Index of tables

CHAPTER I

INTRODUCTION

I.1. Cancer immunotherapy

Cancer is one of the leading causes of mortality in the developed world. It remains a major problem as at least one in three people will develop cancer, one in four men and one in five women will die from it (Franks *et al.* 1997;Pisani *et al.* 1999). Thus, despite advances in surgical, radiotherapy and chemotherapy techniques, the prognosis for many cancers remains poor. This has motivated attempts for alternative approaches to cancer therapy. Gene therapy for cancer treatment is an attractive approach as the origin of cancer is also often related to oncogenic mutations. Due to the severity of the disease, most clinical trials using gene therapy (62%) are directed to the treatment of cancer using a number of different approaches (www.wiley.co.uk). Originally, gene therapy was conceived as a means of replacing defective genes with normal or 'wild-type' genes in diseases resulting from an inherited single gene disorder. Thus, efforts were made to use corrective or replacement gene therapy for cancer treatment, such as delivery of the p53 tumor suppressor gene targeted directly to tumor cells in order to restore the genetic defect (Habib *et al.* 1996). A second technique involves the use of suicide genes to stimulate prodrugs to destroy tumor cells (e.g. herpes simplex thymidine kinase gene followed by ganciclovir – (Stockhammer *et al.* 1997)). A third approach has been to use gene therapy to stimulate host immunity against tumor cells. This latter approach, which currently represents a large proportion of clinical trials in cancer gene therapy (48%), has been termed 'immunotherapy' (www.wiley.co.uk).

The immune system provides surveillance against tumor growth. However, tumors may escape immune surveillance and develop because of a variety of possible interactions or lack of interaction between tumor cells and the immune system which is outlined below. In order to potentially restore impairments between the tumor and the immune system, many cancer gene therapy trials have been directed towards immunotherapy. The objective of immunotherapy is to render the tumor more immunogenic on one hand, and on the other hand to stimulate the immune system in such a way that it efficiently recognises the tumor cells and destroys them. It is

important to understand some of the proposed mechanisms of tumor escape in order to find suitable ways for the potential treatment of cancer via immunotherapy.

It is interesting to observe that tumors may develop in an environment where the immune system is not necessarily deficient (Doherty *et al.* 1984). This suggests that the tumor cell itself alters signaling in order to evade immune surveillance. Several possibilities allowing this to occur have been proposed (Vile 1996):

- It is possible that the population of tumor cells is immunogenic but that the rate of growth of the tumor cells is higher than the rate of immune clearance. Surgical, chemotherapy and radiation therapy may contribute to tumor regression and/or clearance in these cases.
- Another possibility is that the tumor cells do not express specific non-self antigens and thus are invisible to the immune system. In this case, it would be difficult to direct immunotherapy against such tumor cells.
- A third option, is that the tumor cells express the antigen which can be a 'self' antigen that has been over-expressed or a 'non-self' antigen that has either been virally introduced or results from mutations. It is thought that in these type of cancers, where the tumor cells are antigenic but are not immunogenic, various immunotherapy approaches could potentially be used in order to restore the impairments (Rosenberg *et al.* 1993).

When tumors provide weak responses, it may be partly because tumor cells express 'self' molecules and thus the immune system has become tolerant to the cells but also because they possess the ability to down regulate the immune system by various means. These include:

- Production of immunosuppressive factors (Gilboa 1999) .
- Down-regulation of cytokines that stimulate immunity resulting in defects in T cell stimulation and cytokine production (Pardoll 1992) (e.g.

interleukin-2 (IL-2) or granulocyte- macrophage colony-stimulating factor (GM-CSF) (Dranoff *et al.* 1993)).

- Down-regulation or loss of MHC molecules affecting peptide presentation (Restifo *et al.* 1991).
- Down-regulation of co-stimulatory molecules affecting activation of adaptive immunity.

However, T cells also play a critical role and defects in the T-cell receptor (TCR) could potentially affect recognition of tumors (Greenberg *et al.*, 1981). In addition, the immune system may be affected by lack of recognition between tumor cells and B cells, natural killer cells (NK) and/or macrophages. Thus, by ingenious and complex mechanisms that have not been fully elucidated, the tumor cells find ways to escape the body's immune system. A possible scheme of tumor escape from adaptive immunity is shown in figure I.1.

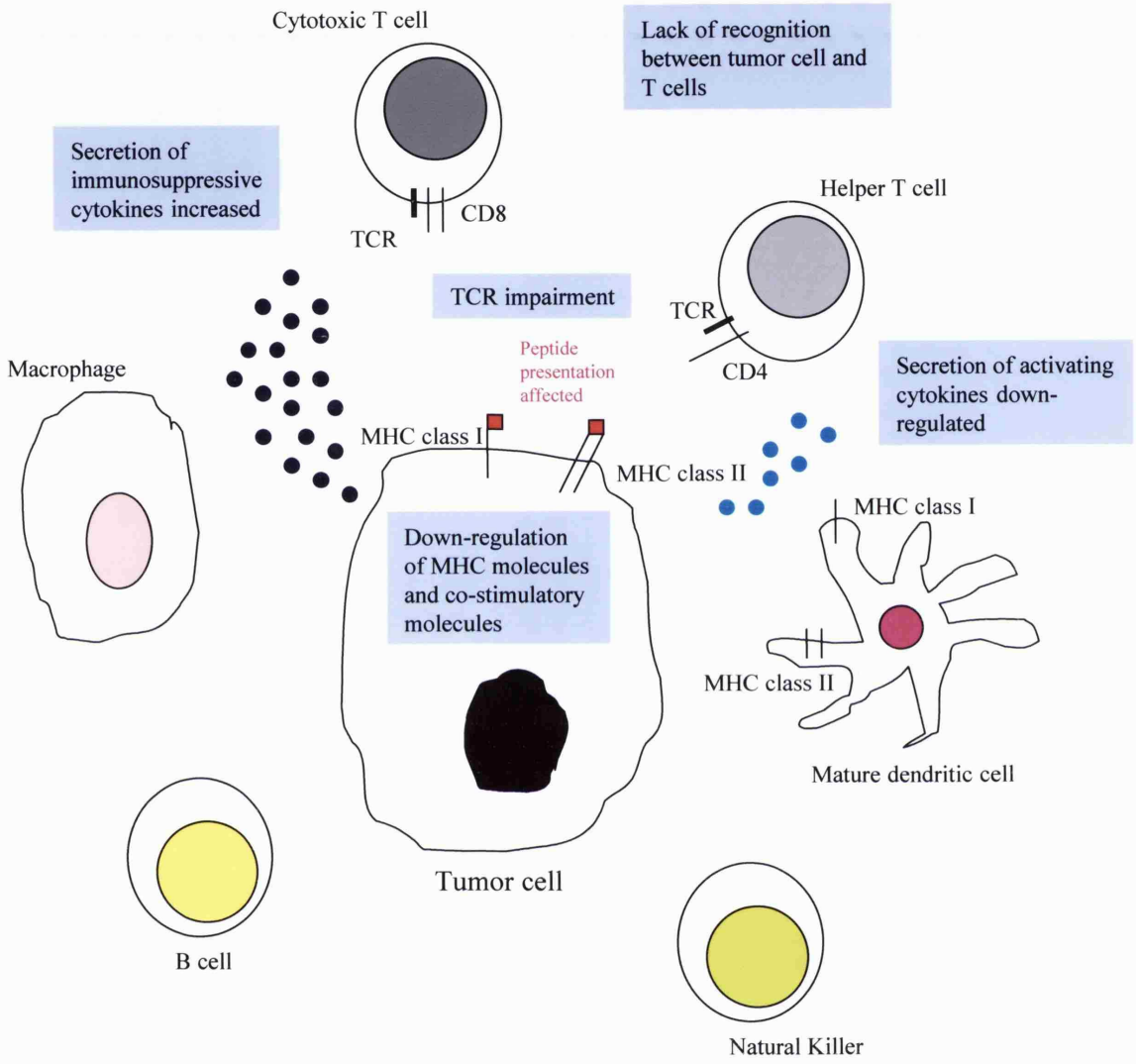


Figure I.1: A possible scheme of tumor escape from adaptive immunity.

The greatest challenge in cancer immunotherapy is to find a strategy to overcome the fact that the immune system has become tolerant to the cancer and thus does not elicit an effective immune response to it. Many strategies have been employed either separately or in combination with surgical, chemotherapy and radiation therapy. These include:

- Direct targeting of tumor cells by gene delivery
  - such that they release cytokines important for an immune response e.g. GM-CSF, IL-2 or MIP-3alpha (Dranoff *et al.* 1993), (Fushimi *et al.* 2000)
  - such that they up-regulate MHC-class I molecules important for presenting antigen to cytotoxic T cells (Tanaka *et al.*, 1988). However, transfection and expression of HLA class I genes into tumor cell lines that had lost class I expression was associated with reduced susceptibility to natural killer-mediated lysis (Maio *et al.*, 1991) indicating that the relationship between the tumor cell, MHC class I, and the immune response is more complex than might be expected
  - such that they up-regulate co-stimulatory molecules of the B7 family. These are crucial for T cell activation (Townsend and Allison 1993)
- Targeting of effector cells such as T cells and restore any impairments in the TCR for recognition of antigenic peptide (Greenberg *et al.*, 1981)
- Targeting of professional antigen presenting cells to express high levels of tumor-associated antigens recognisable by T cells in order to restore possible defects of tumor cells to present the peptide to T cells and thus stimulate the immune system (Pardoll 1993) ; (Rosenberg *et al.* 1993).

Human tumor immunotherapy has met with only limited success, to date. Among the reasons for this have been the limited availability of tumor-associated antigens and the inability to deliver such antigens in a manner that renders them

immunogenic in patients with cancer (Fong and Engleman 2000a). Recent insights in the role of dendritic cells (DC) as the pivotal antigen-presenting cells that initiate immune responses may provide the basis for more effective antitumor immune responses. The role of DC in initiating or "priming" immune responses to viral and bacterial antigens *in vivo* is well established (Hengel *et al.* 1987), (Kast *et al.* 1988), (Nonacs *et al.* 1992). By harnessing the capacity of DC to present tumor antigens to T cells, DC may serve as the centerpiece of an immunotherapeutic approach to cancer. The ability of the immune system to recognize and attack tumors has been shown previously. Both humoral and cellular effector mechanisms can contribute to tumor lysis, although it appears that the cellular response allows tumor regression in the majority of cases. CD8+ cytotoxic T lymphocytes, in particular, have been demonstrated to recognise and kill cancer cells in various tumor models (Feltkamp *et al.* 1993), (Celluzzi *et al.* 1996), (Boon *et al.* 1994). The ability of DC to prime T cells capable of recognising and killing tumor cells has also been demonstrated in various animal models (Zitvogel *et al.* 1996), (Paglia *et al.* 1996), (Flamand *et al.* 1994), (Mayordomo *et al.* 1995a). Furthermore, DC-based immunisation can generate an immunologic memory with protection against subsequent tumor challenges (Hart 1997).

Human clinical trials using DC loaded with tumor antigens are underway at several institutions (Fong and Engleman 2000a). In the first reported DC trial, the effect of autologous DC pulsed *in vitro* with tumor-specific antigen was investigated in patients with malignant B cell lymphoma for whom conventional chemotherapy had failed. The neoplastic cells for these patients express surface immunoglobulin receptors, and because B cell lymphomas are monoclonal, all the cells of a given tumor express identical surface immunoglobulin. To prepare idiotype proteins for this clinical study, patients underwent tumor biopsies and the immunoglobulin produced by each tumor was purified from hybridoma supernatants (Maloney *et al.* 1985), (Carroll *et al.* 1986). Peripheral blood leukocyte preparations containing the DC precursors were obtained from patients by leukapheresis, and monocyte-depleted mononuclear cells were incubated for 36 hours in the presence of either the idiotype protein or a control protein, keyhole limpet hemocyanin (KLH). This incubation

technique not only enabled the DC precursors to take up and process the exogenous proteins, but also induced their maturation into potent antigen presenting cells. After incubation, the idiotype and KLH-pulsed DC were separated from contaminating lymphocytes on the basis of their differences in buoyant density. The DC were then extensively washed to temove free protein, resuspended in sterile saline, and administered to the patients by intravenous infusion. This procedure was repeated three times at monthly intervals with a booster immunization given four to six months later. Two weeks after each DC infusion, the patients received subcutaneous injections of idiotype, which in the absence of DC or a chemical adjuvant would not be expected to induce an immune response (Kwak *et al.* 1992). Through the trial the patients were followed for the development of an immune response to the idiotype and their tumor burden was monitored. All of these treated patients tolerated their infusions and the majority developed T-cell mediated anti-idiotype and anti-KLH proliferative responses that were not observed prior to the treatment initiation.

A phase I DC trial in prostate cancer has also been reported (Tjoa *et al.* 1997a). DC were derived from peripheral blood-derived monocytes in cultured in GM-CSF and IL-4. Subsets of the 51 patients were treated with unloaded DC or DC pulsed with HLA-restricted peptides derived from prostate-specific membrane antigen (PSMA). Seven patients were scored as partial responders and follow-up studies have been taken place. Two vaccine trials for melanoma have also been reported using DC pulsed with a panel of melanoma-derived peptides. One study has reported a complete response in one of six patients (Lotze *et al.* 1997). DC vaccination has been shown to induce tumor-specific immune responses and also tumor regression in clinical trials for malignant melanoma (Nestle *et al.* 1998a), (Thurner *et al.* 1999). A recent study has shown for the first time that DC vaccination with HLA-restricted MAGE-3 peptide is a novel therapeutic approach for patients with gastrointestinal carcinoma (Sadanaga *et al.* 2001). No toxicity was found in any patients and the immune response for MAGE-3 peptide and tumor regression was observed in some patients who had advanced metastatic gastrointestinal carcinoma. The results of another study have shown the efficient use of DC-based vaccination in a phase I clinical trial of glioma patients with fusions of DC and glioma cells

(Kikuchi *et al.* 2001). Clinical results showed that there were no serious adverse effects and two partial responses.

DC-vaccination leads to tumor regression in selected advanced cancer patients but current clinical trials are still in phase I, with many differences in study design, execution, and monitoring (Nestle *et al.* 2001). There is still much debate about DC preparation, antigens, loading, dosing, injection sites and dose scheduling as well as the monitoring of immune and clinical responses. Our goal has been to develop a gene delivery system to target DC for the immunotherapy of cancer. Ongoing pre-clinical and clinical studies conducted in many centres and laboratories worldwide have shown the crucial role of DC as key effectors of the immune system which may be effectively exploited for the immunotherapy of cancer (see table I.1).

Malignancy	Antigen (Ag)	Ag structure	Institution	Investigator
Breast, colorectal, pancreas, lung, melanoma	Tumor derived	Autologous tumor cell lysate	University of Michigan	Mule et al, 1998
Breast, colorectal, lung	Mutant ras derived	Ras peptides	Vanderbilt	Carbone et al[1]
Breast, colorectal, lung	Mutant p53	P53 DNA	Vanderbilt	Carbone et al[1]
Breast, colorectal	CEA	Peptide CAP-1	Stanford	Weber et al[1]
Lung, colorectal	CEA	Peptide CAP-1	Duke	Fong, Engleman et al[1]
Lung	CEA	Peptide CAP-1	Duke	Lyerly, Gilboa et al[1]
Lung	CEA	CEA RNA	Duke	Lyerly, Gilboa et al[1]
Melanoma	Gp100, Mart-1, tyrosinase	Peptides	University of Zurich	Nestle et al, 1998
Melanoma	Gp100, Mart-1, tyrosinase	Peptides	University of Pittsburgh	Lotze et al, 1997
Melanoma	Gp100, Mart-1, tyrosinase	Peptides	NCI	Marincola et al[1]
Melanoma	Gp100, Mart-1, Mage-3, tyrosinase	Peptides	Baylor	Banchereau et al[1]
Melanoma	Gp100, tyrosinase	Peptides	USC	Weber et al[1]
Multiple myeloma	Ig idiotype	Purified protein	Stanford	Levy, Engleman et al[1]
Multiple myeloma	Ig idiotype	Purified protein	Dendreon	Valone et al[1]
Non Hoddkin's lymphoma	Ig idiotype	Recombinant protein	Stanford	Levy, Engleman et al, 1996
Prostate	Xenogeneic PAP	Recombinant protein	Stanford	Fong, Engleman et al[1]
Prostate	PSMA	Peptides	Northwest Biotherapeutics	Murphy et al, 1998
Prostate	PAP	Recombinant fusion protein	Dendreon	Valone et al, 1998
Renal	Tumor derived	Autologous tumor cell lysate	UCLA	Figlin, Belldegrun et al[1]

Table I.1: Current dendritic cell clinical trials for the immunotherapy of cancer. Source: (Fong and Engleman 2000b) 1. Personal communication to Fong & Engleman.

I.2. Why do dendritic cells (DC) offer a potential tool for the immunotherapy of cancer?

Dendritic cells reside in pluristratified epithelia of the oral mucosa, vagina, cervix and the rectum and are refered to as immature DC (de Fraissinette *et al.* 1989). They correspond to epidermal Langerhans cells, characterised by the presence of Birbeck granules and potent endocytic properties (Kaiserlian and Dubois 2001). DC are also present in the pseudostratified epithelium of the respiratory tract (Holt *et al.* 1994) and in the intestine, where they reside beneath the dome epithelium of Peyer's patches as well as in the lamina propria of intestinal villi, in contact with T cells (Kelsall and Strober 1996). Epithelial DC constantly control their environment for antigens by phagocytosis, macropinocytosis and efficient receptor-mediated pinocytosis. Appropriate inflammatory signals provided by infectious agents can trigger the release of cytokines and chemokines. The regulated expression of chemokine receptors on the surface of DC allows them to migrate to lymph nodes while maturing. DC maturation involves the down-regulation of antigen processing properties and the up-regulation of MHC and co-stimulatory molecules to activate antigen-specific T cells (Banchereau and Steinman 1998a).

As DC present antigen to lymphocytes, they are in a central position for the control of immunity (Watts 1997). The role of DC as the pivotal antigen presenting cells that initiate immune responses may provide the basis for generating more effective antitumor immune responses. The use of DC for immunotherapy has motivated interest in better understanding their immunobiology and their interactions with T cells. DC in tissues capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs to interact with T cells and secrete cytokines in order to initiate immune responses (Finkelman 1995). Thus, DC distribution throughout the body allows efficient antigen capture and migration to lymphoid organs for stimulation of helper T cells and cytotoxic T cells. DC are highly potent at stimulating T cells as small numbers of cells and low levels of antigen can induce strong T cell responses. The complexity of the DC population and the lack of a

means to isolate them from the body have long hindered attempts to use DC for immunotherapy. However, as human DC can now be generated in large quantities *in vitro* (e.g. from peripheral blood mononuclear cells), a powerful tool to manipulate the immune system in such a way that it recognises pathogens (i.e. viruses) or diseases (i.e. cancer) is now potentially available (Banchereau and Steinman 1998b).

I.2.1. Developmental biology and lifecycle

It is thought that human DC derive either from a myeloid (blood) or lymphoid (bone marrow) origin and are composed of many different subsets of cells that are mobile and scattered throughout the body. Although phenotypic differences have been identified between these various subsets, their lineage origins, maturation stages and functional differences have not been fully elucidated (Banchereau *et al.* 2000a).

Lymphoid and myeloid DC have been characterised, although the existence of human lymphoid DC is controversial. DC derived from myeloid CD34+ progenitors in the bone marrow can differentiate down one of two precursor pathways. CD34+ progenitors may develop into CD14+ CD11c+ CD1- monocytes, from which immature DC (interstitial DC) can be produced in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, whereas exposure to macrophage colony-stimulating factor (M-CSF) leads to macrophage differentiation. The CD34+ myeloid progenitors can also differentiate into CD14- CD11c+ precursors that yield Langerhans dendritic cells in response to GM-CSF, IL-4, and transforming growth factor-β (TGF–β), or "Langerhans" macrophages in response to M-CSF (Peebles, Jr. and Graham 2001a). The CD34+ lymphoid progenitors can differentiate into CD14- CD11c- IL-3Rα+ precursors that give rise to "lymphoid" DC in response to IL-3 (Banchereau *et al.* 2000b), (Cella *et al.* 1999a). These cells die rapidly after isolation and are dependent on IL-3 for survival and CD40-ligand (CD40L) for maturation. Monocytes and CD11c- IL-3Rα+ DC precursors display different phenotypic differences: monocytes, but not CD11c- IL3Rα+ DC, express significant levels of CD11b, CD13, CD14, CD33 and CD45RO. Monocytes also

express high GM-CSFRα and low IL-3Rα, whereas CD11c- DC precursors display reciprocal patterns of cytokine receptor expression, low GM-CSFRα and high IL-3Rα (Banchereau *et al.* 2000b). Immature monocyte-derived DC display high endocytic/phagocytic capacity, contrary to immature CD11c- DC.

Immunophenotypic and functional analyses have defined the different DC populations. The interrelationship of non-lymphoid and lymphoid tissue DC has been inferred from the behavior of murine Langerhans cells both *in vitro* and *in vivo*, the homing of DC injected into recipients and antigen tracking studies. The ability of DC to migrate into tissues and from there to the lymph nodes is critical to their overall function as antigen presenting cells (Hart 1997). Lymphoid DC have different properties from the myeloid DC, which is immunostimulatory in most circumstances. The surveillance tissue-based DC:Langerhans cells in the skin, DC in the respiratory tract, gut or other nonlymphoid tissues, migrate after exposure to infection, tissue damage/inflammation, or antigen (ie. danger signals) via the afferent lymphatics to the T-lymphocyte dependent areas of the draining lymph nodes. It is possible that epithelial based CD1+ DC have an independent derivation from the stem cell. The ability of monocytes and macrophages to convert to DC *in vivo* has yet to be established (Hart 1997).

DC heterogeneity in humans is thus reflected at four levels (Banchereau *et al.* 2000b):

- Precursor populations:
  In humans, at least two subsets of DC precursors circulate in the blood: CD14+ CD11c+ monocytes and lineage-negative (LINneg) CD11c- IL-3Rα+ precursors DC (Grouard *et al.* 1997), (Olweus *et al.* 1997). The LINneg CD11c+ cells may represent a third precursor, although these cells are more committed because they can spontaneously differentiate into DC when put into culture.

- Anatomical localisation:

  The level of heterogeneity reflected by anatomical localisation includes skin epidermal langerhans cells, dermal (interstitial) DC, splenic marginal DC, T-zone interdigitating cells, germinal-center DC, thymic DC, liver DC and blood DC (Olweus *et al.* 1997).
- Function:

  Both murine and human DC subsets exert different functions, markedly in the regulation of B cell proliferation and differentiation of T cells into type 1 or type 2.
- The final outcome of immune response:

  The final outcome of immune response refers to the induction of tolerance or immunity

The generation of interstitial DC and Langerhans DC from CD34+ hematopoietic progenitors is regulated by the same cytokines that drive differentiation of blood precursors (Caux *et al.* 1997). Although the corresponding mature DC progeny are equally potent in stimulating the proliferation of naive T cells, only interstitial DC induce differentiation of naive B cells *in vitro* following in response to IL-2 (Caux *et al.* 1997). Both subsets express IL-12 after CD40 ligation, but only interstitial DC express IL-10. Interstitial DC show a 10 fold increase in antigen capture compared to Langerhans DC. Currently, no biological function specific to Langerhans DC has been identified. Studies have shown that CD34+ derived DC, composed of interstitial DC and Langerhans cells, are highly potent in the priming of Melan-A/MART-1-specific cytotoxic T lymphocytes, more than DC generated from monocytes (Mortarini *et al.* 1997). Thus, a hypothesis could be that the primary function of Langerhans cells may be the priming of CD8+ T cells (Banchereau *et al.* 2000b). Circulating DC precursors play a critical role in the immediate reaction to pathogens and in the shaping of the immune system. DC precursors can respond to different pathogens, virus, or bacteria. These cells have the dual function at two distinct stages of differentiation of:

- secreting large amounts of pro-inflammatory and anti-viral cytokines (such as CD11c- IL3Rα+ blood DC precursors which produce interferon alpha IFNα in response to virus (Palucka and Banchereau 1999)
- activating and modulating T cell responses when mature cells.

Immature DC are particularly adept in their ability to capture antigen, either by macropinocytosis, by endocytosis through C-type lectin receptors, FcγRI or FcγRII, or by phagocytosis. The process of antigen capture changes the immature DC both in phenotype and function, transforming the DC into an antigen-presenting cell. The maturation of the DC leads to migration of the cell from the peripheral tissues into the draining lymphoid organs. CD40 ligand, and tumor necrosis factor (TNF)-α and IL-1β, activate DC, and are important in this process (Peebles, Jr. and Graham 2001b).

An important attribute of DC at various stages in their lifecycle is their mobility (Austyn *et al.* 1988). When in contact with an antigen, DC precursors in tissues present the antigen to immature DC which then migrate towards lymph nodes in areas rich in T cells for antigen presentation. Once DC were identified and purified from contaminating lymphocytes and macrophages, their distinct function as antigen presenting cells became apparent(Banchereau and Steinman 1998b). This trafficking to tissues is directed by the expression of chemokine receptors as well as adhesion molecules (Sozzani *et al.* 1988). Mature DC have a distinct morphology defined by the presence of numerous membrane processes that can extend to hundreds of micrometers and allow increased surface contact with T cells for antigen presentation. Although DC phenotype varies with different stages of maturation and activation, mature DC show a distinct phenotype:

- the absence of lineage markers (e.g. CD14 present on monocytes, CD3 on T cells, CD19, CD20, CD24 on B cells, CD56 on NK cells, CD66b on granulocytes) (Fearnley *et al.* 1997)

- the expression of molecules involved in antigen presentation to T cells and maturation of DC:

  - 'self' molecules that bind to peptide antigens to allow antigen presentation (e.g. MHC class I, HLA-DR/MHC class II, HLA-DQ/MHC class II) (Fearnley *et al.* 1997)
  - co-stimulatory molecules (e.g. CD80/B7.1, CD86/B7.2, CD40 for regulation of T cell stimulation) (Banchereau *et al.* 1994), (Fagnoni *et al.* 1995)
  - adhesion molecules - although these can also be found on monocytes and macrophages (e.g. CD11a/LFA-1, CD11c – also thought to be DC specific, CD50/ICAM-2, CD54/ICAM-1, CD58/LFA-3, CD102/ICAM-3) (Hart and Prickett 1993)

In addition, immature DC are also characterised by high levels of intracellular structures related to antigen processing (e.g. endosomes, lysosomes and proteasomes). Taken together, the presence of such key molecules provide the basis for the functioning of DC as professional antigen presenting cells and are crucial elements that favor the use of DC for immunotherapy.

I.2.2. Processing and presentation of antigens to T cells

Immune responses are restricted. That is, a T lymphocyte can specifically recognise a target cell only if it bears not only the antigen but also the appropriate MHC molecule (Zinkernagel and Doherty 1974). Thus, for a foreign protein antigen to be recognised by T cells, it must be degraded into small antigenic peptides that form complexes with MHC class I or MHC class II molecules as these molecules are receptors for the peptide antigen (Rammensee *et al.* 1993). This conversion of proteins into MHC-associated peptide fragments is termed 'antigen processing and presentation'. DC are very well equipped to capture, process and present antigens to T cells in this manner. A number of molecules involved in these processes have been

identified. Evaluation of 3000 sequences cloned out of a library constructed from CD34-derived DC showed that 20% of the sequences were related to antigen processing and presentation including 7% for major histocompatibility complex (MHC) and another 6% of sequences constituted enzymes from the serine and metalloprotease families (Banchereau *et al.* 2000a).

After being in contact with antigens from viruses, bacteria or apoptotic bodies, immature DC capture the antigen and process it for presentation to T cells (Hengel *et al.* 1987), (Albert *et al.* 1998a), (Svensson *et al.* 1997). It is thought that a particular antigen will be processed, bound to MHC class I or MHC class II molecules and presented to T cells depending on the antigen itself and the route by which it entered the cell. Antigenic peptides are bound to MHC class I or class II molecules for presentation respectively to cytotoxic or helper T cells, and this loading onto MHC molecules can occur via either the so-called endogenous or the exogenous pathways (Banchereau *et al.* 2000a). In the endogenous pathway, peptides from 'self' and intracellular pathogens (e.g. introduced with a virus into DC) are presented to T cells bound to MHC class I molecules. DC can also load peptides on MHC class I molecules via the exogenous pathway with peptides originating from phagocytosed antigens or immune complexes. Peptides are generated in the proteasome, transferred into the endoplasmic reticulum, and loaded onto the nascent MHC class I molecules (Pamer and Cresswell 1998). For loading to MHC class II molecules, soluble and particulate antigens are captured by DC through endocytosis by the exogenous pathway, degraded in endosomes and loaded on the nascent MHC class II molecules while the DC mature (Sallusto and Lanzavecchia 1994).

In addition to professionally presenting antigen to T cells via MHC molecules, DC further activate T cells by release of soluble mediators. Thus, for optimal effects on T cells, DC employ events mediated by two mechanisms:

- Direct membrane-membrane contact involving binding between cell surface proteins present on DC and T cells

- Secretion of soluble mediators by DC (e.g. cytokines) which bind to specific cell surface receptors on T cells

Mature DC express higher levels of CD86 than other antigen presenting cells, a molecule that is highly important for T-cell activation (Caux *et al.* 1994). CD86 expression is further up-regulated on DC by the cross-linking of CD40 by CD40 ligand found on T cells (Cella *et al.* 1996). Blocking CD86 with antibodies leads to a dramatic inhibition of T-cell proliferation (Caux *et al.* 1994). DC also produce cytokines such as interleukin-12 (IL-12) which is important in generating a cell-mediated immune response (Reis *et al.* 1997). Therefore, DC possess the critical tools that allow efficient antigen presentation to T cells and consequently generation of a vigorous immune response. Thus, the properties of DC make these cells very attractive for use in immunotherapy. This indicates that DC might potentially be used to stimulate immunity against tumor cells.

I.3. Gene delivery methods to DC

The development of effective gene delivery systems to target specific cells has proven to be a major problem for the advancement of gene therapies. While highly promising in immunotherapy, DC are generally hard to load with antigens. Various attempts have been made or are in progress to load DC or their progenitors for use in cancer immunotherapy in order to introduce tumor-associated antigens or molecules to render them more immunogenic for priming of T-cells. Various approaches used to make and utilise genetically engineered DC-based cancer vaccines are presented in this section (Bubenik 2001). Delivery methods include:

- non-viral gene delivery methods (e.g. direct peptide or protein loading, nucleic acid gene transfer, the use of bacteria)
- viral gene delivery methods (e.g. retroviral vectors, lentiviruses, adenoviral vectors, adeno-associated viral vectors, vaccinia virus-based vectors, influenza virus, herpes simplex virus-based vectors).

I.3.1. Non-viral gene delivery methods

The efficiency of non-viral gene delivery systems is currently low compared to viral systems. However, the success of some non-viral delivery methods has shown that with further development these could be used for gene therapy in the future. Several methods used for gene delivery to DC are described below.

I.3.1.1. Direct protein or peptide loading

In 1990, it was shown that isolated murine DC could be incubated *in vitro* with tumor-specific peptides following which they retained the ability to present the antigen to T cells *in situ* for a period of several days (Inaba *et al.* 1990). In 1995, it was shown that murine DC pulsed with synthetic tumor peptides could elicit protective and therapeutic antitumor immunity *in vivo* (Mayordomo *et al.* 1995b).

Indeed, in sarcoma and lung carcinoma murine models, treatment of animals bearing established tumors with tumor peptide-pulsed DC resulted in sustained tumor regression. These encouraging results showed that murine DC could be pulsed *in vitro* with specific peptide antigens and thus be used for adoptive immunotherapy.

The use of antigen-pulsed DC was demonstrated in humans by the vaccination of patients with B-cell lymphoma (Hsu *et al.* 1996). In all four patients, humoral and cellular responses developed as early as two weeks after a single infusion of DC. These responses were stronger after repeated infusions of pulsed-DC due to the weak immunogenicity of the chosen protein and were induced with only two or three million DC, showing the potency of this form of treatment. These results indicated that antigen-pulsed DC represent a powerful new vaccine type capable of inducing cellular immune responses against weak antigens such as tumor proteins. However, the inconvenience of this particular approach for B-cell lymphoma is its limitation to each patient for which an idiotype protein characteristic of each patient's tumor had to be made.

A phase I clinical trial was conducted on advanced prostate cancer patients using DC pulsed with prostate-specific membrane antigen (PMSA) peptides (Tjoa *et al.* 1997b). The responses observed were significant in terms of hematological studies and showed that an effective immunotherapy could be achieved when patients were repeatedly infused with DC pulsed with two types of peptides (PMSA-P1 and PMSA-P2). In another study, DC isolated from patients with advanced malignancies were loaded with carcinoembryonic antigen (CEA) peptide (CAP-1) *in vitro* in order to test for their ability to lyse CEA-expressing malignant cells (Nair *et al.* 1999). The results showed that the majority of patients possessed functional DC and that it was possible to stimulate CEA-specific cytotoxic T-lymphocyte (CTL) activity.

The generation of tumor specific CTL was also shown *in vitro* by incubation and weekly re-stimulation of DC from healthy donors with human papilloma virus (HPV-16) E7 peptide (Schoell *et al.* 1999). After 6 weeks in culture, peptide specific CTL

lines were established, implying that DC pulsed several times with cervical cancer tumor-associated antigens were potent antigen presenting cells and might be used for adoptive immunotherapy of cervical cancer.

Thus the use of peptides or purified proteins to pulse DC *in vitro* was shown to be safe for clinical applications but was limited by:

- The identification of specific peptides from tumor-antigens or tumor-associated antigens that could generate a strong T cell response
- The sustained ability of DC to process and present the pulsed peptide or protein to T cells
- The ability of DC loaded with exogenous antigen to enter the MHC class I pathway for presentation to CTL
- The prior knowledge of the patient's MHC haplotype as only some peptides will comprise a CTL epitope
- The uncertainty regarding the longevity of antigen presentation
- The need for long exposure of DC to the peptide or protein in order to prime T cells efficiently

In recent trials, DC were loaded with MHC class I-specific synthetic peptides or with whole tumor cell preparations (Thurner *et al.* 1999), (Kugler *et al.* 2000). The disadvantages of using DC pulsed with synthetic peptides from tumor-associated antigens include the uncertainty regarding the longevity of antigen presentation, the restriction by the patient's haplotype and the relatively low number of known MHC-class I and MHC-class II helper cell-related epitopes. Whole tumor cell preparations such as tumor lysates (Nestle *et al.* 1998a), apoptotic tumor cells (Jenne *et al.* 2000a) or DC-tumor cell fusions (Kugler *et al.* 2000) depend on the availability of tumor cells.

In order to overcome some of these disadvantages, several approaches have been employed. In a clinical study, DC generated *in vitro* from melanoma patients

blood were pulsed with tumor lysate or a cocktail of peptides known to be recognised by CTL (Nestle *et al.* 1998b). This method gave responses in 5 out of 16 evaluated patients with regression of metastases indicating a safe and promising approach in the treatment of melanoma. However, no information was given on patient survival. In order to further optimise peptide loading, DC were matured with CD40-ligand (CD40L), which is usually present on activated T cells, before exposure to CEA peptide in order to increase their ability to process and present the antigen to T cells(Morse *et al.* 1998). The binding of CD40L to CD40 on DC induces the surface expression of B7.1 (CD80) and B7.2 (CD86), further driving the T-cell response. Thus here, DC isolated from cancer patients were artificially matured (with CD40L) prior to peptide loading, and this allowed a specific CTL response.

As protein-based vaccines that induce class I-restricted CTL responses have proved difficult to develop, a further strategy tested was to conjugate the target protein with a peptide derived from HIV tat protein and mix with DC in order that the cells process and present the peptides in association with MHC class I molecules and stimulate a CTL response(Kim *et al.* 1997). The HIV tat protein facilitates entry of peptides in these circumstances into cells.

Although considerable efforts have been made to improve the efficacy of loading of synthetic peptide from tumor-associated antigens into DC, disadvantages remain such as the short duration of antigen presentation, the need to determine the patient's haplotype as well as the profile of the individual tumor antigen, and the lack of CD4+ T helper cell-related epitopes.

I.3.1.2. Direct nucleic acid transfer

An alternative approach would be the genetic *in vitro* manipulation of DC with DNA/RNA to express full length tumor-associated antigens. Potential advantages of this approach include the presentation of MHC class I and II epitopes,the inclusion of previously unknown epitopes of a given tumor-associated

antigen in any human MHC context and the ability to include sequences of immunomodulators or costimulatory molecules.

In 1997, naked DNA encoding two proteins of herpes simplex virus (HSV) was used to transfect murine DC both following intramuscular injection and *in vitro* in order to enhance an immune response to HSV (Manickan *et al.* 1997). Although encouraging results were obtained, relatively low efficiency of transfection was observed implying that more work was required. In order to improve the delivery of DNA into DC, Tuting and colleagues evaluated the feasibility of DNA vaccination for the induction of CTL reactivity to five different melanoma antigens *in vitro* after transient transfection of plasmid DNA by particle bombardment (Alters *et al.* 1998). The insertion of genes encoding the cytokines IL-12 and IFN-alpha enhanced the specific CTL response to melanoma antigens. Thus, this method allowed an alternative to peptide-based approaches for antigen delivery to DC.

Other strategies have involved the use of liposomes for gene delivery as combining lipid complexes with plasmid DNA or RNA vectors generates a product that can interact directly with the membranes of target cells. DC have successfully been transfected with RNA using this method. In 1996, Boczkowski and colleagues used the cationic lipid DOTAP to deliver tumor-derived RNA into DC *in vitro* in order to induce a CTL response to the tumor (Boczkowski *et al.* 1996). The treatment of mice bearing pre-existing lung metastases with DC pulsed with tumor-derived RNA led to a significant reduction in the extent of these metastases. This was also shown in humans as DC generated from a patient with metastatic cancer transfected with CEA RNA and the lipid DMRIE stimulated an *in vitro* CEA-specific CTL in 5 out of 8 advanced cancer patients (Nair *et al.* 1998). One advantage of this type of vaccination with RNA-transfected DC over vaccination with peptide-pulsed DC, is that it is not limited to the prior knowledge of the patient's haplotype. Also, these results indicated that vaccination with total tumor RNA transfected DC could provide a strategy to induce tumor immunity in patients against a variety of tumor antigens. However, the major inconvenience of this method using unfractionated tumor-derived

RNA, is the risk of generating an autoimmune response to the potent vaccine developed.

With respect to this approach, physical transfection methods including liposome complexes or electroporation have a relatively low efficiency in monocyte-derived DC (Zhong *et al.* 1999), (Arthur *et al.* 1997). So far, the best transfection efficiency was reported using a combination of *in vitro*-transcribed RNA and liposomes, where up to 20% of DC were transduced (Strobel *et al.* 2000a). Recently, a gene gun approach was used to transfect human skin organ cultures with a particular goal of expressing transgenic antigens in resident cutaneous DC (Larregina *et al.* 2001). Gold particles, when delivered to the skin, are primarily observed in the epidermis, even when high helium delivery pressures are used. Langerhans cells resident in the basal epidermis were transfected and this was sufficient to stimulate the activation and migration of skin DC. RT-PCR analysis of DC, which have migrated from transfected skin, demonstrates the presence of transgenic mRNA, indicating direct transfection of cutaneous DC. Furthermore, it was shown that Langerhans cells can efficiently present a tumor-associeted antigen derived from MART-1 to a MART-1-specific CTL. These results demonstrate direct transfection using a gene gun, activation and antigen-specific stimulatory function of transduced human Langerhans cells in situ (Larregina *et al.* 2001).

I.3.1.3. Bacteria

In 1997, the use of bacterial toxins was proposed as an alternative approach for the delivery of antigens to DC via the MHC class I pathway (Goletz *et al.* 1997). Of the toxins studied, *diphteria*, *pertussis*, *pseudomonas* and *anthrax* appeared to be efficient for delivering large proteins to DC *in vitro*. Other studies have shown the ability of murine bone-marrow-derived DC to process attenuated bacteria (e.g. *Salmonella typhimurium* and *Escherishia coli*) containing a model antigen for peptide presentation and priming of both CD4+ and CD8+ T cells to bacterial antigens and to the specific antigen used (Svensson *et al.* 1997), (Svensson and Wick 1999). Thus,

this approach for gene transfer appears to be particularly suited for the delivery of antigens presented by MHC class I and class II molecules.

I.3.2. Viral gene delivery methods

Viruses are small infective agents that require living cells for multiplication. Many viruses are known to be responsible for diseases but others are benign intracellular parasites. The elucidation of viral life cycles shows their potential for use as vectors for gene delivery. Viruses have developed ingenious ways to:

- enter the cells
- evade host immunity
- deliver their viral genome to the nucleus

Thus, considerable efforts have been made to use attenuated viruses in order to safely deliver foreign genes to target cells. A number of these viruses and the strategies used to genetically modify them in order to obtain safe but efficient gene delivery to DC are described in this section (see table I.2 for the comparative properties of some of these viruses). Recently, several viral vectors have been introduced successfully by the *in vitro* transduction of DC. Because antigen concentration seems to be a crucial parameter for the induction of potent immune responses, the high expression levels found *in vitro* transduced DC might offer a clear advantage over *in vitro* DNA-transfected DC. Furthermore, it has been reported that several of these viruses enhance costimulatory molecule expression, antigen presentation and the T-cell stimulatory capacity of transduced DC (Jenne *et al.* 2001).

Two types of viral vectors can be produced: recombinant viral vectors and defective vectors. Recombinant vectors are produced by homologous recombination of the transgene into the viral genome usually after removal of viral genes essential for replication. The viruses are then grown on complementing cell lines that produce

the deleted viral gene in trans. Examples of recombinant vectors include, adenoviral vectors and some herpes viral vectors (Hermens and Verhaagen 1998). Defective viral vectors are produced by inserting the transgene into a plasmid that contains cis-acting sequences that allow replication and packaging of the plasmid into a viral protein coat in the presence of a helper virus or packaging cell line. This type of vector includes retrovirus, adeno-associated viral vectors and amplicon HSV-based vectors.

Virus	Retrovirus	Adenovirus	Adeno-associated virus (AAV)	Herpes simplex virus (HSV)
Structure	Enveloped	Non-enveloped	Non-enveloped	Enveloped
Maximum insert capacity (kb)	9-10	7-8 helper independent 37 helper dependent	4.5	Large capacity Up to 150: gutless-helper-dependent
Infects quiescent cells	No (Lentiviruses: Yes)	Yes	Yes	Yes
Integrating	Yes	No	Yes	No
Theoretical titers	10^8-10^9	10^{11}-10^{12}	10^9-10^{10}	10^8-10^9
Genome	SsRNA	DsDNA linear	SsDNA linear	DsDNA linear
Genome size (kb)	10	36	4.7	152
Particle size (nm)	Pleomorphic	60-90	18-26	110

Table I.2: The major properties of the four most reported gene therapy viral vectors. Source: Viral gene delivery systems for gene therapy (Murphy 1999).

I.3.2.1. Retroviral vectors

Retroviruses (RV) are small enveloped RNA viruses that replicate through DNA intermediates. Following attachment of a viral protein envelope to the cell surface receptor, the virus is internalised. The reverse transcribed dsDNA is subsequently integrated into the host chromosomes as a provirus, directed by the virus-encoded integrase protein, and this DNA is transcribed using the host machinery (Boris-Lawrie and Temin 1994). Retroviral genomes contain three core genes termed *gag, pol* and *env*, which are flanked by long terminal repeat (LTR) sequences and a packaging signal which directs the assembly of the genome into the viral particle. The virus is then released from the cell by budding through the env-coated cell membrane.

The viral RNA backbone of these agents has been engineered in such a way that essential structural genes coding for capsid proteins (*gag*), reverse transcriptase (*pol*) and envelope glycoproteins (*env*) have been removed. The essential components to the virus life cycle are provided in trans by packaging cell lines into which these viral genes have been introduced (McLachlin *et al.* 1990). Packaging cell lines, transfected with recombinant retroviral backbone DNA, will produce infectious but replication incompetent RNA retroviral vectors. These vectors provide for stable integration of proviral sequences into primary and established cells, provided the target cells are undergoing replication (Temin 1989). The introduction of safety features into both the viral and packaging systems has increased the safety of these agents. However, recombination events and generation of wild-type virus is still possible (Donahue *et al.* 1992).

Most current retroviral vectors for gene therapy have been based on the retroviral oncogenic subgroup Moloney murine leukemia virus (MoMuLV) but multiply attenuated lentiviral vectors have also been used (Vile *et al.* 1991), (Zufferey *et al.* 1997). However, for gene transfer to DC, which are non-dividing cells, MoMuLV-based retroviral vectors are poor candidates as these require dividing cells

to replicate. Thus, one approach has been to use such vectors to transduce human CD34+ haematopoetic cells which are DC progenitors. Indeed, after retroviral transduction with a melanoma tumor associated antigen gene (MART-1), human CD34+ cells were then differentiated into dendritic cells *in vitro* and function assessed. A total of 22-28% of cells expressing the DC phenotype also expressed a transduced marker gene and were able to stimulate tumor-infiltrating lymphocytes to raise a specific anti-tumor immune response *in vitro* (Reeves *et al.* 1996). Another study showed that CD34+ derived dendritic cells retrovirally transduced with the human epithelial tumor antigen mucin (MUC-1) were able to express the tumor associated antigen *in vitro* (Henderson *et al.* 1996). It was also shown that transduced human dendritic cells expressed a normal phenotype and were potent T-cell stimulators (Szabolcs *et al.* 1997). This further showed the potential of this method for *in vivo* immunisation against tumor associated antigens.

Retroviral vectors derived from the lentiviruses group which contain a number of additional accessory genes to the conventional retroviruses (such as MoMuLV) are currently being developed. DC are a natural target for the lentiviruses human immunodeficiency virus HIV and simian immunodeficiency virus (SIV). Thus, an attenuated variant might constitute a suitable vector that could genetically modify DC. In contrast to retroviral vectors which are not able to infect non-dividing cells, lentiviral vectors, including HIV and SIV-derived vectors, can infect non-dividing cells, including monocyte-derived DC (Gruber *et al.* 2000), (Negre *et al.* 2000). Using an HIVΔEN vector, both immature and mature DC could be transduced. At an MOI of 5, 26% of immature DC and 5% of mature DC expressed the gene encoding the green fluorescent protein GFP (Gruber *et al.* 2000). At an MOI of 50, the transduction efficacy increased to 50% when immature DC were analysed. At an MOI of 5, the viability and phenotype of the transduced DC were not significantly altered. Furthermore, immature DC were still able to mature and these matured DC stimulated allogeneic T cells as well as CTL clones. In addition, SIVmac251-derived lentiviral vectors have been reported to transduce DC (Negre *et al.* 2000). At an MOI of 10, 10% of immature and 32% of mature DC were GFP positive. Phenotypically,

these DC were comparable to uninfected DC. These data indicate that lentiviral based vector systems might be useful tool for genetically modifying DC.

Because lentiviral vectors are pseudotyped with vesicular stomatitis virus G glycoprotein, they can transduce a broad range of tissues and cell types (Kafri *et al.* 1997). Recent studies have shown that a single-cycle form of HIV-1 efficiently infected an immature stage of DC development (monocytes). This vector was prepared by pseudotyping envelope-defective HIV plasmids with the envelope from vesicular stomatitis virus G protein (VSV-G), to which most humans lack pre-existing immunity. The infected populations were further matured with CD40 ligand which resulted in strong CTL responses from HIV-1-infected individuals (Granelli-Piperno *et al.* 2000a). This novel strategy using non-replicating vaccines to target immature DC which upon maturation stimulate T cells provide powerful tools for use in immunotherapy.

A similar system was used to infect DC directly showing that DC derived either from healthy donors or from melanoma patients were transduced at 70 to 90% efficiency compared to 2 to 8% using MoMuLV-based vectors. Transduced cells showed characteristic DC phenotype and were able to stimulate strong T cell responses (Chinnasamy *et al.* 2000). Efficient gene transfer to DC was also obtained using a human immunodeficiency virus 1-derived lentiviral vector deleted of all structural and accessory genes. 30 to 40% of DC transduced with such a virus at multiplicity of infection of 20 expressed the delivered gene and were fully functional (Dyall *et al.* 2001). The availability of a stable gene delivery system based on a multiply disabled lentivirus that does not encode any viral protein will be useful for the improvement of immunotherapeutic strategies using DC.

Despite these advances, a number of obstacles generally limit the effectiveness of retrovirus gene transfer:

- Although lentiviral vectors can infect dividing or non-dividing cells, most retroviral-based vectors (e.g. MoMuLV-based vectors) are unable to infect post-mitotic cells
- Size limitations for the incorporation of foreign DNA
- The fairly low titers obtained after *in vitro* growth
- The possibility of recombination to produce replication-competent virus
- Integration into the host genome may activate cellular proto-oncogenes or inactivate a cellular tumor repressor gene causing the cell to become malignant

Thus, further refinements of *in vivo* strategies are required before effective clinical trials of retrovirus-mediated gene therapy are possible. Safety concerns should be addressed, before these vectors can be used in clinical applications.

I.3.2.2. Adenoviral vectors (Ad)

Adenovirus-based vectors are currently in use for many *in vivo* gene transfer applications. Adenoviruses combine the characteristics of high titer, infection of non-dividing cells, a broad host range, and a tropism for epithelial tissue. These vectors are double-stranded DNA viruses of about 36 kb which allow the insertion of up to 7.5 kb of foreign DNA (Graham and Prevec 1992). The genome does not integrate into the host's genome, so there is no risk of insertional activation of cellular proto-oncogenes. The genome is divided in two major noncontiguous overlapping regions, early (E) and late (L), defined by the time of transcription after infection.

The first generation of adenovirus vectors used for gene delivery purposes have a deletion in the *E1* gene which is essential for viral gene expression and replication. Thus, such replication defective viruses were grown on complementing cell lines that produced the *E1* gene in trans. Furthermore, genomic deletions in the E3 and E4 region of the virus led to an increase in the otherwise limited cloning capacity without affecting viral viability (Imler 1995). Both humoral and cellular

immunity can be induced by genetically modified adenoviruses (Xiang *et al.* 1996). Current research into adenovirus vectors is focusing on strategies to avoid host immune responses to the vector.

The uptake of adenoviruses is mediated by $\alpha_v\beta_5$ integrin, known to be expressed preferentially in immature DC. Thus, very high MOI are needed to transduce mature DC (Zhong *et al.* 1999). Nevertheless, the very high transduction rate in the case of immature DC at an MOI of 100, did not lead to the perturbation of DC functions. Adenovirus-based vectors were used to transfer genes to dendritic cells (DC). Initial studies showed that a recombinant adenovirus, Ad5RSVGFP, containing the GFP marker gene gave high gene delivery to human DC but required high multiplicity of infection (5000) and combination with cationic liposomes (Dietz and Vuk-Pavlovic 1998). A similar adenovirus-based vector, Ad5CMVβ-gal, encoding β-galactosidase, was used for gene delivery to DC at multiplicity of infection of 1000, and it was demonstrated that cells remained alive for more than a week in culture with the virus and were able to generate a CTL response (Diao *et al.* 1999). Despite the use of high multiplicity of infection of virus, these results provide an essential link between existing animal data and prospective human clinical trials. Therefore, these viruses provide a valuable gene delivery system.

Recently, genetic immunisation using DC transduced *in vitro* with an adenovirus expressing the ErbB-2/Neu gene (AdNeuTK) induced protective and therapeutic immunity against a breast tumor cell line overexpressing ErbB-2/Neu (Chen *et al.* 2001). Over-expression of ErbB-2/Neu occurs in 20-30% of patients with breast cancer and indicates poor prognosis. Subcutaneous immunisation with the DC vaccine elicited protective immunity inan average of 60% of animals. CTL analysis demonstrated specific cytotoxic activity against breast tumor cells. *In vivo* depletion studies demonstrated both CD4+ and CD8+ T cells were required. Immunisation with the DC vaccine could cure mice with pre-established tumors and efficacy was further enhanced by co-transducing DC with a vector expressing murine IL-12 (AdmIL-12). These studies support DC vaccines as therapeutic strategy for human breast cancer

using adenoviral vectors, while emphasizing the importance of optimising an immune response by combining tumor antigen presentation with cytokines (Chen *et al.* 2001).

It is becoming increasingly more evident that chemokines play an integral role in the initiation of immune response (Sallusto and Lanzavecchia 2000). Chemokines are a family of small secreted molecules that mediate leucocyte migration (Baggiolini 1998). One such molecule, 6-C-kine (SLC) is a chemokine found on high endothelial venules and within the T-cell zones of both spleen and lymph nodes and is an agonist for the MIP-3β receptor (Campbell *et al.* 1998). SLC is capable of recruiting both DC and naïve T cells via the CCR7 receptor found on both cell types (Saeki *et al.* 1999). Because of its expression pattern and that of its receptor, SLC has been postulated to play an important role in the priming of naïve T cells by DC (Cyster 1999). A recent study has shown the augmentation of DC-based immunotherapy using an adenovirus vector encoding SLC to modify DC to express high levels of this chemokine (Kirk *et al.* 2001). Indeed, the SLC gene was used to modify DC as a treatment of established tumors in mice and SLC was shown to both induce antitumor responses and enhance the antitumor immunity elicited by DC. This indicates that the use of chemokine can increase the efficiency of DC-based immunisation.

I.3.2.3. Adeno-associated viral vectors (AAV)

Adeno-associated viruses are single stranded DNA parvoviruses of 4.7 kb that are naturally replication incompetent and thus require a helper virus such as adeno-associated or herpes amplicon to replicate. AAV is not pathogenic in humans. These viruses infect and integrate into the host genome at specific regions in chromosome 19 (Muzyczka 1992). AAV may not require target cell replication and high titers of infectious virus can be achieved increasing their potential advantages for *in vivo* gene transfer. The genome consists of two coding regions, *rep* genes encode proteins involved in replication and integration and the *cap* genes encode structural proteins. Both sets of genes are flanked by terminal repeats (TR) that contain the viral origin of replication.

AAV has recently become an attractive candidate for gene transfer (Monahan *et al.* 1998). However, accumulating evidence indicates that administration of AAV vector may initiate cellular and humoral immune response to its transduced neo-antigen *in vivo*. Thus, AAV-based vectors have been developed by the replacement of the *rep* and *cap* genes, retaining the terminal repeats and packaging sequences essential to direct replication and packaging of the genome. Indeed, such a recombinant adeno-associated virus type 2 (rAAV) was used as a vector to deliver genes to mouse bone marrow-derived DC at a multiplicity of infection of 100 and CTL were activated (Zhang 1999).

However, this system has a number of limitations at present for clinical application. These include:

- Laborious preparation for viral growth (stocks are easily contaminated with helper viruses)
- The toxicity associated with the rep proteins and the helper virus proteins prevent the establishment of stable packaging cell lines
- The risk for insertional mutagenesis as the genome integrates in the host's genome
- The limited capacity for foreign DNA insertion

I.3.2.4. Other viral vector systems

Amongst viral vector systems which could be used to generate an anti-tumoral or anti-infection immunity, vaccinia virus was shown to transduce human DC efficiently. The vaccine virus is a member of the orthopox virus family and can carry at least 25 kb of foreign DNA, allowing the simultaneous expression of different heterologous genes. Highly attenuated virus strains or strains deficient for late gene expression have been designed (Sutter and Moss 1992), (Holzer *et al.* 1999). The attenuated vaccine-based strain of the virus did not replicate in DC and no late gene

expression was detected but it was found to inhibit DC maturation (Jenne *et al.* 2000b). This showed that vaccinia virus has developed multiple strategies to interfere with the immune response (Drillien *et al.* 2000). Thus, the development of vaccinia-based vectors for gene delivery to DC have to be further refined by the identification and elimination of the viral genes that modulate DC functions in order to facilitate T cell responses. Similarly to vaccinia-based vectors, measles virus was found to transduce DC efficiently. However, it was also demonstrated to block their allostimulatory properties for CD4+ T cells (Grosjean *et al.* 1997).

In contrast, variants of prototype alphavirus, sindbis virus replicon vectors were efficiently used to transduce human and mouse DC and CTL responses were obtained *in vitro* and *in vivo* demonstrating that infected DC maintained their ability to process and present replicon-encoded antigen (Gardner *et al.* 2000). The results also showed that human and mouse DC were differentially infected by sindbis virus suggesting differences in receptor expression between human and murine DC. These findings indicated that sindbis virus-based vectors offer potential for the direct targeting to DC and for activation of these cells although many more work is required for clinical application.

Influenza virus has also been shown to transduce DC efficiently. Influenza virus is a negative-stranded RNA virus that does not integrate into the host genome. Furthermore, influenza virus strains are sensitive to antiviral pharmaceuticals and fail to establish persistent infections. Recently, it was shown that avian influenza virus provide a very efficient vector system for the delivery of foreign genes into mature human monocyte-derived DC (Strobel *et al.* 2000b). A transduction of more than 90% of DC was observed at an MOI of 1 and the transduced DC retained their characteristic phenotype and were potent immune stimulators. This indicates the versatility of influenza virus-based vector in the development of genetically modified DC.

Thus, each virus has its own effect on DC and this has to be taken into account when using viruses as vectors for *in vivo* vaccination or *in vitro* transduction of DC. A deeper understanding of these interactions and the identification of the factors involved will help in the design of better vector systems and vaccines (Jenne *et al.* 2000b).

I.4. Herpes simplex type 1 viral vectors

HSV-1 is responsible for the symptoms of cold sores. Infection can spread on rare occasions to the central nervous system which causes an often fatal encephalopathy. About 90% of the population test positive for antibodies to HSV-1, although although a considerably lower percentage actually show symptoms of the disease. This indicates that the majority of the population has been exposed to the infection and has developed some sort of immunity against the virus.

Herpes simplex virus-1 (HSV-1) based vectors are attractive for use in gene delivery as HSV-1 can infect a wide-range of dividing or non-dividing cells (i.e. virtually any human cell), can accommodate very large DNA inserts (up to 30 kb if all the non-essential genes are deleted), is capable of delivering genes into the nucleus of the infected cell. The virus can enter a lifelong latent state in neurons. In this section, the biology of HSV-1 is described with its structure and life cycle, followed by a brief review of current HSV-1 based vectors for use in gene delivery to the nervous system, and finally a description of the HSV-1 viral vectors, and their use to deliver genes to dendritic cells (DC).

I.4.1. The Biology of HSV-1

HSV-1 belongs to the herpesvirus family, a diverse group of large double stranded DNA (dsDNA) viruses which have the potential to establish lifelong latent infection (Fink *et al.* 1996), (Roizman and Sears, 1996). The structure of the HSV-1 virion consists of 110 nm diameter particles comprised of an electron-opaque core

containing the HSV-1 genome, surrounded by an icosahedral protein nucleocapsid separated from the surrounding glycolipid-envelop by a layer of proteins, the tegument. The envelop contains eleven glycoproteins (named gB to gM) which mediate virus attachment and entry into cells through fusion of the envelop with the cell membrane. The tegument facilitates virus growth by assisting in the early stages of infection. Amongst these tegument proteins, vmw65 (also called VP16) is known to contribute with other cellular transcription factors to activate immediate early (IE) gene promoters. The virion host shut-off protein (vhs) assists viral replication by degrading cellular mRNA and thus interfering with host protein synthesis (Fink and Glorioso 1997).

The HSV-1 genome consists of a linear dsDNA 152 kb in length encoding 81 known genes, 38 of which are essential for viral production *in vitro*. The HSV-1 genome consists of a unique long (UL) and a unique short (US) region, each flanked by inverted terminal repeats. Following entry into the cell, the linear viral genome circularises and replicates to produce a linear concatameric DNA. Distinct origins of replication have been identified within the viral DNA. The structure of the HSV-1 virion and genome is indicated in figure I.2.

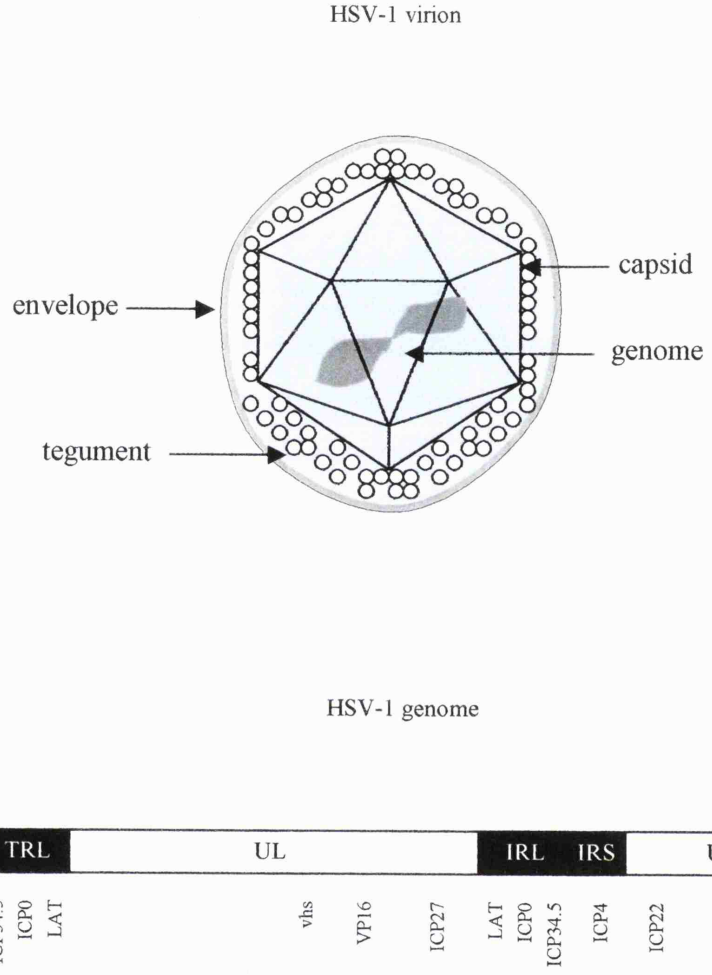


Figure I.2: A schematic representation of the HSV-1 virion and genome. The enveloped HSV virion consists of a double strand DNA genome, surrounded by a capsid and the tegument. The HSV-1 genome consists of the unique long (UL) and short (US) regions respectively flanked by terminal and internal long repeats (TRL and IRL) and short repeats (TRS and IRS). The positions of several essential and non-essential genes are indicated.

Viral infection begins in epithelial cells of the skin or mucous membrane, after which the virus spreads to axons of sensory neurons that innervate the primary site of infection, and is carried by retrograde axonal transport from the site of the original infection to the neuronal perikaryon (Cook and Stevens, 1973). Depending on the cell-type, the virus can either:

- Begin a cycle of lytic replication which results in the death of the infected cell and release of virus
- Enter a latent state in which the viral genome persists without the expression of any viral proteins, often for the life of the host

Initial attachment of the envelope membrane to the cell surface occurs through glycoproteins gB, gC and gD and heparan sulphate present on the cell membrane. The virus capsids are then internalised and transported to the nucleus. One of the tegument proteins, vhs, then plays a key role in down-regulating host protein synthesis while the virus enters a lytic infection. Following penetration and internalisation, several of the tegument proteins have an important function, as mentioned previously . During infection, one of the earliest viral activities is that mediated by the virion host shuttoff (vhs) protein, a product of the UL41 gene. This viral tegument component exerts its effects immediately upon entry into the cell, prior to viral gene expression (Fenwick and Clark 1982). The vhs protein is associated with degradation of both viral and host mRNA and endoribonucleolytic activity which causes an early shut down of host protein synthesis (Kwong and Frenkel 1987), (Elgadi *et al.* 1999). The destabilisation of viral messages mediated by vhs has been theorised to promote the switch from transcription of one kinetic class of viral genes to the next (Strom and Frenkel 1987). Virus capsids are transported to the nucleus by retrograde axonal transport where the viral DNA and at least some tegument proteins enter the nucleoplasm by an unknown mechanism. Here the course of the HSV-1 infection depends on whether the virus enters the lytic cycle or establishes a latent infection.

HSV-1 mutants with vhs deleted have been constructed that allowed detailed investigation of the function of the vhs protein (Jones *et al.* 1995). Moreover, such mutants were instrumental in many studies addressing cell biological and immunological aspects of HSV-1 infection (Geiss *et al.* 2000), (Smith *et al.* 2000). An HSV-1 strain with a mutation in vhs is attenuated in virulence and induces immune responses in mice that are protective against corneal infection with virulent HSV-1, but it has the capacity to establish latency (Walker and Leib 1998). It has been shown that disruption of vhs activity improves the immunogenicity and protective capacity of a replication-incompetent HSV-1 vaccine strain (Geiss *et al.* 2000). Recently, it has been demonstrated that the vhs protein as well as vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1 (Koppers-Lalic *et al.* 2001). At present, a viral gene product of HSV-1 that has been identified to subvert the MHC class I-restricted antigen presentation pathway is the ICP47 protein (York *et al.* 1994).

The tegument protein, vmw65 (also called virion protein 16, VP16 or α-transinducing factor, α-TIF) interacts with cellular factors to transactivate the promoters of the first class of viral genes to be expressed, the immediate early (IE) genes, and consequently initiate an efficient lytic infection (Batterson *et al.* 1983). The initial expression of IE genes is enhanced by the tegument protein, vmw65 which enters the nucleus with the viral genome and collaborates with a cellular factor (octamer binding protein-1, Oct-1) in order to bind to a consensus sequence in the IE gene promoter enhancers. Thus, Vmw65 cannot bind DNA directly but it is dependent on the cellular POU domain protein Oct-1 and at least one other cellular factor, host cell factor (HCF) to form a multicomponent complex on the TAATGARAT motifs which are present in all the IE gene promoters (Gaffney *et al.* 1985). HCF has an important role in transporting vmw65 to the nucleus (La Boissiere *et al.* 1999), Oct-1 binds directly to the TAAT region of the motif, and the recruitment of vmw65 is dependent on the presence of the GARAT half of the sequence (where R is a purine) (Gerster and Roeder 1988). Vmw65 is known to contain a very potent C-terminal transactivation domain (Sadowski *et al.* 1988). The

location of the TAATGARAT motifs just upstream of the TATA box sites of the IE gene promoters and the observation that vmw65 can interact directly with transcription factor TFIID suggests that vmw65 acts directly to activate the basal transcription machinery (Klemm *et al.* 1995).

After this transactivation, the lytic gene cascade takes place. Recurrent infections result from the lytic replication of the virus after reactivation from the latent state. During a productive infection in cultured cells, HSV-1 gene expression proceed in a tightly regulated cascade which allows the controlled expression of three classes of genes: the immediate early (IE) or α genes, the early (E) or β genes and the late (L) genes or γ genes (Honess and Roizman 1974). The α gene products, infected cell polypeptides (ICP) 0, 4, 22 and 27 have regulatory functions, and they cooperatively act to regulate the expression of all classes of viral genes (Roizman and Sears, 1996). β genes which are expressed next encode many of the proteins involved in viral DNA synthesis (Honess and Roizman 1974) and the late (L) genes or γ genes mainly encode virion components (Batterson and Roizman 1983). With the exception of ICP47 (which is also an α gene product), the IE proteins have regulatory effects on E and L gene expression and are thus responsible for directing a well-ordered temporal cascade of viral gene expression and viral replication. This is described below and in figure I.3.

ICP0 is an activator of E and L genes and thus of viral replication, although it does not bind to DNA. ICP0 is not essential for infection but its deletion impairs viral replication. ICP0 transactivates almost any target promoter and this transactivation is enhanced by the presence of ICP4 (Everett 1987), (Everett 1984). The exact mechanism of transactivation by ICP0 has not been fully elucidated. ICP0 does not bind DNA directly but it acts at or before the initiation of mRNA synthesis. ICP4 encodes a nuclear phosphoprotein that is essential for viral growth and controls the expression of E and L genes through both transcriptional and posttranscriptional mechanisms (DeLuca and Schaffer 1985). ICP4 also negatively regulates IE gene expression. ICP4 is thus an essential regulatory IE gene and is the major

transcriptional regulator of HSV-1 (DeLuca *et al.* 1985). ICP4 is a DNA binding protein, recognising the consensus site, where R is purine, Y is pyrimidine, S is C or G, and N is any base (DiDonato *et al.* 1991). ICP4 is able to transactivate E and L genes, and downregulate the expression of some IE genes, especially ICPO and itself. ICP4 is capable of repressing the activity of the latency promoters, either on its own or in combination with ICPO (Goins *et al.* 1994). Although the precise mechanism of action of ICP4 is unclear, the protein has been demonstrated to interact directly with several components of the basal transcription machinery (Carrozza and DeLuca 1996).

ICP27 is an essential regulatory IE gene encoding a nuclear phosphoprotein which performs a number of diverse regulatory functions (Sacks *et al.* 1985). These include repression of IE and E genes, activation of late genes and selection of transcriptional termination site (McCarthy *et al.* 1989). ICP27 also contributes to the shut off of host protein synthesis during lytic infection (McCarthy *et al.* 1989). Mutant recombinant viruses unable to produce functional ICP27 (ICP27 null) showed that ICP27 was essential for the optimal expression of L genes, as well as the synthesis of viral DNA. However, these studies were performed in cultures of Vero cells, which are of African green monkey origin. Thus another study was conducted to determine the role of ICP27 in the replication of HSV-1 in cultured human cells. Infection of at least three separate strains of human cells (Hep-2, HeLa, and 143tk-) with HSV-1 ICP27 null virus did not produce IE or L proteins at the levels observed following infection with wild-type virus of these cells (Aubert and Blaho 1999a). Measurements of cell morphology, chromatin condensation, and genomic DNA fragmentation demonstrated that the human cells died by apoptosis after infection with the ICP27 null virus. It was also shown that while HSV-1 infection induced apoptosis in all cells, viral evasion of the response differed among the cells tested in this study. Thus, ICP27 is required for the prevention of apoptosis in infected human cells. ICP27 also contributes to the shutoff of host protein synthesis seen during lytic infection, through impairment of host cell pre-mRNA splicing (Hardy and Sandri-Goldin 1994).

ICP22 is non-essential for viral growth but has been shown to promote efficient late gene expression in a cell type dependent manner (Sears *et al.* 1985). ICP22 is also implicated in the production of an aberrantly phosphorylated form of cellular RNA polymerase II (Long *et al.* 1999). The HSV-1 ICP22 the product of the unique sequence 1 (US1) gene, is a nucleotidylated and phosphorylated protein with properties of a transcriptional factor required for the expression of a subset of late viral genes (Ogle and Roizman 1999). The coding domain of the US1 gene encodes two proteins, a full length protein, ICP22, and a protein called US1.5 which is initiated from methionine 147 of ICP22 and which is colinear with the remaining portion of the protein. The two corresponding mRNAs are expressed by their own independent promoter (Carter *et al.* 1996). Recent studies have demonstrated that HSV US1 gene expression in lymphoblastoid antigen presenting cells results in a dramatically reduced ability to stimulate CD4+ T cell clone proliferation and cytokine secretion (Barcy and Corey 2001). However, it is not clear yet which of either the ICP22 or US1.5 proteins is responsible for the antigen presenting cell function inhibition. It has also been shown that HSV-1 protein can partially prevent Hep-2 cell-induced apoptosis which could provide an explanation for the reduced susceptibility of CD4+ T lymphocytes to lyse (Aubert *et al.* 1999).

ICP47 is non-essential for viral replication and does not play a role in IE gene expression. However, it has been shown that ICP47 which is a cytosolic protein binds to the transporter associated with antigen processing (TAP) in order to retain MHC class I molecules in the endoplasmic reticulum, and block peptide translocation. This inhibits antigen presentation via the MHC class I pathway and has been suggested to be a mechanism through which the virus escapes host immunity (Hill *et al.* 1995).

Following IE gene expression, the E genes are transcribed. The E gene products include enzymes which are necessary for viral DNA replication. Viral DNA synthesis proceeds via a rolling circle mechanism that produces head-to-tail concatemers of the HSV-1 genome (Jacob *et al.* 1979). L gene expression is triggered

by ICP4 and ICP27 and occurs only after viral DNA synthesis. L gene products include structural proteins of the capsid, tegument and envelope which allow virus assembly. The viral DNA concatemers are cleaved into genome length units and packaged into the capsids as a coupled process within six hours of infection. The newly formed capsids covered with tegument proteins then bud out of the nucleus, acquiring the glycoprotein envelope and are transported to the cell surface via the endoplasmic reticulum. The lytic life cycle is rapid as it results in cell death about ten hours after infection (Fink *et al.* 1996).

Lytic infections can be treated with acyclovir, a drug that inhibits viral DNA synthesis and thus prevents the lytic cycle. However, following treatment, lesions can occur again due to the ability of the virus to reactivate from lifelong latent infections established in neurons at the time of initial infection. Establishment of asymptomatic latent infections can occur in sensory neurons of the trigeminal or dorsal root ganglia. HSV-1 can then reactivate in response to stress, fever or hormonal imbalance by migration of virus in the periphery resulting in recurrent lytic infection (Latchman 1990).

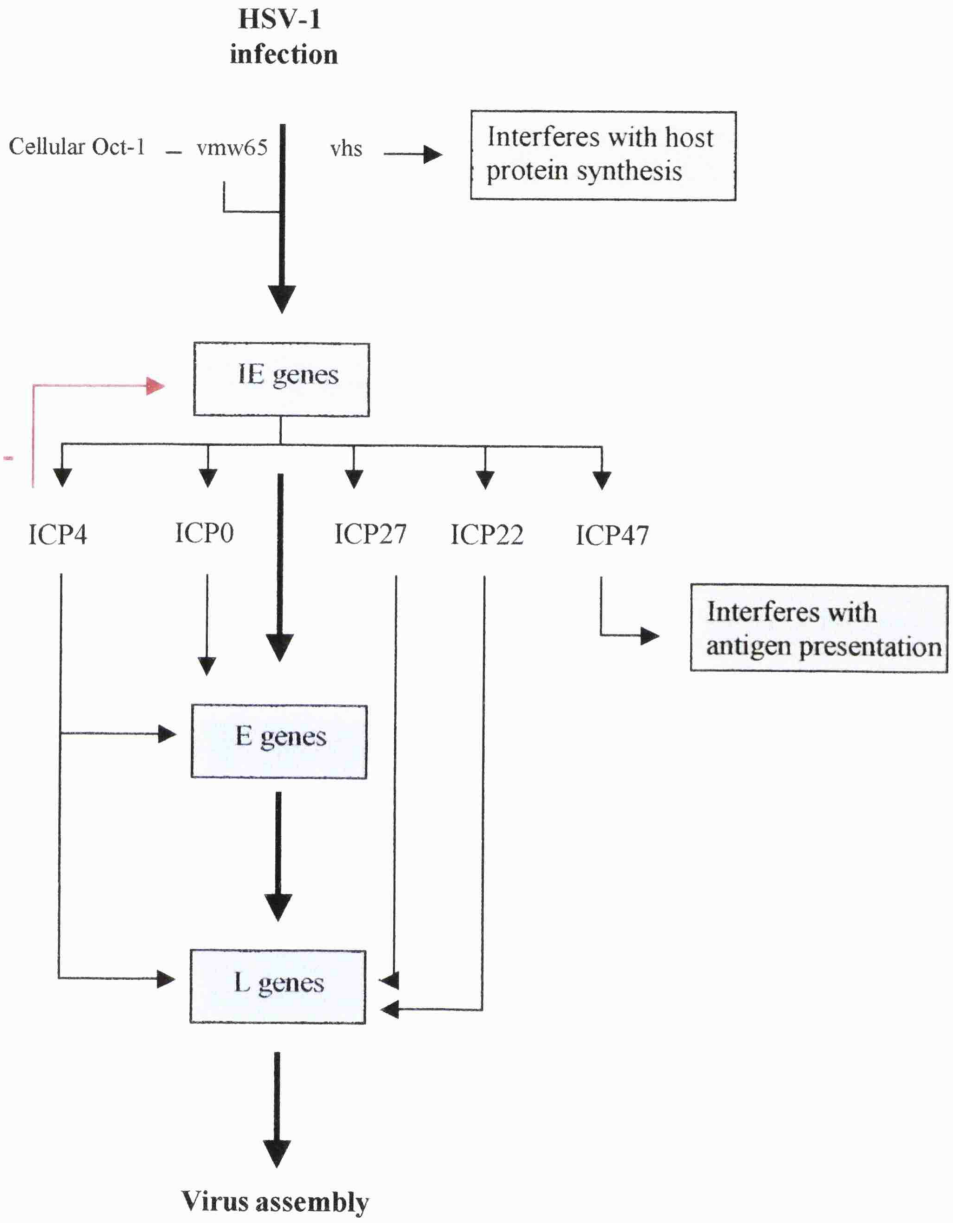


Figure I.3: Temporal regulation of gene expression in the lytic infection of HSV-1 (Honess and Roizman 1974). Interaction of vmw65 with Oct-1 transactivates Immediate Early (IE) gene expression. The IE gene expression is required for the expression of Early (E) and Late (L) genes. Known positive regulatory effects are indicated in black arrows. A red arrow indicates a repressive effect.

HSV-1 inhibits apoptosis of infected cells, presumably to ensure that the infected cell survives long enough to allow completion of viral replication. HSV-1 is a member of a family of cytolytic viruses whose lytic replication cycle ultimately leads to the destruction of cells in culture. The cytopathic effect (CPE) of HSV-1 infection is generally observed as the rounding up of cells almost immediately upon infection, and it tends to become more severe with increasing times of infection (Roizman and Sears, 1996). Manifestations of HSV-1 infection included: the loss of matrix binding proteins on the cell surface (leading to detachment), modifications of membranes, cytoskeletal destabilisations, nucleolar alterations and chromatin margination and aggregation or damage, as well as a decrease in cellular macromolecular synthesis (Aubert and Blaho 1999a), (Blaho *et al.* 1997), (Galvan and Roizman 1998). Though it was clear that HSV-1 infection caused major biochemical alterations within the infected cell, the exact method by which the virus killed the cells was not well understood.

Several other genes of HSV have been shown to be necessary for the inhibition of apoptosis in various experimental systems, including US3 and US5 (Leopardi *et al.* 1997), (Jerome *et al.* 1999), (Aubert and Blaho 1999b). The usual approach in these studies has been to use mutant viruses deleted for individual HSV genes and compare these with viruses in which the deleted gene has been restored. Although this approach demonstrates that the deleted gene is required for viral inhibition of apoptosis, it does not directly show anti-apoptotic activity for the gene in question. The HSV-1 protein kinase US3 is required for protection against apotosis (Leopardi *et al.* 1997). HSV encodes two protein kinases expressed by the genes US3 and UL13, respectively (Purves *et al.* 1987). Whereas UL13 is packaged in the virion, US3 is not. Not all substrates of the US3 gene are known. The most extensively studied substrate of US3 is an essential membrane protein encoded in the UL34 gene (Purves *et al.* 1991). The UL34 protein is not phosphorylated in cells infected with an HSV-1 mutant lacking the US3 kinase. Thus, a functional protein kinase encoded by the HSV-1 US3 is required to block apoptosis induced by infection (Leopardi *et al.* 1997).

Recently, the US5 gene was expressed in isolation in Jurkat cells under the control of a Tet-inducible promoter (Jerome *et al.* 2001). It was demonstrated that expressed US5 rendered transfected cells resistant to Fas- or UV-induced apoptosis, as measured by morphology, caspase activation, membrane permeability changes, or mitochondrial transmembrane potential . This provides the first demonstration of a direct effect by an HSV protein on apoptosis induced by exogenous stimuli. However, the mechanism by which US5/gJ inhibits apoptosis remains unclear. Additional experiments indicate that HSV deleted for US5 could still inhibit Granzyme B-induced apoptosis, which is built in the immune system to counter viral escape mechanisms, implying that other HSV gene products can perform this function in absence of US5 (Jerome *et al.* 2001).

HSV appears to have evolved a variety of mechanisms of evasion of the host defense machinery. They include genes that prevent apoptosis in differentiated cells, that interact with antibody and complement, and that prevent induction of CD8+ cytotoxic T cells. The association of the γ34.5 gene encoding the ICP34.5 protein with neurogrowth is an example. HSV mutants lacking the gene encoding ICP34.5 exhibit an attenuated phenotype in models of pathogenesis and have been used for experimental cancer therapy. It was shown that the HSV ICP34.5 protein functions to prevent the host cell-induced double-stranded RNA-activated protein kinase (PKR)-dependent translational block that normally occurs during viral infection (Randazzo *et al.* 1997). HSV ICP34.5 mutant called HSV-1716 is unable to replicate in the simian kidney cell-derived line CV-1, due to a translational block. It was shown that this block can be overcome by simian virus 40 (SV40). This has been shown directly by infecting CV-1 cells with SV40 and HSV-1764 simultaneously and indirectly via HSV-1764 infection of COS-1 cells. The translational block is restored when infection is done in presence of the phosphatase inhibitor okadaic acid. These results support contentions that ICP34.5 interacts with the PKR pathway to restore translation in non-permissive cells, and that SV40 large T antigen has a similar role, but acts downstream of the site of ICP34.5 interaction (eIF2alpha) in the pathway.

Many viruses have evolved to exploit cell-surface glycosaminoglycans (GAG), particularly heparan sulphate, to facilitate their attachment and infection of host cells (Ibrahim *et al.* 1999). It was shown that HIV-1 infection is facilitated by heparan sulphate GAG in only one of three highly permissive cell lines tested in this study, whereas HSV-1 infection is facilitated to varying extents in all three. Treatment of peripheral blood lymphocytes (the physiological host for HIV-1) with heparitinase, to remove any traces of GAG, did not alter their sensitivity to infection. Thus, heparan sulphate GAG has little physiological role in the infection of lymphocytes by HIV-1(Ibrahim *et al.* 1999).

Another study showed the evidence for a proteoglycan-independent virus entry pathway, using a mouse L-cell mutant cell line defective in the biosynthesis of glycosaminoglycans (Banfield *et al.* 1995). These cells, termed sog9, were derived from mutant parental cells, which are defective in heparan sulphate biosynthesis and 90% resistant to HSV-1 infection compared with control L cells (Gruenheid *et al.* 1993). It was demonstrated that Sog9 cells exhibit a 3-order magnitude reduction in susceptibility to HSV-1 infection compared with control L cells, due to the specific reduction of GAG assembly in these cells. Despite these defects, sog9 cells were fully permissive for HSV replication, assembly and regress. Furthermore, HSV-1 infection of sog9 cells was not significantly reduced by soluble heparan sulphate, indicating that infection was GAG independent. Infection was inhibited by soluble gD-1, which suggests that glycoprotein gD plays a key role in the infection of this cell line (Banfield *et al.* 1995). Glycoprotein gD is a structural component of the HSV envelope which is essential for virus entry into host cells. Chinese hamster ovary (CHO K1) cells are one of the few cell types which are non-permissive for the entry of many HSV strains. However, when these cells are transformed with the gene for the herpesvirus entry mediator (HVEM), the resulting cells, CHO-HVEM12, are permissive for many HSV strains. HVEM is a member of the tumor necrosis factor receptor superfamily which has been shown to directly interact with glycoprotein gD, suggesting that it is a receptor for virion gD and that the interaction between these

two molecules is a step in viral entry into HVEM-expressing cells (Whitbeck *et al.* 1997).

It has also been shown that there are marked differences in the outcome of HSV infection among different populations of primary sensory neurons following peripheral inoculation (Yang *et al.* 2000). Indeed, three weeks after ocular inoculation with HSV strain KOS, 81% of latency-associated transcripts (LAT)-positive trigeminal ganglion (TG) neurons coexpressed SSEA3, 71% coexpressed Trk (A) (the high affinity nerve growth factor receptor), and 68% coexpressed antigen recognised by Mab KH10. It was shown that the distribution of LAT-positive, latently infected TG neurons contrasted sharply with the overall distribution of neuronal phenotypes in latently infected TG and the neuronal distribution of viral antigen in productively infected TG. Thus, although all neuronal populations within primary sensory ganglia appear to be capable of supporting a productive infection with HSV, some neuronal phenotypes are more permissive for establishment of a latent infection with LAT expression than others. However, expression of HSV LAT does not appear to play a key role in this process. These findings highlight the key role that the host neurons may play in regulating viral gene expression during the establishment of HSV latency (Yang *et al.* 2000).

The interaction of HSV with mononuclear phagocytes (MP), i.e. monocytes and macrophages, is of importance for the pathogenesis of HSV infections. MP are known to play a significant role in the cellular defence against infections with HSV, but it has also been shown that HSV-1 affects MP. The infection of these cells at different stages of differentiation has various outcomes, and may result in the alteration of important cellular functions. HSV-1 inhibits the morphological differentiation of human monocytes and this inhibition occurs in spite of the fact that human monocytes are non-permissive to HSV-1. It was shown that there is a relation between permissiveness and cytokine response in mononuclear phagocytes infected with HSV-1. Such a relation may be of importance to both intrinsic and extrinsic defence mechanisms of MP against HSV-1. In addition, it was demonstrated that the

functions of non-permissive cells such as blood-derived monocytes may be affected by viral infection (Bruun *et al.* 1998).

The importance of HSV as human pathogens and the emerging prospect of using mutant derivatives of HSV-1 as potential anti-cancer therapeutics have necessitated a thorough investigation into the molecular basis of host-cell permissiveness to HSV (Farassati *et al.* 2001). It was recently shown that NIH-3T3 cells transformed with the oncogenes v-erbB, activated sos or activated ras become significantly more permissive to HSV-1. Inhibition of the ras signalling pathway, such as farnesyl transferase inhibitor 1, effectively suppressed HSV-1 infection of ras-transformed cells. Enhanced permissiveness of the transformed cells was linked to the inhibition of virus-induced activation (phosphorylation) of the double-stranded RNA-activated protein kinase (PKR), allowing viral transcripts to be translated in these cells. Thus, it was demonstrated that HSV-1 specifically targets cells with an activated ras signalling pathway, and this may have important implications in the use of HSV as a vector for cancer therapy, the development of strategies against HSV infections and the controversial role of HSV in human cancers (Farassati *et al.* 2001).

During latency, the HSV-1 genome exists in an episomal extrachromosomal state within the nucleus. *In situ* hybridisation studies have shown that RNA encoding the IE proteins are not detectable in latently infected trigeminal or spinal ganglia in humans and mice which implies that latency is due to the absence of IE mRNA and thus of their corresponding proteins (Deatly *et al.*, 1987). However, one region of the HSV-1 genome remains transcriptionally active during latency. A series of transcripts called the latency-associated transcripts (LATs) are transcribed from the terminal repeats which flank the UL region of the HSV-1 genome (Stevens *et al.* , 1987). Further studies of the LAT RNA have detected these transcripts in latently infected neurons. The major LATs are 2 and 1.5 kb non-polyadenylated RNAs that remain intranuclear and appear to be stable introns spliced from an unstable 8.3 kb primary transcript (Devi-Rao *et al.* 1991).

The function of the LATs has not been fully elucidated. Initially, as the major 8.3 kb LAT RNA contains 750 bp sequence complementary to the ICP0 RNA, it was thought that the LAT RNA plays an anti-sense role. However, other studies have shown that deletion of the LAT region from HSV-1 does not appear to have an effect on the establishment of latency but affects the efficiency of reactivation, contradicting this first hypothesis (Leib *et al.* 1989). However, now seems probable that the LAT region has several functions, including a role in virus reactivation and/or maintenance of latency.

The natural ability of HSV-1 to infect neurons, together with the study of its lytic infection, induction of apoptosis and latent life cycle have stimulated efforts to produce viral vectors with reduced toxicity for long term gene delivery to the nervous system and also to other cell types.

I.4.2. Engineering HSV-1 for use as a gene delivery vector

Two types of HSV-1 vectors have been produced for use in gene delivery, amplicon vectors which are replication defective versions of the virus and recombinant disabled HSV-1 vectors.

Amplicon vectors cannot replicate without helper functions and consist of a plasmid containing the gene of interest, an HSV-1 origin of replication and an HSV-1 packaging signal (Spaete and Frenkel 1982). The plasmid is co-transfected with an HSV-1 helper virus into a cell line which favors growth of the helper virus. The plasmid DNA then replicates in concatamers of up to 152 kb (the size of the HSV-1 genome), and is packaged into virus particles. Thus, a mixture of two populations, replication defective and helper virus is obtained which cannot be separated. Helper viruses that are commonly used contain temperature sensitive mutations or a deletion in the ICP4 IE gene so that the virus is replication defective. The advantages of the amplicon system are that the only manipulation required is that of the amplicon plasmid and each replication defective vector contains multiple copies of the gene of

interest. In addition, efficiency of gene delivery to cells is very high and toxicity is low.

Replication-defective vectors containing a reporter gene driven by the IE 4/5 promoter of HSV-1 have been used to infect cultured rat peripheral nervous system (PNS) and central nervous system (CNS) neurons (Geller and Breakefield 1988), (Geller and Freese 1990), as well as a number of cultured human cell types (Boothman *et al.* 1989). Disadvantages of the HSV amplicon system include:

- The fact that packaging of plasmid DNA may be less efficient than packaging of viral DNA
- The inability to separate helper virus from defective virus in the final stock which may increase toxicity to target cells. However, current amplicon systems allow the production of stocks that are not contaminated with helper virus (Fraefel *et al.* 1996)
- The production of low titer of replication-defective virus due to the difficulty in transfecting several plasmids together (Fraefel *et al.* 1996)

The second method for producing HSV-1 based vectors involves the genetic manipulation of the HSV-1 genome in order to introduce the transgene of interest in specific loci within the genome. As the HSV-1 genome is very large, it is difficult to clone restriction fragments of foreign DNA directly into the viral genome. Thus, other techniques were designed for the genetic engineering of large genomes. Amongst these, homologous recombination was developed (Roizman and Jenkins 1985). The transgene is inserted into a fragment of HSV DNA within a plasmid (shuttle vector) and co-transfected with infectious viral DNA into complementing cells. Homologous recombination then occurs between the plasmid and the viral DNA at the insertion site in the HSV-1 genome corresponding to the HSV DNA in the shuttle vector. This allows insertion of transgene into a specific site of HSV-1 genome. Following homologous recombination, the recombinant progeny is selected, plaque purified and grown on complementing cells in order to produce a pure stock of virus. However,

several limitations have tempered the use of HSV-1 as a vector for gene delivery. These include:

- The toxicity of the virus
- The transient expression of transgenes usually achieved with HSV

Thus, in order to use HSV as a gene delivery vector, it is necessary to disable the wild-type virus so that it is non-pathogenic to target cells, but can still infect and be grown *in vitro* for production of virus stocks.

Strategies used to disable HSV-1 in order to reduce toxicity and allow insertion of foreign DNA include the deletion of non-essential and/or essential genes. Non-essential genes provide accessory functions which may induce toxicity *in vivo* but are not required for viral growth in culture. However, essential genes are indispensable for viral growth and pathogenicity. Various viruses containing deletions or inactivations in the non-essential genes ICP6, ICP0, thymidine kinase (TK), gC, vmw65, ICP34.5 or ICP34.5 and vmw65 together have been described (Coffin *et al.* 1996). These viruses have been used for gene delivery to the peripheral nervous system and reasonable levels of at least short term gene delivery were obtained and/or the ability to enter latency and express a transgene after inoculation of mice by the footpad or ear route (Palmer *et al.* 2000). However, such vectors still retain some level of viral replication as no essential genes have been deleted. A replication incompetent virus with ICP4 and ICP27 deleted has been used in mice but has shown low gene delivery efficiency in dorsal root ganglia following footpad inoculation (Palmer *et al.* 2000). An ideal vector would have regulatory IE genes encoding ICP4, ICP27, ICP22 and ICP0 deleted giving low toxicity. However, initial such viruses were very difficult to grow and gave only low gene delivery efficiency (Wu *et al.* 1996). Newer such viruses containing reporter genes (i.e. Green fluorescent protein *GFP* and/or *LacZ*) have now been developed which can be produced effectively and give very high efficiency long term gene delivery *in vitro* and *in vivo* system (Lilley *et al.* 2001).

The *GFP* gene from Aequorea victoriae codes for a protein with a strong intrinsic fluorescence and has been used as a reporter gene in various eukaryotes and prokaryotes. In contrast to other reporter genes, the *GFP* gene product offers the advantage that no additional substrate are needed for *in situ* monitoring of expression (Schilde *et al.* 2001). GFP is, to date, the most appropriate tool for direct monitoring of the fate of plasmids. The search for stabilising genetic elements and for experimental treatments that promote stable integration is thus highly facilitated. GFP is widely used as a reporter molecule for monitoring gene expression *in vivo*, *in vitro* and using real-time assays (Ehrmann *et al.* 2001), (Chalfie *et al.* 1994).

GFP is known to be exceptionally resistant to heat, alkaline pH, detergents, chaotropic salts, organic solvents and many proteases. Although GFP was originally expressed by a marine living organism, its behaviour under high pressure conditions has only recently been tested. Indeed, a study showed the stability and expression of GFP *in vitro* under high pressure conditions (Ehrmann *et al.* 2001). The influence of extreme pH on fluorescence has also been reported. Wild-type GFP is quenched by acidic pH values, with an apparent pKa near 4.5, and several of the mutants with enhanced spectral properties were reported to be more acid-sensitive than wild-type GFP. For example, EGFP is 50% quenched at acidic pH values (Patterson *et al.* 1997). The replacement of Ser65 in the chromophore sequence by Thr (EGFP) or other aliphatic residues, such as Ala or Cys (GFP mut2 and rsGFP), produces variants with high brightness, and simple excitation and emission spectra peaking at wavelengths very similar to fluorescein (Tsien 1998). Moreover, improved bacterial expression and a significantly faster oxidation of the mature fluorophore is achieved by Leu64, as is the case in rsGFP and EGFP, or Ala72 in GFPmut2 (Cormack *et al.* 1996). The contributions of these amino acyl substitutions to high pressure stability remain unclear. However, the fluorescence intensity of GFP and its mutant forms is not affected *in vitro* by high pressure conditions which allows this reporter gene to be used for expression studies at sublethal conditions. Another study showed that GFP variants fold differentially in prokaryotic and eukaryiotic cells (Sacchetti *et al.* 2001). The specificity of this differential folding supports a role of chaperones in guiding the

folding of GFP *in vivo*. No relationship was detected between GFP folding efficiency and expression levels, or protein stability.

As of today, GFP has been used in many studies to visualise organs or cell populations in live animals (Okabe *et al.* 1997), (Chan *et al.* 2000). However, one study reported cardiomyopathy associated with GFP over-expression in the heart of mice (Huang *et al.* 2000) and cytotoxic effects were also described for strongly GFP-expressing cell lines (Liu *et al.* 1999). Recently, it was shown that a GFP-containing bi-directional expression module provides a useful tool for Doxycycline-regulated gene expression in the mouse (Krestel *et al.* 2001). It was also found that high responder animals developed a lethal phenotype but it is not clear if the phenotype results from GFP toxicity.

The effects of formulation buffer and processing parameters were studied with monomeric and tetrameric β-gal during freeze-thawing in sodium and potassium phosphate buffer solutions (Pikal-Cleland *et al.* 2000).The monomeric protein, β-gal derived from *Aspergillus oryzae*, has a molecular weight of 105 kDa (Tanaka *et al*, 1975). The tetrameric protein, β-gal from Escherichia coli, has a molecular weight of 540 kDa, and loses activity upon dissociation (Marchesi *et al*, 1965). The monomeric form has been studied under freeze-thaw conditions using a rapid cooling process (Izutsu *et al.* 1993). These studies indicated that monomeric β-gal stability during freeze-thaw conditions increased with increasing protein concentration. Inactivation of this protein was also observed with increasing buffer concentration during exposure to relatively high temperatures (50-55°C) in aqueous solutions, indicating that the buffer concentration may affect the conformational stability of the protein. In addition, it was demonstrated loss of activity of the protein at low pH (<4) in solution (Tanaka Y *et al*, 1975). Similar studies have been performed for the tetrameric form, by measuring changes in the protein's secondary structure. Taken together, the previous research suggests that β-gal stability during freeze-thawing is dependent upon protein concentration, buffer concentration and solution pH.

Furthermore, a recent study addresses an issue that is critical to the success of any multi-center gene therapy clinical therapy, the maintenance of vector viability during shipping and storage at remote sites (Croyle *et al.* 2001). The formulations that enhance physical stability of viral vectors for gene therapy have been identified, which include appropriate buffer systems, cryoprotectants and storage conditions. For this purpose, adenovirus and adeno-associated virus expressing β-gal were resuspended in blends complex carbohydrates, cyclodextrins and various surfactants. It was shown that in preparations lyophilised in sucrose and stored at 4°C for one year, only a negligible loss of titer was observed. This provides a solution to the current problem of distribution of viral vectors for clinical trials.

I.4.3. HSV-1 based vectors for gene delivery to dendritic cells

Following infection with HSV-1, an initial acute peripheral infection in the skin or mucosa allows initial interaction with host defense and stimulation of a vigorous immune response. However, this phase is characteristically followed by a state of chronic infection, in which virus co-exists with an on-going host immune response over long periods (often throughout the life of the host). During this period of chronic infection, the virus remains dormant (in a latent state) within sensory neurons. A variety of external and internal factors can initiate virus re-activation. During that time, viral replication occurs, but the spread of virus is limited by the on-going host immune response, and in most cases virus latency is re-established. HSV, as well as infecting neurons, has also on a number of occasions shown to infect dendritic cells (DC) at high efficiency. This has suggested that HSV may naturally infect DC as part of its life cycle and that HSV might be used as an effective vector for gene delivery to DC.

DC are the body's most potent antigen presenting cells and as such have attracted considerable interest for the development of anti-tumor specific antigens by various means (see section I.3). Several groups including ours has used HSV-1 vectors for gene delivery to dendritic cells (DC). An essentially wild-type vector

based on the 17+ strain of HSV-1 containing one deletion in a non-essential gene UL43 and the reporter genes encoding *GFP* and *LacZ* was the first HSV vector used to transduce human DC *in vitro* (Coffin *et al.* 1998). At a multiplicity of infection (MOI) of one, about 40% gene delivery was achieved which is a higher efficiency of transduction than had previously been achieved with other vectors at such low vector dose. This implied that HSV-1 may be used as a potential vector for gene delivery to DC.

However, further studies indicated that an HSV-1 mutant derived from the SC16 strain, deleted in gH and containing GFP, inhibits DC maturation (Salio *et al.* 1999). A consequence of HSV infection is the loss of host cell protein synthesis due to the disruption of polysomes and degradation of mRNA. Thus, the viral protein, vhs responsible for the shut off process would be implicated. However, it was shown that inhibition of DC function following infection was independent of *vhs* expression. Furthermore, it has been shown that HSV-1 interfere with antigen presentation, DC cytokine production and that HSV-1 infected DC could not stimulate T cells (Kruse *et al.* 2000). Thus, interference with maturation and functions of DC could be a powerful mechanism for the virus to escape host immune responses. When viral vectors are used to transduce DC, the viral mechanisms of immune evasion must be taken into account. Viral escape strategies can either be designed to limit the effect of the pre-existing immune response or to hamper the generation of an anti-viral immunity.

I.5. DC and viral immunity

Not all viral vectors are suitable for the *in vitro* transduction of DC (Jenne *et al.* 2001). One of the main obstacles is the induction of cellular immune responses against viral antigens and the transgene itself, which leads to the destruction of genetically modified cells (Christ *et al.* 1997), and the pre-existing or newly generated neutralising antibodies, which interfere with the initial booster immunisation (Molnar-Kimber *et al.* 1998). The aim of DC-based immunotherapy is

the induction of strong cellular and humoral immunity against an inserted tumor associated antigen. In order to reach this goal, the avoidance of DC function appears to be crucial. Proposed escape mechanisms of several virus strains that have considerable potential for use in immunotherapy using *in vitro*-transduced DC are presented in this section.

Viruses have developed a broad range of mechanisms to escape the host immune response (Alcami and Koszinowski 2000), (Tortorella *et al.* 2000). These include:

- reduced expression of critical antigen epitopes (such as in latent EBV infection)
- genetic variation of MHC epitopes (HIV-1 mutation)
- clonal exhaustion of CTL (HIV-1 and LCMV)
- down regulation of MHC class I and MHC/peptide complex expression (HSV, adenovirus, cytomegalovirus)
- production of immunosuppressive cytokines and homologous cytokine receptors
- down-regulation of critical cytokines

Most viral infections are initiated by virus penetration through epithelial surfaces of mucosae and skin, which have rich networks of DC. Thus, it would appear that many of the viral escape mechanisms specifically exploit DC functions. The outcome of DC interaction with viruses could explain why viral infection can lead to an immune response that is not necessarily protective against viral challenge (Kaiserlian and Dubois 2001). Because DC have the ability to capture and process antigen in tissue in the initial stages of a viral infection, they are particularly suited to prime antiviral immunity. CD8+ T lymphocytes are also very important in host immunity to viral infections. CD8+ lymphocytes recognise 8 or 9 amino acid peptide epitopes presented in the context of MHC class I molecules, leading to lysis of the infected target cell.

DC are effective in antigen presentation, costimulation and activation of CD8+ cytotoxic T lymphocytes. For example, DC infected with polyomavirus, but not infected macrophages, have the capability to prime polyomavirus-specific CD8+ T lymphocytes *in vivo* (Drake, III *et al.* 2001). DC infected with other viruses (e.g. influenza and lymphocytic choriomeningitis virus - LCMV) also induce antigen specific CD8+ T lymphocytes (Ridge *et al.* 1998), (Borrow *et al.* 1995). In a model of influenza A virus infection, mature DC are superior to immature DC in stimulating INF-γ production from CD8+ effector cells (Larsson *et al.* 2000). Furthermore, only mature DC (and not immature DC) have the ability to stimulate expansion and differentiation of cytotoxic T lymphocyte effectors over a one-week period. IL-4 was found to have an effect on DC antigen presentation of LCMV and the T lymphocyte response to viral infection (King *et al.* 2001). Furthermore analysis revealed that IL-4 increased the number of antigen-specific CD8+ cells, but inhibited the differentiation of cytotoxic precursors by LCMV-pulsed DC by increasing B7.2 and decreasing B7.1 expression. Changes in DC antigen presentation and costimulation may also explain recent findings that showed that IL-4 inhibits virus-specific CD8+ cytolytic activity (Aung *et al.* 1999).

Some viral infections, such as influenza, promote DC maturation after uptake, improving the ability of the host to kill the virus (Banchereau and Steinman 1998a), (Cella *et al.* 1999b), (Cella *et al.* 2000). In contrast, poxviruses have developed several different mechanisms to evade immune recognition. Vaccinia virus, for example, inhibits DC maturation and leads to impaired allostimulatory properties of the infected DC (Engelmayer *et al.* 1999). Inhibition of DC maturation occurs within one day after infection, particularly in immature DC, which are susceptible for vaccine infection, while mature DC are more likely to act as antigen presenting cells. Immature DC infected with vaccinia have decreased expression of CD25, CD83, CD86 and HLA-DR (markers of mature DC) as compared to non-infected immature DC. A decrease in CD86 on the DC cell surface could lead to antigenic tolerance, whereas decreased HLA-DR expression results in decreased antigen presentation. Vaccinia infection of DC also leads to abortive replication and induction of DC

apoptosis, which further suppress the immune response to vaccine. The poxviruses also encode receptor homologs of cytokines involved in the induction of DC maturation (such as IL-1β,TNF-α, INF-α/β and IFN-γ), which are important in host defence against viral infections (Moss B 1996).

Even though vaccinia virus has developed many immune escape mechanisms, it still induces a strong cellular and antibody-mediated response. However, the precise mechanism for the induction of this immune response still requires elucidation. A possible explanation may be provided by cross-presentation. Here, apoptotic vaccine virus-infected DC may be taken up by uninfected bystander DC, and the bystander cells could then present viral antigens to virus-specific T cells. This has already been described for influenza virus-infected apoptotic cells (Albert *et al.* 1998b). These findings clearly demonstrate the dual role of DC during viral infections. On one hand, they are able to induce a specific immune response, while on the other hand, they contribute to pathogenic effects and immune suppression.

There is increasing clinical evidence in humans that natural infection with most viruses is deleterious to the host, not only because of substantial tissue destruction affecting the efficacy of the immune response, but also because some viruses escape the immune response by various strategies, or induce immunosuppression (Kaiserlian and Dubois 2001). The induction of immune suppression is potentially advantageous for the virus, being one of the mechanisms by which viruses may escape clearance and establish a persistent infection *in vivo*. Examples of virus infection in humans associated with clinically important immune suppression include human immunodeficiency virus type 1 (HIV-1) infection, which results in AIDS, an expanding pandemic that has caused almost 2 million deaths in the last decade (Merson 1993), and measles virus infection, which results in the death of more than 1 million children per year (Borrow and Oldstone 1995). Despite its clinical importance, the pathogenesis of virus-induced immunosuppression is poorly understood.

Measles virus mediates immunosuppression at the level of the antigen presenting cell by a variety of mechanisms, including down-regulation of IL-12 production and delayed-type hypersensitivity response (Karp *et al.* 1996), (Marie *et al.* 2001). IL-12 is a critical cytokine in the differentiation of CD4+ type 0 cells to become CD4+ type 1 cells that produce the anti-viral cytokine IFN-γ and the potent T cell stimulatory cytokine IL-2 (Abbas *et al.* 1996). Measles virus has also been shown to inhibit DC maturation, and thus the ability of DC to present antigen to T lymphocytes. Measles virus infection can also induce T lymphocyte apoptosis, further impairing the immune response (Engelmayer *et al.* 1999), (Fugier-Vivier *et al.* 1997). These results may explain why measles virus infections are often accompanied by a dramatic suppression of the immune system. Other viruses, such as dengue virus can also infect DC and evoke a variety of influences on antigen processing and presentation (Wu *et al.* 2000).

The dual role of DC has also been reported following HIV-1 infections. In this case, DC not only elicit virus-specific CTL responses but also transport the virus from the periphery to the T cells in the lymph nodes, where HIV-1 replicates (Geijtenbeek *et al.* 2000). These reports all clearly demonstrate that DC are important targets in virus struggle for survival against the host immune response. Productive infection of DC requires activation signals provided by activated or memory T cells. DC constitute a viral reservoir and can efficiently pass the virus to T cells during the cellular interactions required for antigen presentation, resulting in apoptosis of both DC and T cells (Cameron *et al.* 1992). Thus, virus replication in DC may be induced by interaction with T cells after migration to lymph nodes, but can also take place in mucosa enriched in activated T cells under normal or inflammatory conditions. Contact with activated T cells could enhance virus replication in DC, possibly through CD40 engagement as shown for Measles virus or in the case of HIV, through co-expression of transcription factors such as NF-κB and Sp1 within DC/T cell syncytia, allowing virus transcription and replication (Servet-Delprat *et al.* 2000), (Granelli-Piperno *et al.* 2000b).

DC can also represent latent reservoirs for viruses responsible for chronic infections, including cytomegalovirus (Hahn *et al.* 1998), hepatitis C virus (which can induce hepatocarcinoma) (Bain *et al.* 2001) and human herpes virus 8 (responsible for Kaposi sarcoma) (Rettig *et al.* 1997). In this respect, recent studies in chronic hepatitis C infected patients reported that DC derived *in vitro* from patient's monocytes had a normal phenotype and maturation capacity but impaired allostimulatory capacity, which could be restored by exogenous IL-2 and IL-12 (Kanto *et al.* 1999). Thus, in contrast to measles virus infection, DC infected with hepatitis C virus could not induce active suppression of mixed lymphocyte reaction, which is compatible with the fact that generalised immunosuppression is not a clinical feature of hepatitis C infection. A defect in antigen presenting function and/or in production of cytokines by DC could account for the poor anti-hepatitis C immune response, leading to viral persistence (Kaiserlian and Dubois 2001).

The evasive functions of four HSV-1 proteins have been demonstrated so far, although none of these have been shown to affect DC function. HSV-1 expresses an immediate early protein, infected cell protein, ICP47 (encoded by the US12 gene) which blocks presentation of viral peptides to MHC-class I restricted cells, thus blocking CD8+ T cell response (Hill *et al.* 1995). ICP47 binds to a transporter associated with antigen processing (TAP) and prevents peptide translocation into the endoplasmic reticulum. Thus, ICP47 enhances HSV-1 virulence and helps the virus escape from immune clearance (Goldsmith *et al.* 1998). A complex of glycoprotein E (gE) encoded by the US8 gene and gI encoded by the US7 gene forms an Fc receptor on the surface of infected cells and virions. This Fc receptor forms antibody polar bridges resulting in the escape from neutralisation of virions and infected cells by complement and antibody-dependent killer cells (Para et al. 1982). Also, complement activity is suppressed by gC (encoded by UL44), which is a C3b receptor (Friedman *et al.* 1984). Furthermore, the vhs protein has been suggested to be a fifth protein mediating evasion of host defence mechanisms, although this was demonstrated to affect non-specific defence mechanisms (Suzutani *et al.* 2000).

Viruses have adapted to exploit the flexibility of DC function with diverse escape mechanisms that are just beginning to be explored. Continued work on fundamental aspects of the virus interference with DC function is necessary in order to develop therapeutic strategies to counteract these evasive mechanisms. Identification of virus receptors on DC and mechanisms of receptor-mediated signalling, as well as how viruses modulate antigen presentation is necessary to understand better viral pathology and viral escape mechanisms. This will be important for developing preventative vaccines targeting the DC and for development of viral vectors engineered to express tumor antigens for DC-based immunotherapy. To minimize anti-vector responses and to boost therapeutic antitumor reactions, it might be advantageous to combine different vector systems expressing the same tumor associated antigen in sequential immunisation protocols or boost with DC loaded with peptide or whole tumor cell preparations for antigen presentation (Jenne et al 2001).

In order to use HSV-1 as a gene delivery vector for human DC for potential use in immunotherapy several areas of study and development are required:

- Reduction of toxicity of the virus. Only essentially wild-type vectors have been tested so far which may cause toxicity
- Definition of parameters that allow safe and efficient gene delivery to DC
- Definition of the mechanisms that interfere with DC functioning.

I.6. Aim

The aim of this thesis is to define the optimal HSV-1 based vector for transduction of DC *in vitro* for use in cancer immunotherapy.

The first part of the project involved the optimisation of gene delivery to DC *in vitro* using a panel of HSV-1 vectors. For this purpose, gene delivery and cell survival were monitored, as well as viral growth and the expression of several IE genes in DC. Based on these results, selected vectors were further studied for their

effect on the phenotype, activation state and functioning of DC. The phenotype and activation of DC was monitored following transduction with wild-type vectors and replication incompetent vectors with or without the virion host shutoff (vhs) protein inactivated. Furthermore, the T-cell stimulatory capacity of transduced DC was studied. As a model to study antigen presentation to T cells, hepatitis B surface antigen (HBsAg) encoding vectors were constructed and used to transduce DC isolated from hepatitis B virus (HBV) vaccinated or unvaccinated healthy donors. After identification of the best HSV-1 vector, a vector encoding the human epithelial tumor associated antigen mucin-1 (MUC-1) was also constructed for potential application in breast/ovarian cancer immunotherapy using DC.

CHAPTER II

MATERIALS AND METHODS

II.1. Materials

II.1.1. Suppliers

- General chemicals : **Merck Ltd.** (Poole, Dorset, UK),
  Boehringer Mannheim (Lewes, East Sussex, UK)
  or **Sigma chemical Company Ltd.** (Poole, Dorset, UK)
- General disposable plasticware : **Greiner** (Stonehouse, Gloucester, UK)
  or **Sterilin** (Stone, Staffordshire, UK)
- All tissue culture plasticware : **Nunc** (Roskilde, Denmark)
- All restriction and modifying enzymes and buffers : **Promega Corporation** (Madison, Wisconsin, USA)
- 4-chloro, 5-bromo, 3-indolyl-b-galactosidase (X-gal) : **Insight Biotechnology Ltd.**
- α-^{32}P-dCTP (3000 Ci/mM), Rainbow^{TM} coloured protein molecular weight markers, Hybond^{TM}-C and Hybond^{TM}-N membranes : **Amersham International plc.** (Little Chalfont, Bucks)
- Qiagen 'midi-prep' plasmid DNA extraction kits : **Qiagen** (Chatsworth, USA)
- Ammonium persulphate, N, N'-methylene-bis-acrylamide, N,N,N',N'-tetra methylethylene-diamine (TEMED) : **Bio-Rad** (Hemel Hempstead, Herts)
- Fluorescein or peroxidase conjugated secondary antibodies : **Dako Ltd.** (High Wycombe, Bucks)
- Bacto-agar, Bacto-tryptone,Yeast extract : **Difco Laboratories** (Basingstoke, Hants)
- 1kb DNA ladder : **Gibco-BRL Life Technologies** (Renfrewshire, Scotland, UK)
- 3MM chromatography paper : **Whatman International Ltd.** (Maidstone,Kent, UK)
- Disposable 0.45 and 0.2 μm filters : **Gelman Life Sciences** (Ann Arbor, Michigan, USA)
- Hexanucleotides [pd(N)6] for random prime labelling, dNTPs : **Pharmacia Biotechnology Ltd** (St. Albans, UK)
- Kodak X-OMAT imaging photographic film, Kodak Professional 64T colour film : **Sigma Chemical Company Ltd.** (Poole, Dorset, UK)
- Zeocin^{TM} (phleomycin derivative) : Cayla (Toulouse, France)

II.1.2. Standard buffers and solutions

The following buffers and solutions used are all expressed at 1x concentration:

PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM disodium orthophosphate dihydrate Na2HPO4.7H2O, 1.4 mM potassium dihydrogen orthophosphate KH2PO4, pH 7

TE: 10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0

TAE: 400 mM Tris base, 200 mM sodium acetate, 20 mM EDTA pH 8.3

TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0

SSC: 150 mM NaCl, 15 mM sodium citrate

Luria Bertani media (LB): 1% (w/v) Bacto^{R}-tryptone, 1% (w/v) NaCl, 0.5% Bacto$^{®}$-yeast extract; autoclaved at 120 C

Chloroform/Isoamylalcohol (IAA): 96% (v/v) chloroform, 4% (v/v) IAA

Tris equilibrated phenol: phenol was equilibrated twice with excess 0.1 M Tris-HCl pH 8.0 removing the aqueous layer each time

II.1.3. Plasmids

Name	Description	Source/Reference
pSP72	Cloning vector	Promega Corporation, Madison, Wisconsin, USA
Pbluescript	Expression vector containing the β-actin gene	Stratagene Ltd., Cambridge, UK
PcDNA3	Expression vector containing the *GFP* gene	Invitrogen Corporation, Carlsbad, California, USA

pCH110	Eukaryotic assay vector containing the *LacZ* gene	Pharmacia Biotechnology Ltd, St.Albans, Herts, UK
pHBV130	Expression vector containing the HBsAg	Kenneth Murray, University of Edinburgh
pBS-PEM	pBluescript containing the PEM transcriptional start, MUC-1, polyadenylation signal	Ian McFarlane, ICRF Immunotherapy lab, Guy's Hospital, London, UK

Table II.1.: Description and sources of plasmids

II.1.4. Antibodies

Antibody	Dilution	Detection	Source
Anti-HSV-1 ICP0	1:1000 Western blot	1:1000 dilution of HRP conjugated anti-mouse Ig	Autogen Bioclear, Caine, Wilts, UK
Anti-HSV-1 ICP22	1:500 Western blot	1:1000 dilution of HRP-conjugated anti-rabbit Ig	Bernard Roizman, University of Chicago, Illinois
Anti-HSV-1 ICP47	1:500 Western blot	1:1000 dilution of FITC-conjugated anti-rabbit Ig	David Johnson, Oregon Health Sciences University, Portland, Oregon
Anti-GFP	1:1000 Western blot	1:1000 dilution of HRP-conjugated anti-mouse Ig	Quantum Biotechnologies inc.
Anti-HBV HbsAg	1:100 Western blot	1:1000 dilution of HRP-conjugated anti-mouse Ig	Samreen T, Virology dept., Windeyer Institute, London

Anti-Muc-1 (PEM)	1:300 Western blot	1:1000 dilution of HRP-conjugated anti-rabbit Ig	Dr. Jonathan Lederman, Oncology dept., UCL Medical school, London
Anti-CD1a (NA1/34)	Neat FACS staining	Supernatant mouse monoclonal Ab	Dr. Peter Lydyard, Immunology dept., UCL
Anti-CD2	1:20 DC Depletion	Mouse monoclonal Ab	Harlan – Sera Lab
Anti-CD3	1:20 DC depletion	Mouse monoclonal Ab	Harlan – Sera Lab
Anti-CD4 (Q243)	1:25 FACS staining	Supernatant mouse monoclonal Ab	Immunology dept., UCL
Anti-CD8 (UCHT4)	1:25 FACS staining	Supernatant mouse monoclonal Ab	Immunology dept., UCL
Anti-CD14 (HB246)	Neat FACS staining	Sheep anti-mouse IgG1	Gift of Prof. PCL Beverley to Immunology dept, UCL
Anti-CD19 (BU12)	Neat DC depletion	Sheep anti-mouse IgG1	Gift of Dr. Hardie to Immunology dept., UCL
Anti-CD40	Neat FACS analysis	PE-conjugated Anti-mouse IgG1	Dr. Pippa Newton, Immunology dept., UCL - Immunotech
Anti-CD83	Neat FACS analysis	PE-conjugated Anti-mouse IgG1	Dr. Pippa Newton, Immunology dept., UCL - Immunotech
Anti-CD86	Neat FACS analysis	PE-conjugated anti-mouse IgG2	Dr. Pippa Newton, Immunology dept., UCL - Immunotech
Anti-MHC Class I (Serotec)	1:200 FACS staining	PE-conjugated Anti-mouse IgG1	Immunology dept, UCL

Anti-HLA-DQ (LNDQ)	5 µg/ml FACS staining	PE-conjugated anti-mouse IgG1	Gift of Prof. R. Winchester to Immunology dept., UCL
Anti-HLA-DR (L243)	Neat FACS staining DC depletion	PE-conjugated anti-mouse IgG1	Gift of Prof. PCL. Beverley to Immunology dept., UCL
Anti-mouse IgG1 (FITC)	1:20 FACS staining	PE-conjugated rabbit anti-mouse IgG1	Dako, Glostrup, Denmark

Table II.2: Antibodies (Ab) used in western blot, dendritic cell preparation and FACS staining. For DC and T cell depletion, cells were incubated with the appropriate Ab followed by anti-mouse IgG coated Dynabeads (Dynal, Merseyside, UK) were used.

II.1.5. Proteins and antigens

Tuberculin purified protein derivative (PPD) recombinant:

Source: Evans Medical Limited, Leatherhead, KT22 7PQ, UK

Hepatitis B surface antigen (HBsAg) recombinant:

Source: Austral Biologicals distributed by Insight Biotechnology Limited, PO box 520 Wembley, Middlesex, HA9 7YN, UK

Proteasome inhibitors:

Z-Leu-Leu-Leu-H (aldehyde)

MG132 – Carbobenzoxy-L-Leucyl-L-Leucyl-L-Leucinal

Source: Peptides International, Inc., Louisville, Kentucky 40224, USA

Lipopolysaccharide (LPS):

Source: Sigma Chemical Company Ltd., Poole, Dorset, UK

II.1.6. Bacterial strains

Escherischia Coli XL-1 Blue:

Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'proAB LacI*^{q} *ZΔM15, Tn 10* (Tet)^{r}]

Source: Stratagene Ltd., Cambridge, UK

Escherischia Coli SCS110:

Genotype: *rpsL* (Str^{r}) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44Δ (lac-proAB) [F' traD36 proAB lacI*^{q} *ZΔM15]*

Source: Stratagene Ltd., Cambridge, UK

II.1.7. Cell lines: characteristics and growth conditions

BHK 21: Baby Hamster Kidney 21 clone 13 cells – permissive for HSV infection (Macpherson and Stoker, 1962) ATCC*CCL 10 .
BHK cells were grown in 1 x Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) Foetal Calf Serum, 5% (v/v) tryptose phosphate broth (TBP), 100 U/ml penicillin and 100 μg/ml streptomycin.

B130/2: BHK cell line stably transfected with pMAM neo and pSGB130 (ICP27 promoter, coding region and pA) (Howard *et al.* 1997).
B130/2 cells were grown in the same conditions as BHK cells with the addition of 800 μg/ml of Geneticin-G418 sulphate to the media to maintain selection.

27/4/M:4: BHK cells which complement mutations in ICP4, ICP27 and vmw65 (Thomas *et al.* 1999).
27/4/M:4 cells were grown in the same conditions as BHK cells with the addition of 800 μg/ml of Geneticin-G418 sulphate and 750 μg/ml of Zeocin to the media to maintain selection.

II.2. Molecular Biology

II.2.1. Bacterial growth conditions

II.2.1.1 Propagation of bacteria

Bacteria were grown in liquid LB or on plates from LB media containing 2% Bacto®-agar. Liquid cultures were grown onvernight at 37°C in an orbital shaker at 200 rpm. Bacterial plates were incubated overnight at 37°C in a standard incubator. Both media contained antibiotic selection. Ampicillin was used at a final concentration of 100 μg/ml and tetracyclin was used at a final concentration of 12.5 μg/ml in plates and 10 μg/ml in liquid culture. Zeocin was used at a final concentration of 20 μg/ml.

II.2.1.2 Transformation of bacteria

The method used for preparation and transformation of competent cells is based on the calcium chloride method described by Sambrook et al., 1989. A single bacterial colony was grown overnight in 10 ml of LB (without antibiotic). 100 ml of this culture was used to inoculate 100 ml of LB (without antibiotic) and the culture was grown to an OD_{580} of about 0.5 units. The bacteria were pelleted in 50 ml sterile tubes by centrifugation at 3000 rpm for 10 minutes. The supernatant was discarded and any excess LB was removed. The pellets were then resuspended in 10 ml of ice-cold 100 mM $CaCl_2$. The bacteria were pelleted again and resuspended in 4 ml of ice-cold $CaCl_2$. The competent cells were then incubated on ice for at least 30 minutes and used within the next 72 hours.

Competent cells were transformed by addition of DNA and further 30 minute incubation on ice. The cells were then heat shocked at 42°C for 90 seconds and then left on ice for another 2 minutes. 800 μl of LB was added and the cells incubated in an orbital shaker for 1 hour at 37°C/200 rpm. The cells were then pelleted,

resuspended in about 100 µl of LB and plated onto LB agar plates (with the appropriate antibiotic selection).

II.2.2. DNA isolation and analysis

II.2.2.1. Small scale 'mini-prep' plasmid DNA extraction from transformed bacteria

This method of extraction and precipitation of recombinant DNA from *E.coli* transformants is based on the alkaline lysis method described by (Birnboim and Doly 1979). Individual bacterial colonies were used to inoculate 3 ml of LB containing the appropriate antibiotic selection. Cultures were incubated overnight at 37°C/200 rpm. The cells from 1.5 ml of culture were pelleted by centrifugation in a bench top centrifuge at 1300 rpm for 2 minutes and the supernatant was removed by aspiration. The pellet was resuspended in 100 µl of resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8, 100 µg/ml Rnase-A). Bacteria were then lysed by addition of 200 µl of lysis buffer (200 mM NaOH, 1% (v/v) Triton X-100) and neutralised by the addition of 150 µl of neutralisation buffer (3 M sodium acetate pH 5.5). The cell lysate was centrifuged for 3 minutes at 13000 rpm and the pelleted precipitate was removed and discarded. 500 µl of isopropanol was then added to the supernatant which was vortexed and centrifuged for 5 minutes at 1300 rpm in order to pellet the DNA. The supernatant was removed and the DNA was washed with 500 µl of 70% ethanol, dried under vacuum and resuspended in 100 µl of double distilled water (ddH_20) containing 20 mg/ml RNase A. Plasmid DNA was then stored at 20°C.

II.2.2.2. Large scale 'midi-prep' plasmid DNA extraction from transformed bacteria

For extraction and precipitation of large scale plasmid DNA, a single colony from bacterial plate (or 50 µl of bacterial culture) was used to inoculate 400 ml of LB containing the appropriate antibiotic selection. This was then incubated at 37°C/200

rpm overnight. 100 ml of this culture was then centrifuged at 3000 rpm for 10 minutes. Plasmid DNA was then extracted using the Qiagen 'midi-prep' kit. The subsequent dried, purified DNA was resuspended to approximately 100 ml of ddH_2O and the average yield using this method was 100 μg.

II.2.2.3. Restriction enzyme digestion

Small scale digests were carried out to characterise the structure of an isolated plasmid. Analytical digests were usually performed in 20 μl containing either 2 μl of mini-prep DNA or 1 ml of midi-prep DNA (about 1 μg DNA). The amount of enzyme did not exceed 0.1 volumes and the appropriate enzyme buffer was used at 1x concentration as specified by the manufacturer. The digested DNA was electrophoresed on a 1% agarose gel (see section II.2.2.7) and bands were visualised on a UV transilluminator.

For large scale digests, restriction digests were carried out in a total volume of 100 ml containing approximately 5 μg of midi-prep DNA and not more than 0.1 volumes of restriction enzyme(s). The enzyme buffer was used at 1x concentration. Digests were incubated for 1-16 hours at the appropriate temperature.

II.2.2.4. Blunt ending reactions

If there were no compatible sticky ends for cloning, restriction enzyme cleaved overhangs were filled (at the 5' end) or blunted (at the 3' end) using T4 DNA polymerase. After restriction digest, 1 μl of a 25 mM stock of dNTPs (dATP, dCTP, dTTP, dGTP) and 15 units of T4 DNA polymerase were added to the reaction. The reaction was then incubated for 1 hour at 37°C. For subsequent manipulation of DNA, the reaction was heat inactivated at 80°C for 20 minutes and then cooled on ice for 5 minutes prior to the addition of enzymes.

II.2.2.5. Phosphatase treatment of plasmid DNA

To prevent religation of compatible ends of vector during insert/vector ligation, the vector DNA was treated with calf intestinal alkaline phosphatase (CIAP). Restriction enzyme/blunt ending reactions were made up to 400 µl with a final concentration of 1x alkaline phosphatase buffer, 10 units of CIAP and ddH_20. The reaction was incubated at 37°C for no longer than 30 minutes and the CIP was inactivated by incubation for 20 minutes at 65°C.

II.2.2.6. Phenol/chloroform extraction and precipitation of DNA

To purify DNA after a restriction enzyme or blunt ending reaction, the reaction mix was made up to 400 ml with ddH_20, and the same volume of tris-equilibrated phenol was added. The mixture was vortexed, centrifuged at 13000 rpm for 2 minutes and the aqueous phase removed and re-extracted with one volume of chloroform/IAA. The aqueous phase was removed and the DNA precipated by addition of 0.1 volumes of 3 M sodium acetate pH 5.5 and 2 volumes of 100% ethanol (previously chilled at –20°C).

II.2.2.7 Agarose gel electrophoresis

Agarose (Biometra) was melted and dissolved at 1% (w/v) in 1x TAE gels in a microwave. Ethidium bromide was added to a final concentration of 0.5 µg/ml. The melted agarose was poured into a gel caster and left to set with a comb. The DNA marker e.g. 1 kilobase (kb) DNA ladder and samples containing 1x loading buffer (10x stock: 1x TAE, 50% v/v glycerol, 0.025% bromophenol blue) were loaded. DNA was electrophoresed at approximately 100 V at room temperature until the DNA fragments were seperated. Bands were then visualised on a UV transilluminator and photographed onto polaroid film.

Low melting point gels for DNA fragment isolation and excision straight from the gel were performed as above using low melting point agarose (Biometra). Gels were run at 50 V until the desired fragment of DNA was separated from other fragments. The band was then quickly excised, removing all excess agarose on a UV transilluminator and stored at –20°C.

II.2.2.8. Ligations of DNA

Ligations were carried out in a total volume of 30 µl. Gel fragments were melted at 80°C for 5 minutes and added straight to the ligation without any further purification step. Reactions contained 5 ml of each gel fragment, 1x ligase buffer and 1-3 units of T4 DNA ligase in ddH_20. The reaction was left at room temperature for 2 hours, then heated to 80°C for 5 minutes and an equal volume of ddH_20 was added. The reaction mix was then transformed into competent cells (see section II.2.1.2).

II.2.2.9. DNA analysis by southern blot

Southern blots (Southern, 1975) were performed on viral DNA to confirm genome structures of recombinant HSV-1 vectors constructed. 10 µl of a viral DNA preparation was digested overnight with the appropriate enzymes and buffers in a total volume of 50 µl. 0.1 µg of plasmid DNA was also digested in a total volume of 20 µl and used as a control. The digest reactions were then loaded and electrophoresed on a 1% agarose gel (see section II.2.2.7).

The DNA was visualised on a UV transilluminator and photographed against a fluorescent ruler. The gel was left on the transilluminator for 2 minutes to nick the DNA afterwhich it was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 45 minutes. The gel was then transferred to the neutralising solution (2 M NaCl, 1 M Tris pH 5.5) for 45 minutes. The gel was then placed upside-down on a plastic support which was previously layered with 3 MM Whatman paper and placed in a recipient filled with 20x SSC. A piece of Hybond N nylon membrane pre-soaked in

the neutralising solution was then carefully cut and placed on top of the gel. 10 pieces of 3 MM Whatman paper pre-soaked in 20 x SSC 3 MM were also placed on the nylon membrane followed by some dry paper towels. The DNA was then transferred overnight by capillary action onto the nylon membrane. The next day, the membrane was removed carefully, washed in 6 x SSC and dried for 30 minutes at room temperature. The DNA was then cross-linked to the membrane using a UV Stratalinker 2400.

II.2.2.10. Radiolabelling of DNA

The method used for radiolabelling of DNA was based on the random labelling reaction described by Feinberg and Vogelstein, 1983. 1 μg of DNA (for use as the probe) was digested (see section II.2.2.3) and run on a low melting point gel (see section II.2.2.7). The required DNA fragment was then excised from the gel and 3 volumes of ddH_20 were added. The gel slice was heated to 100°C for 5 minutes cooled by placing directly on ice for 2 minutes. 7 ml of melted gel slice was then added to 10 μl of oligolabelling buffer*, 5 units of DNA polymerase large fragment (Klenow) and 50 μCi of α-^{32}P-dCTP in a total volume of 50 μl. The reaction was then incubated at 37°C for 1-2 hours (optimum enzyme temperature). The probe was filtered through a G50 Sephadex column in order to remove any unincorporated radioactivity, heated for 5 minutes at 100°C and then cooled immediately on ice for 2 minutes. The denatured labelled probe was then added to the hybridisation solution.

*Oligolabelling buffer composition was made by mixing the solutions A, B and C described below in a ratio of 100:250:150.

- Solution O: 1.25 M Tris HCl pH 8.0; 0.125 M $MgCl_2$
- Solution A: 1 ml solution O; 18 μl β-mercaptoethanol; 5 μl 0.1 M dATP; 5 μl 0.1 M dGTP; 5 μl 0.1 M dTTP
- Solution B: 2 M HEPES pH 6.6
- Solution C: 90 units/ml random hexamers [$pd(N)_6$] dissolved in TE pH 8.0

II.2.2.11. Hybridisation

For DNA blots, the membranes were pre-hybridised for 1 hour at 65°C in 5x SSC, 5x Denhardt's reagent*, 0.5% (w/v) SDS in ddH_20 containing 100 μg/ml of denatured (97°C for 5-10 minutes, snap cooled on ice) herring sperm DNA. The volume of the pre-hybridisation solution was reduced to approximately 5 ml prior to adding the denatured (97°C for 5-10 minutes, snap cooled on ice) probe. The probe was hybridised to the membrane at 65°C overnight. The hybridisation solution was discarded and the membranes were washed twice for 10 minutes in 2x SSC/0.1% w/v SDS. The membranes were wrapped in cling film and exposed to X-ray film for 4h-overnight at -70°C. The film was subsequently developed.

For RNA blots, the membranes were pre-hybridised for 2 hours at 42°C in 5x SSPE, 50% (w/v) deionised formamide, 5x Denhardt's reagent, 0.5% (w/v) SDS and 100 mg/ml of denatured salmon sperm DNA and made up to desired volume with DEPC-treated ddH_20. The denatured probe was then added to about 5 ml of prehybridisation solution and the membrane was incubated overnight at 42°C. The hybridisation solution was then discarded. The membranes were first washed for 30 minutes at 65°C in 3x SSC/0.1% (w/v) SDS and for a further 30 minutes in 1x SSC/0.1% (w/v) SDS. The membranes were wrapped in cling film and exposed to an X-ray film overnight in an autoradiography cassette at –70°C. The film was subsequently developed. Slot blot filters were wrapped in cling film, incubated for 4h-overnight against a ^{32}P imaging screen and the detected bands quantified using a BioRad GS-250 Molecular Imager™.

*The 5x Denhardt's solution was prepared from a 100x Denhardt's stock reagent composed of: 2% w/v bovine serum albumin, 2% Ficoll® (type 400), 2% w/v polyvinylpyrrolidone in ddH_20 or DEPC-treated ddH_20.

II.2.3. RNA isolation and analysis

Solutions used for the isolation of RNA were made up in diethylpyrocarbonate (DEPC)-treated ddH_20 and autoclaved prior to use in order to inactivate contaminating RNases.

II.2.3.1. RNA isolation

The method for the isolation of RNA was based on the protocol described by (Chomczynski and Sacchi 1987). For the isolation of RNA cells in suspension (from dendritic cells), 1 x 10^6 cells were centrifuged at 12000 g for 10 minutes at 4°C and cells were resuspended in 1 ml of Tri reagent (guanidine thiocyanate - Sigma) by gentle pipetting. For isolation of RNA from a monolayer of cells (BHK cells), 1 ml of Tri reagent was directly added to the cells and the mix was transferred to a fresh tube. Samples were allowed to stand for 5 minutes at room temperature in order to ensure complete dissociation of nucleoprotein complexes. 200 µl of chloroform was then added and the mix was shaken vigorously for 15 seconds. Samples were then left at room temperature for 2-15 minutes and centrifuged at 12000 g for 15 minutes at 4°C. Centrifugation separates the mixture in three phases: a red organic phase (containing the protein), an inter-phase (containing DNA) and an upper aqueous phase (containing the RNA). The aqueous phase was transferred to a fresh tube and 500 µl of isopropanol were added and mixed together by inversion. Samples were allowed to stand for 5-10 minutes at room temperature and then centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. The sample was vortexed and then centrifuged at 7500 g for 5 minutes at 4°C. The RNA pellet was dried for 5-10 minutes under a vacuum and resuspended in 100 µl of DEPC-treated water. Samples were stored at –70°C until required for use.

II.2.3.2. Quantitation of RNA

10 µl of sample was diluted 50 times in DEPC treated ddH_20 and absorbance was measured at A_{260} and A_{280} on a spectrophotometer. Ratio of A_{260}:A_{280} should be 2:1 for a pure sample of RNA. Total RNA concentration was calculated in µg/ml:

$$[RNA] = A_{260} \times 40 \times 50 \times (90/1000)$$

where:

- absorbance at wavelength 260 nm = A_{260}
- 1 unit optical density = 40 µg/ml RNA
- dilution factor = 50
- volume of the remaining sample of RNA = 90/1000 µl

II.2.3.3. RNA analysis by slot blot

RNA samples from II.2.3. were centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was discarded and the pellets resuspended in DEPC treated ddH_20 to a final concentration of 3.2 µg/µl (32 µg). 10 µl was added to 190 µl DEPC treated ddH_20 in a 1.5 ml microfuge tube and mixed by gentle pipetting. 400 µl of slot buffer (7.5 x SSC, 25% (v/v) formaldehyde, made up with DEPC treated ddH_20) were added to each sample and vortexed. The samples were then denatured for 10 minutes at 65°C and snap cooled on ice. Hybond-N membrane was cut to the size of slot apparatus and soaked in ddH_2O followed by 3 x SSC. The apparatus was assembled, 240 µl of each sample was loaded into each slot and a gentle vacuum was applied until the solution had passed through. RNA was cross-linked using a UV Stratlinker 2400. Membrane was wrapped in cling film and stored at 4°C for subsequent hybridisation (see II.2.2.11.).

II.2.4. Protein isolation and analysis

II.2.4.1. Protein extraction

1 x 10^6 cells (monolayer – BHK or BHK-based cells) were washed in 1 ml of 1x PBS and harvested in 1.5 ml eppendorf tubes. After removal of the supernatant, the pellet was thoroughly resuspended in 100 µl of standard protein sample buffer ice-cold [5% b-mercaptoethanol, 50 mM Tris-HCl pH 8.0, 6% (v/v) glycerol, 2% (w/v) SDS and 0.005% (w/v) bromophenol blue]. For cells in suspension (dendritic cells), cells were first spun for 2 minutes in a microfuge, the supernatant discarded and the pellet resuspended in 100 µl protein sample buffer. The samples were immediately placed on ice and then heated at 100°C for 5 minutes. The samples were either run directly on a SDS-polyacrylamide gel (II.2.4.2.) or stored at –20°C.

II.2.4.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The resolving gel was poured and immediately layered with isopropanol. After the gel had set, the isopropanol was removed, the upper part of the gel dried and the stacking gel was added to form the upper layer. An suitable comb was added prior to the polymerisation of the stacking gel. The composition of the resolving and stacking gel buffers is described in table II.3.

Total protein samples were separated into polypeptide units by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli, 1970. Protein samples were denatured at 100°C for 5 minutes immediately before loading on to the gel. 10 µl of sample or 3 µl of coloured molecular weight protein marker (Rainbow markers) were loaded. SDS-polyacrylamide gels were prepared in a vertical gel electrophoresis system and run in 1x running buffer [25 mM Tris, 250 mM glycine and 0.1% (w/v) SDS, pH 8.3] at 40 mA/gel for 4-6 hours (or until the dye reached the bottom of the plate).

Gel type / composition	10% Resolving gel (25 ml)	12% Resolving gel (25 ml)	15% Resolving gel (25 ml)	5% Stacking gel (10 ml)
Acrylamide[1]	8.3 ml	10 ml	12.5 ml	1.7 ml
Buffer[2]	6.3 ml	6.3	6.3 ml	1.25 ml
10% APS[3]	250 µl	250 µl	250 µl	100 µl
TEMED[4]	10 µl	10 µl	10 µl	10 µl
ddH_20 to	9.9 ml	8.2 ml	5.7 ml	6.8 ml

Table II.3: Composition of resolving and stacking gels for SDS-PAGE. [1]Acrylamide bis = N,N'-Methylene-bis-acrylamide. [2]Resolving gel buffer (100 ml) was composed of 18.15 g Tris, 0.4 g SDS, pH 8.9 and Stacking gel buffer (100 ml) was composed of 5.1 g Tris, 0.4 g SDS, pH 6.7. [3]APS = ammonium persulphate. [4]TEMED = NNNN-tetraethylethalinediamine.

II.2.4.3. Equalisation of protein loading

To equalise protein loading in each lane, duplicate protein samples were separated by SDS-PAGE (II.2.4.2) and one of the gel was placed in Coomassie stain solution [2% (w/v) Coomassie brilliant blue R250, 50% (w/v) methanol, 50% (v/v) glacial acetic acid] for 1 hour at room temperature with constant shaking. Any unbound stain was removed by repeated exposure of the gel to destain solution [10% (w/v) glacial acetic acid, 30% (v/v) methanol].

II.2.4.4. Transfer of protein to nitrocellulose by western blotting

Proteins separated on SDS-PAGE gels were transferred to Hybond C membranes using a wet-transfer method described by Towbin, 1979. The gel and the nitrocellulose membrane were pre-soaked in transfer buffer [50 mM Tris, 180 mM glycine, 0.1% (w/v) SDS and 20% methanol] and blotted overnight at 200 mA/4°C in a Trans-blot™ Cell (BioRad) according to the manufacturer's instructions. The

membrane was subsequently removed, dried and the Rainbow molecular weight marker highlighted.

II.2.4.5. Immunodetection of proteins on western blots

Membranes were blocked in blocking buffer [1x PBS, 5% (w/v) skimmed milk powder, 0.1% polyoxyethylene sorbitan monolaurate – Tween 20] shaking gently in a platform for 1 hour at room temperature. The membrane was then incubated with primary antibody diluted in 3% (w/v) skimmed milk powder in 1x PBS shaking in a platform for 1-2 hours at room temperature (see table II.2 for details of primary antibodies used). Unbound antibody was rinsed off by washing the membrane three times for 10 minutes in wash buffer [1x PBS, 0.1% Tween 20] at room temperature with constant shaking. The membrane was then incubated in an appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody diluted to the required concentration in 3% block buffer at room temperature with constant shaking (see table II.2 for details of secondary antibodies used). Unbound secondary antibody was removed by washing the membrane three times for 10 minutes in wash buffer at room temperature with on a shaking platform. The bound horseradish peroxidase was then detected using enhanced chemiluminescence (ECL™, Amersham International Plc, Little Chalfont, Bucks, UK) and the resultant light emissions exposed to X-ray film for 5 seconds-1 hour, depending on the strength of the signal.

II.3. Cell Biology

Cell lines were stored for long term in liquid nitrogen and were maintained at 37°C while in culture in either a 5% CO_2 incubator in a humidified atmosphere or on a rotary roller apparatus (for roller bottles). All manipulations of cells were carried out under sterile conditions using standard aseptic techniques.

II.3.1. Growth conditions and storage of cell lines

For long term storage, cells from one 175 cm^2 flask were suspended in 1.8 ml of 8% (v/v) dimethylsulphoxide (DMSO), 30% FCS, 68% appropriate medium (see section II.1.8). The freezing vials were slowly cooled to –70°C and then immersed in liquid nitrogen. For recovery, cells from one vial were rapidly thawed and transferred into a 25 cm^2 flask containing the appropriate selection medium. Cells were passaged the next day.

Cells were grown in 175 cm^2 flasks, 500 cm^2 plates or 800 cm^2 roller bottles in the appropriate growth medium (see section II.1.8) . All cell lines were passaged when they were 80-90% confluent. Cells were washed in Hanks Balanced Salt Solution (HBSS) at room temperature, detached from the surface of the flask with trypsin/versene (1:10) at room temperature and disaggregated. The reaction was stopped by adding growth media and cells were aliquoted in 1:10 ratio in clean flasks containing growth media.

II.3.2. Transfection of plasmids for transient expression

BHK or BHK-based cells were transfected with HSV-1 DNA by a standard calcium phosphate transfection method based on the protocol described by Stow and Wilkie, 1976. 5-10 μg supercoiled plasmid DNA was transiently transfected per well of a 6-well plate.

- HEBES transfection buffer: 140 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 5.5 mM D-glucose; 20 mM Hepes; pH 7.05 with NaOH; buffer was filter sterilised with a 0.2 μm filter and stored at 4°C.
- BHK or BHK-based cells were grown until they were at 70% confluency
- Two tubes were set up in 1.5 ml sterile microfuge tubes:

Tube A: 31 ml 2 M CaCl2

10 mg plasmid DNA

20 mg herring sperm DNA (phenol/chloroform extracted)

Tube B: 400 µl HEBES transfection buffer

The content of tube A was mixed and then added dropwise to tube B while it was constantly vortexed. The mixture was then left for 20-40 minutes at room temperature to allow the DNA to precipitate. After removing media from the cells, the DNA mixture was slowly added. The cells were then incubated for 20-40 minutes at 37°C. 1 ml of growth media (without selection) was added onto the cells and the plate incubated for 4 hours at 37°C. The media was then removed and cells were washed twice with 2 ml of growth media. The cells were then shocked with 1 ml of 25% (v/v) DMSO in HEBES transfection buffer which was left on the cells for no more than 2.5 minutes. The cells were immediately washed twice in 2 ml growth media/well. 2 ml of growth media was added to each well and the cells incubated at 37°C/5% CO_2.

II.3.3. Virus construction

II.3.3.1. Homologous recombination into the HSV-1 genome

Transfections were carried out as described in II.3.2. except 10-30 µg of purified viral DNA (II.3.4.3) was added to tube A and tubes were mixed more gently at each step. The DNA precipitate was left on cells for 7 hours prior to the DMSO shock. For transfections using viral DNA from HSV-1 mutants with an inactivated vmw65, the media was supplemented with 3 mM hexamethylene bisacetamide (HMBA) in order to induce immediate early gene transcription (McFarlane *et al.* 1992). Homologous recombination were left for 3-5 days until complete cytophathic effect (CPE) was observed. The well was then harvested and freeze-thawed. The cells

were then titred and the efficiency of transfection assessed by the presence or absence or cells expressing a reporter gene.

II.3.3.2. Viral infectivity (plaque) assay

BHK or BHK-based cells were grown in a 6-well dish at 37°C/5% CO_2 until 80% confluent. Serial 1:10 dilutions of virus starting from $1x10^{-3}$ to $1x10^{-8}$ pfu were prepared in DMEM without FCS (serum free DMEM media, SFM) and added to 500 µl of SFM previously layered on the cells. After 1 hour incubation at 37°C/5% CO_2, 2ml of 1:2 (v/v) of 1.6% (w/v) carboxymethyl cellulose: growth media were added to each well (with 3 mM HMBA if necessary). Cells were then incubated for 48 hours at 37°C/5% CO_2 and the number of plaques in each well were counted in order to determine the virus titer in plaque forming units per ml (pfu/ml).

II.3.3.3. Growth Curves

Dendritic cells (DC) were transduced with various HSV-1 mutants at an MOI of 0.1, 0.5, 1, 5 and 10 as described in II.3.7 and incubated in 24-well plates at 37°C/CO_2 for four days. Aliquots of transduced cells were then harvested at 0, 24, 48, 72 and 96 hours post-infection. Harvested samples were freeze-thawed in order to disrupt the cells and virus yield was assessed by viral infectivity assay (II.3.3.2.).

II.3.3.4. Purification of viral recombinants by plaque selection

Harvested wells from homologous recombination transfections were freeze-thawed to disrupt the cells and titred (see II.3.2.3.) in order to assess the efficiency of transfection. For visualisation of blue or white plaques resulting in the gain or loss of *LacZ*, then plaques were stained following the X-gal staining procedure without fixing cells (II.3.8) For visualisation of green or white plaques resulting in the gain or loss of green fluorescent protein *GFP*, then plaques were directly visualised under an inverted fluorescent microscope (II.3.8). The well was harvested by scraping and

freeze-thawed. 100 µl of the mixture was added to a monolayer of BHK or BHK-based cells and left for two days at 37°C/CO_2. Cells were then examined under microscopy for the appearance of any viral plaques indicating replication. Recombinants were identified as green, blue, white plaques and were picked from the monolayer in a minimum volume using a P20 Gilson micropipette. Plaques were then transferred to an eppendorf tube containing 100 µl of serum free media (SFM) and freeze-thawed. 10 µl and 90 µl of each plaque suspension was then used to infect a 6-well plate of BHK or BHK-based cells at 80% confluency. The virus was allowed to adsorb for 40-60 minutes at 37°C/CO_2. The monolayer of cells was then covered with 2 ml of 1:2 (v/v) mix of 1.6% (w/v) carboxymethyl cellulose: growth media without selection (if necessary, 3 mM HMBA was added). The plaque purification steps were repeated until a pure population of virus was obtained. Each well was then harvested and this initial stock was used to propagate virus at a larger scale.

II.3.4. Virus production

II.3.4.1. Production of high titre stock of recombinant virus

A 175 cm^2 tissue culture flask containing the appropriate cell line grown at 37°C/5% CO_2 until 80% confluent was infected with $1x10^5$ plaque forming units (pfu) of an initial stock of virus (from II.3.3.4). Infection occurred in 5 ml serum free DMEM Media for 1 hour after which viral replication was allowed by addition of 20 ml growth media. Cells were harvested 3-5 days later and stored at –70°C as an intermediate viral stock.

For large scale viral culture of wild-type and partially disabled vectors, 10x175 cm^2 flasks of 100% confluent cells were split into 10x850 cm^2 roller bottles (Corning Glass Works, Corning, New York, USA) and grown in 100 ml of growth media without selection at 37°C/5% CO_2. At 90% confluency, cells were infected at MOI of 0.01 with $1x10^6$ pfu of virus per RB (from the intermediate viral stock) and grown in fresh growth media without selection at 32°C/0.5 rpm for 3-5 days until

complete CPE was observed (supplemented with 3 mM HMBA if necessary). Cells were then harvested by vigorous shaking of the roller bottle and immediately frozen at –80°C. After defrosting, the cellular debris was removed by centrifugation at 3500 rpm for 45 minutes. The supernatant was then immediately removed, filtered through a 5 μm followed by a 0.45 μm filter and spun at 12000 rpm for 2 hours at 4°C in a GS12 rotor. The supernatant was discarded and the subsequent viral pellet was gently resuspended in DMEM without any supplements. The resuspension volume depended on the level of disablement of the virus (1-3 ml/10 roller bottle). The resuspended pellet was then sonicated for 5 times 10 seconds in a water bath sonicator and chilled on ice between each sonication. Virus was aliquoted and stored in liquid nitrogen. Virus stock was titred by the viral infectivity assay (II.3.3.2.).

For large scale viral culture of replication incompetent viruses (containing deletions in essential genes), 10 x 500 cm^2 plates were seeded with the appropriate complementing cell line in 50 ml of growth media without selection. At 90% confluency, cells were infected at an MOI of 0.05 (3 x 10^6 pfu/ml) in a total volume of 50 ml of fresh growth media without selection (supplemented with 3 mM HMBA if necessary). The plates were then incubated at 37°C for 2-4 days until complete CPE was observed. The whole plate was then directly frozen at –80°C. After defrosting, the protocol was the same as for the growth of less disabled viruses except that the resuspension of the viral pellet before sonication was made in 500 μl/10 plates.

II.3.4.2. Small scale viral DNA extraction

In order to obtain viral DNA for southern blot analysis or PCR, small scale viral DNA extractions were carried out based on the method described by Feldman, 1996. One well of a 6-well plate of virally infected cells at complete CPE was harvested and cells were centrifuged at 100 rpm for 10 minutes. The cells were resuspended in 200 μl of TES buffer (50 mM Tris pH 7.8, 1 mM EDTA, 30% (v/v) sucrose). 200 μl of proteinase K buffer (2% (w/v) SDS, 100 mM β-mercaptoethanol) was added and the mix was incubated on ice 30 minutes. 10 μl of proteinase K (stock

at 20 mg/ml in 10 mM $CaCl_2$) was then added and the reaction was incubated at 55°C overnight. Extractions were performed twice using phenol/chloroform (1:1 v/v) and then once with chloroform/isoamyl alcohol (24:1 v/v). Viral DNA was precipitated by the addition of 75 µl of 7.5 M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol and pelleted by microcentrifugation at 13000 rpm for 10 minutes. The DNA pellet was then washed with 500 µl of 70% ethanol and air dried. Viral DNA was then resuspended in 20-50 µl of ddH_2O depending on the level of disablement of the virus.

II.3.4.3. Large scale viral DNA extraction

For use of viral DNA in homologous recombination transfection, large scale viral DNA extractions were performed. 8 x 175 cm^2 flasks at complete CPE were harvested and centrifuged for 2 hours at 12000 rpm at 4°C in order to pellet the cells and virus. The pellet was then transferred to 15 ml of proteinase K buffer (0.01 M Tris pH 8.0, 5 mM EDTA, 0.5% SDS) and proteinase K (Boehringer Manheim) was added to a final concentration of 50 µg/ml. The mix was then incubated at 37°C/200 rpm overnight and 15 ml of ddH_2O were added. Extractions were carried out by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) with gentle inversion of the tube for 10 minutes followed by centrifugation at 15000 rpm for 10 minutes. The aqueous layer was extracted at least three times until the white interface disappeared. For the final extraction, an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was performed. Two volumes of ethanol were carefully layered on the aqueous phase and the layers were gently mixed by inversion of the tube. After centrifugation at 3000 rpm for 10 minutes in order to pellet the precipitated viral DNA, the viral DNA was then washed with 5 ml of 70% ethanol. The pellet was air dried overnight and resuspended in 0.5-3 ml of ddH_20 depending on the level of disablement of the virus.

II.3.5. Dendritic cell preparation

Dendritic cell (DC) preparation was performed following the protocol described by (Romani *et al.* 1994), (Morse *et al.* 1997).

On day 1:

60 ml PBMC (Peripheral Blood Mononuclear Cells) from healthy donors containing anticoagulant (heparin) were diluted by adding an equal volume of Hank's Buffered Saline Solution (HBSS). 30 ml were carefully layered onto 20 ml of lymphoprep (Nycomed) in a 50 ml tube (Falcon) and centrifuged at 1600 rpm, for 30 minutes, at room temperature (RT), brake off. After centrifugation, mononuclear cells in the interface were removed using a Pasteur pipette. The density of the solution was reduced by diluting it in HBSS and cells were then centrifuged at 1600 rpm, for 10 minutes, at RT. Cells were pooled, washed twice in HBSS and centrifuged at 1400 rpm, for 5 minutes, at RT. The cell pellet was resuspended in 10 ml red blood cell lysis buffer (Sigma) for 5 minutes, at RT. Cells were washed twice more in HBSS and the resultant pellet was resuspended in 18 ml of complete RPMI medium (Gibco) (10% foetal calf serum (FCS), 2-mercaptoethanol (0.05 mM), L-glutamine (2 mM), Penicillin/Streptomycin (100 IU/ml)). Cells were aliquoted at 3 ml/well in a 6-well plate and incubated for 2 hours at 37°C, 5% CO_2.

Non-adherent cells (mainly T cells and B cells) were removed, washed in HBSS and centrifuged at 1400 rpm, 5 minutes, RT. The cell pellet was resuspended in a 2 ml 90% FCS:10% dimethylsulphoxide (DMSO) mix, aliquoted and stored at –80°C for subsequent T cell isolation. Adherent cells (mainly monocytes and macrophages) were cultured in 3 ml complete RPMI medium supplemented with GM-CSF (0.1 µg/ml) and IL-4 (0.05 µg/ml) and incubated for 7 days, at 37°C, 5% CO_2.

On Day 8:

6 ml cell suspension (containing the non-adherent cells) were carefully layered onto 4 ml of lymphoprep in a 15 ml tube (Falcon) and centrifuged at 1600 rpm, for 30 minutes, at RT, brake off. Cells from the interface were removed, washed 3 times in HBSS and centrifuged as described above. Cells were resuspended in complete RPMI medium, counted (as large cells - DC and smaller cells - contaminating cells), incubated with anti-CD19 (BU12 – 200 µl neat, Immunology Dept., UCL), anti-CD2 (Harlan) and anti-CD3 (Harlan) mAb and left on ice for 30 minutes. The cells were washed in HBSS, resuspended in 2 ml complete RPMI medium, mixed with sheep anti-mouse antibodies bound to magnetic beads (Dynabeads, Dynal) at a ratio of 10 µl beads/10^6 contaminating cells and incubated on a rotor mixer at 4°C, for 45 minutes. DC were then depleted by removing the supernatant after placing the cell suspension/magnetic bead mix in contact with a magnet, for 10 minutes, on ice. DC were counted, resuspended in complete RPMI medium at the appropriate concentration and left on ice for immediate use.

II.3.6. T cell isolation

T and B cells were frozen at –80°C as described above from 60 ml PBMC of healthy donors on day 1 of the cell preparation. On day 8, cells were defrosted quickly, washed in HBSS and centrifuged at 1400 rpm, for 5 minutes. Cells were resuspended in 2 ml complete RPMI medium, counted and incubated with anti-CD19 (BU12 - 200 µl neat, Immunology dept, UCL), anti-CD14 (HB246 – 200 µl neat, Immunology dept, UCL) and anti-HLA-DR (L243 – 200 µl neat, Immunology dept, UCL) mAb and left on ice for 30 minutes. The cells were washed in HBSS, resuspended in 2 ml complete RPMI medium, mixed with sheep anti-mouse antibodies bound to magnetic beads (Dynabeads, Dynal) at a ratio of 10 µl beads/10^6 contaminating cells and incubated on a rotor mixer at 4°C, for 45 minutes. CD4+ T cells were then depleted by removing the supernatant after placing the cell suspension/magnetic bead mix in contact with a magnet, for 10 minutes, on ice. CD4+ T cells were counted, resuspended in complete RPMI medium at the

appropriate concentration, left on ice or cultured overnight at 37°C, 5% CO_2 for subsequent use.

II.3.7. Transduction of dendritic cells with HSV-1

5 x 10^5 DC in suspension were transferred to a 15 ml tube (Falcon) and centrifuged at 1400 rpm, for 5 minutes, at RT. The cell pellet was then infected at MOI of 1 with an HSV-1 vector in 200 µl of complete RPMI (Gibco) medium (10% foetal calf serum (FCS), 2-mercaptoethanol (0.05 mM), L-glutamine (2 mM), Penicillin/Streptomycin (100 IU/ml)) for 1 hour at 37°C, 5% CO_2. Cells were resuspended in 1 ml of complete RPMI medium supplemented with GM-CSF (0.1 µg/ml) and IL-4 (0.05 µg/ml). DC were transferred in a 24-well plate and left overnight at 37°C, 5% CO_2. Gene delivery was assessed every 24 hours over a period of four days.

For treatment of DC with lipopolysaccharides (LPS), cells were resuspended in complete RPMI medium containing GM-CSF/IL-4 supplemented with LPS at 100 ng/ml. Cells were then transferred in a 96-well flat-bottom plate and left overnight at 37°C, 5% CO_2.

For treatment of DC with the proteasome inhibitor MG132, cells were pre-treated for half an hour with the inhibitor (10 µM for 10^5 cells), pelleted at 1400 rpm for 5 minutes, infected for an hour with an HSV-1 vector, resuspended in complete RPMI medium containing GM-CSF/IL-4 supplemented with the inhibitor. Cells were then transferred in a 24-well plate and left overnight at 37°C, 5% CO_2.

II.3.8. Assessment of gene delivery

Gene delivery in cells infected with viruses containing green fluorescent protein *GFP* reporter gene was detected under an inverted fluorescent microscope at a wavelength of 500 nm. The infected green cells expressing GFP were counted in

relation to the total number of cells present in the well. The percentage gene delivery was then calculated.

For cells infected with viruses containing *LacZ* reporter gene, gene delivery was assessed by X-gal staining. For cells in suspension (Dendritic cells), cells were centrifuged at 1400 rpm, 4°C for 4 min and the cell pellet was washed in 1 ml of 1xPBS. The cells were centrifuged again and the pellet was fixed for 10 min at room temperature in 500 µl of 1xPBS/ 0.1% glutaraldehyde. After centrifugation, the subsequent cell pellet was resuspended gently in 2 ml of X-Gal solution ([150 µg/ml 4-Cl,5-bromo, 3-indolyl-β-galactosidase in DMSO]; [1xPBS; 10 mM sodium phosphate; 1 mM $MgCl_2$; 150 mM NaCl; 3.3 mM $K_4Fe(CN)_6$; 3.3 mM $K_3Fe(CN)_6$]). The 24-well dish was then returned to the 37°C incubator for 2 h-overnight depending on the strength of the promoter driving the *lacZ* reporter gene. Cells expressing the *lacZ* reporter gene were observed under an inverted microscope. Blue cells indicating β-galactosidase activity were counted in relation to the total number of cells present in the well. The percentage gene delivery was then assessed.

II.3.9. Trypan blue exclusion assay

Cell survival was assessed by the trypan blue exclusion assay. Dead cells take up the blue dye in which they have been resuspended and thus turn blue. Live cells remain white. 10 µl of DC suspension were diluted in an equal volume of 0.04% trypan blue solution. 10 µl of this mix were then loaded on a haemocytometer counting chamber placed under the microscope and white live cells were counted within a 4 x 4 square grid. The number of DC alive in 1 ml cell suspension was calculated by multiplying the number of white cells counted within the grid by $2x10^4$.

II.3.10. Flow cytometry

DC were washed in 30 ml of block buffer (HBSS containing 10% normal rabbit serum and 0.1% sodium azide), centrifuged at 1400 rpm, for 5 minutes, at 4 C, resuspended in block buffer at a concentration of $1x10^6$ cells/ml and left on ice for 20-

30 minutes. 50 µl of each cell suspension were added to wells of a 96-well round bottom plate previously containing PE (phycoerythrin)-conjugated mouse antibodies diluted in the block buffer: anti-IgG1, anti-CD40, anti-CD83, anti-CD86 and anti-HLA-DR. A negative control well was set up with block buffer alone. Cells were incubated in dark on ice, for 30 minutes and washed 4 times with HBSS containing 0.1% sodium azide. DC were then resuspended in 200 µl of HBSS containing 0.1% sodium azide, placed on ice, in dark for immediate analysis or in 200 µl 2% formaldehyde, kept at 4°C, in dark until analysis. The acquisition was carried out on a FACSCAN (Becton Dickinson, Mountain View, CA) and the data was analysed by WinMDI software.

II.3.11. CD4+ proliferation assay

DC and CD4+ T cells were isolated from 60 ml PBMC of healthy donors. DC were serially diluted from $1x10^5$ DC/ml, to $5x10^4$ DC/ml and down to $1x10^4$ DC/ml and CD4+ T cells were diluted to the fixed concentration of $1x10^6$ cells/ml. Each of DC concentration was set up in triplicate. 100 µl of DC and 100 µl of CD4+ T cells were added to each assay well. 10 µl recall antigen - purified protein derivative (PPD) at 10000 IU/ml or Hepatitis B surface antigen (HBsAg) recombinant (Austral) at 100 µg/ml – were added to wells at a final concentration of 100 IU PPD/well or 1 µg HBsAg/well. For all experiments, negative controls which provided the background were set up such that each missed one component, the antigen (DC and T cells alone), DC (T cells and antigen alone) or CD4+ T cells (DC and antigen alone). HSV infected and mock-infected DC were cultured with CD4+ T cells in a 96-well flat-bottom plate and incubated for 6 days at 37°C, 5% CO_2. Cells were pulsed with radioactive [^{3}H] thymidine (ICN Biomedical, High Wycombe,UK) at 1 µCi/well for 16-18 hours and harvested. The T-cell proliferation response was then measured in counts per minute (cpm) by [^{3}H] thymidine incorporation using a betaplate counter (Trilux).

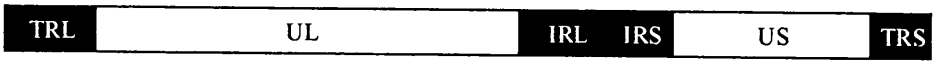
II.4. Characteristics of viruses used for gene delivery to DC

Various HSV-1 constructs have been developed in our laboratory based on the manipulation of the wild type virus from which some genes have been knocked-out and others inserted after transfection and homologous recombination (Coffin *et al.* 1996), (Howard *et al.* 1998), (Palmer *et al.* 2000), (Lilley *et al.* 2001). These constructs are based on the 17+ strain of HSV-1. The vectors fall into three categories depending on their level of disablement: minimally disabled vectors contain a deletion in one non-essential gene, partially disabled vectors contain more than one deletion and/or inactivation in non-essential genes and replication incompetent vectors contain deletions and/or inactivations in essential and non-essential genes. A schematic diagram of viral constructs used in this thesis is shown below.

II.4.1. Minimally disabled vectors

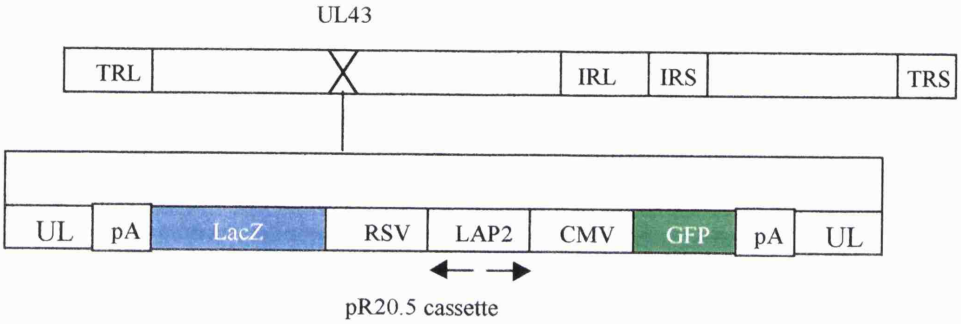
17+:

- The wild-type backbone virus. The HSV-1 genome consists of the unique long (UL) and short (US) regions respectively flanked by terminal and internal long repeats (TRL and IRL) and short repeats (TRS and IRS).



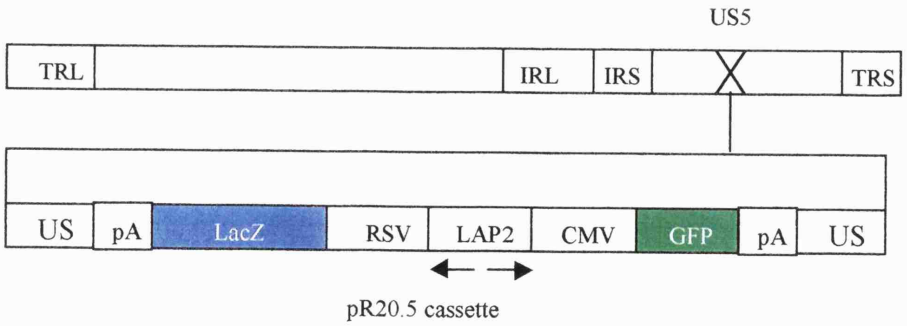
17+/pR20.5/UL43:

- One inactivation in the non-essential gene UL43 (coding for a membrane channel protein).
- The pR20.5 cassette composed of the LAP2 promoter linked in a bi-directional fashion to the cytomegalovirus (CMV) promoter driving *GFP* and to the rous sarcoma virus RSV promoter driving *LacZ* is inserted in the UL43 locus of the HSV-1 genome (Thomas *et al.* 1999).



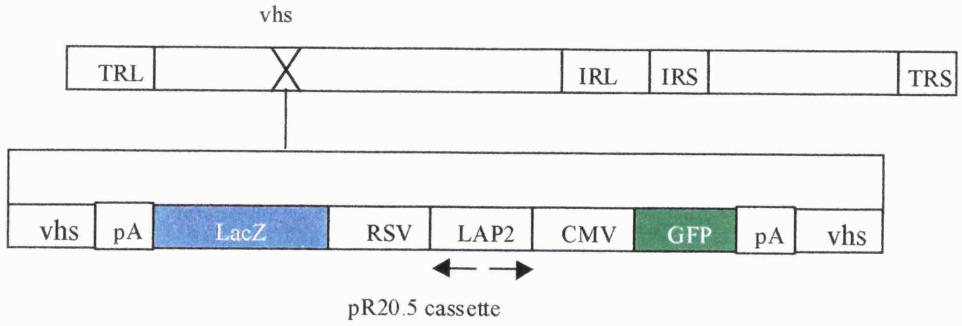
17+/pR20.5/US5:

- One deletion in the non-essential gene US5 (coding for the viral envelope glycoprotein gJ).
- The pR20.5 cassette is inserted in the US5 locus (Thomas *et al.* 1999).



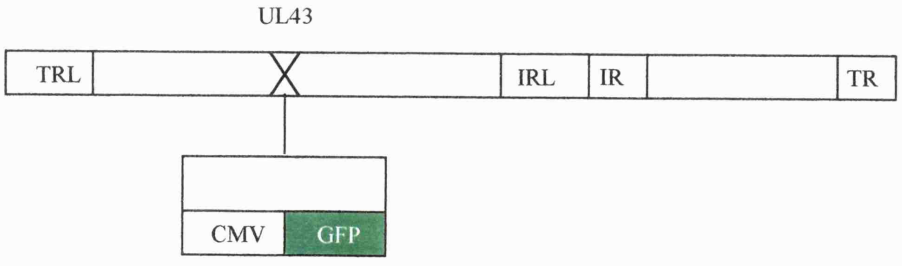
17+/pR20.5/vhs:

- One deletion in the non-essential gene UL41 (coding for the tegument protein virion host shut-off, vhs).
- The pR20.5 cassette is inserted in the UL41 locus.



17+/UL43CMVGFP:

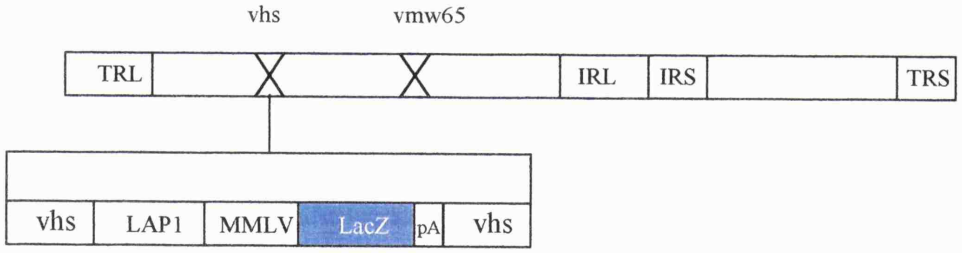
- One insertional inactivation in the non-essential gene UL43.
- The CMV promoter driving GFP is inserted in the unique NsiI site of the UL43 gene.



II.4.2. Partially disabled HSV-1 based vectors

17+/vmw65/pR15:

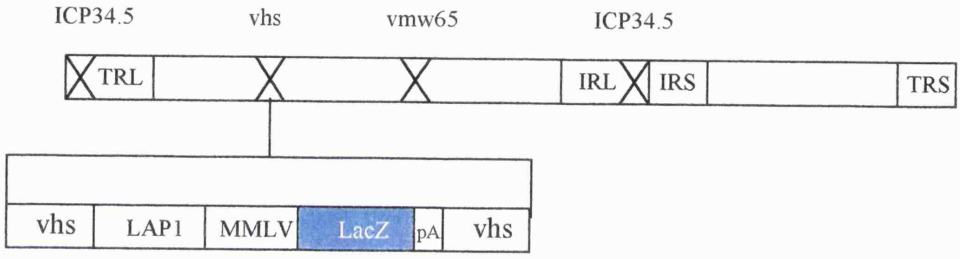
- The inactivating mutation *in1814* (12 bp inserted in the region containing the activation domain) is inserted in vmw65. This reduces toxicity of the virus by reducing immediate early IE gene expression (Ace *et al.* 1989). vmw65 codes for a structural protein that is essential for capsid assembly and thus cannot be deleted. The inactivation is compensated *in vitro* by the addition of 3 mM HMBA in the culture media (McFarlane *et al.* 1992). The UL41 gene is also inactivated.
- The pR15 cassette consisting of LAP1 linked to the moloney murine leukaemia virus long terminal repeat (MMLV-LTR) promoter driving *LacZ* is inserted in the unique NruI site (nt 91854) in the UL41 locus (Palmer *et al.* 2000).



pR15 cassette

1764/pR15:

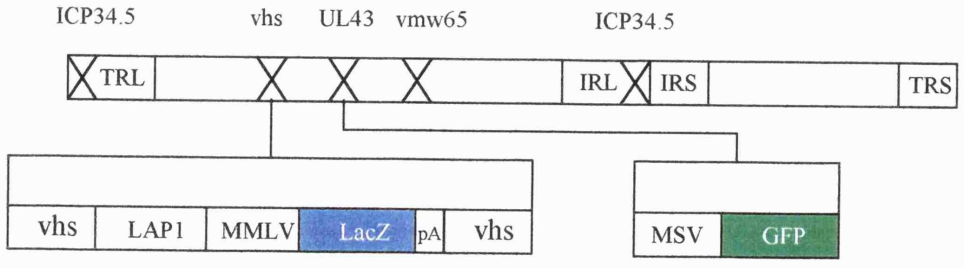
- One deletion in the non-essential gene γ34.5 (coding for infected cell polypeptide ICP34.5, a neuro-virulence factor) which considerably reduces toxicity of the virus. Inactivating mutation *in1814* in vmw65 (this combination of deletion and inactivation is termed 1764) (Coffin *et al.* 1996). Insertional inactivation in the UL41 gene.
- The pR15 is inserted in the UL41 locus (Palmer *et al.* 2000).



pR15 cassette

1764/pR15/UL43MSVGFP:

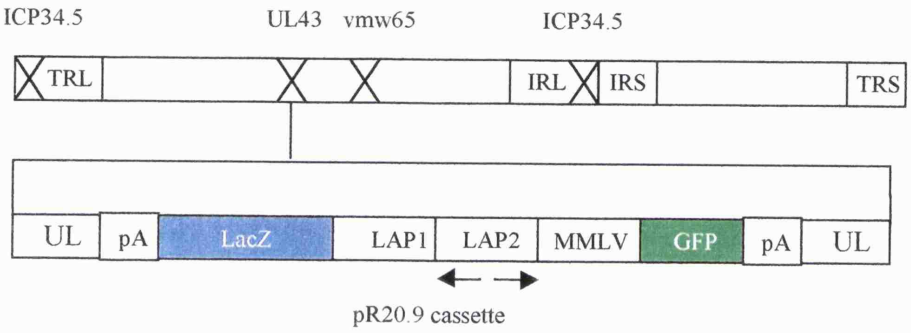
- One deletion in the non-essential gene γ34.5. Inactivating mutation *in1814* in vmw65. Insertional inactivation in the UL41 gene. Insertional inactivation in the UL43 gene.
- The pR15 cassette is inserted in the UL41 locus. The murine simian virus (MSV) promoter driving *GFP* is inserted in the UL43 gene.



pR15 cassette

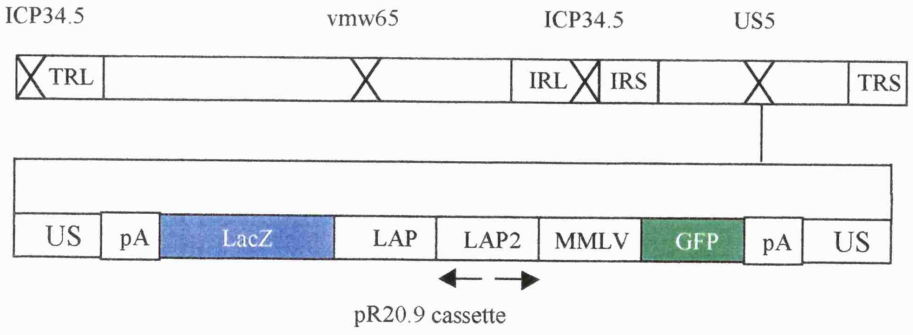
1764/pR20.9/UL43:

- One deletion in γ34. Inactivating mutation *in1814* in vmw65. Inactivation in the non-essential gene UL43.
- The pR20.9 cassette consisting of the LAP2 promoter linked in a bi-directional fashion to MMLV-LTR promoter driving *GFP* and to LAP1 promoter driving *LacZ* is inserted in the UL43 locus (Palmer *et al.* 2000).



1764/pR20.9/US5:

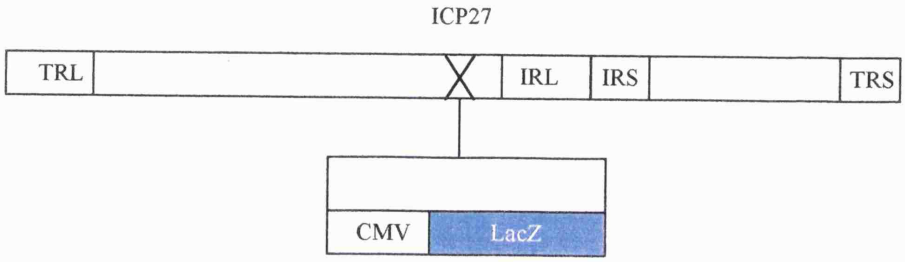
- One deletion in γ34.5. Inactivating mutation *in1814* in vmw65. Insertional inactivation of the non-essential gene US5.
- The pR20.9 cassette is inserted in the US5 locus.



II.4.3. Replication incompetent HSV-1 based vectors

17+/27-/pR20:

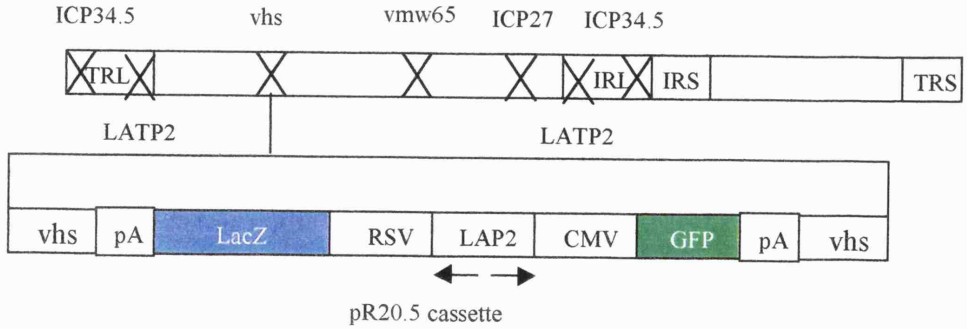
- One deletion in the essential gene ICP27. Such viruses are grown on cell-lines which complement for the ICP27 deletion.
- The pR20 cassette consisting of the CMV promoter driving *LacZ* is inserted in the ICP27 locus of HSV-1 genome (Howard *et al.* 1998).



pR20 cassette

1764/27-/pR20.5/vhs:

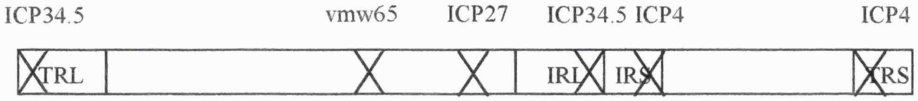
- One deletion in ICP27. Deletion of γ34.5. Inactivating mutation *in1814* in vmw65. Insertional inactivation of the UL41 gene (coding for vhs). Deletion in the LAT P2 region to prevent recombinational instability which is otherwise caused by the presence of LAP2 promoter contained in the pR20.5 cassette (Thomas *et al.* 1999).
- The pR20.5 cassette is inserted in the unique NruI site of the UL41 gene (Thomas *et al.* 1999).



pR20.5 cassette

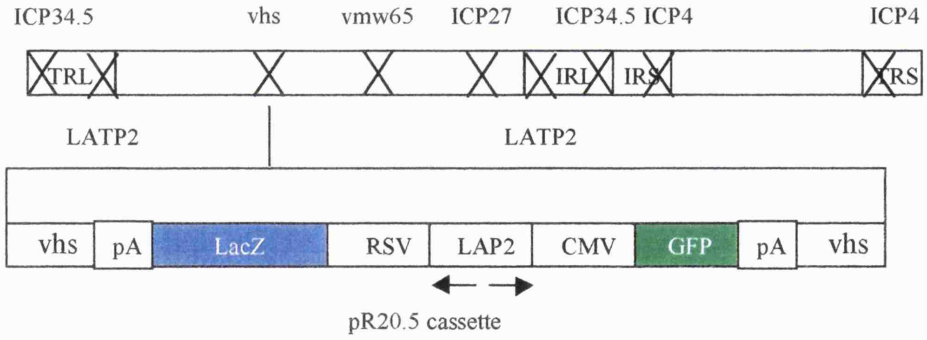
1764/27-/4-:

- Deletion of ICP27. Deletion of ICP4. Deletion of γ34.5. Inactivating mutation *in1814* in vmw65.
- No cassette has been inserted in this virus which is thus 'white'.



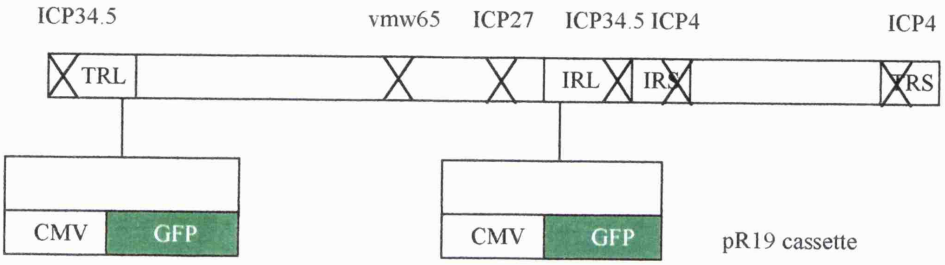
1764/27-/4-/pR20.5/vhs:

- Deletion of ICP27. Deletion of ICP4. Deletion of γ34.5. Inactivating mutation *in1814* in vmw65. Insertional inactivation in the UL41 gene. Deletion in the LAT P2 region.
- The pR20.5 cassette is inserted in the unique NruI site of the UL41 gene (Thomas *et al.* 1999).



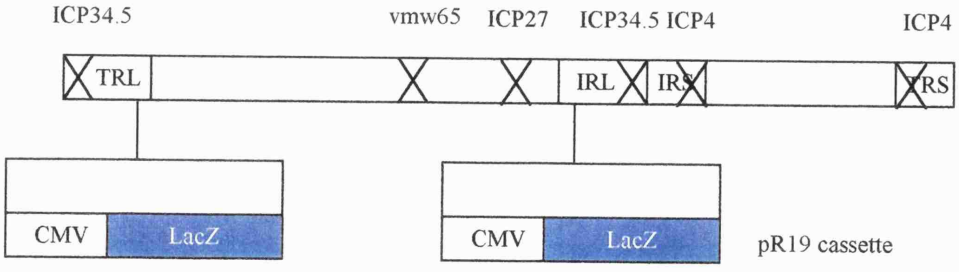
1764/27-/4-/pR19GFP:

- Deletion of ICP27. Deletion of ICP4. Deletion of γ34.5. Inactivating mutation *in1814* in vmw65.
- The pR19 cassette composed of the LAP1 promoter linked to the LAP2 promoter, in turn linked to the CMV promoter driving *GFP* is inserted in the LAT region (Thomas *et al.* 1999).



1764/27-/4-/pR19LacZ:

- Deletion of ICP27. Deletion of ICP4. Deletion of γ34.5. Inactivating mutation *in1814* in vmw65.
- The pR19 cassette driving *LacZ* is inserted in the LAT region (Thomas *et al.* 1999).



RESULTS

PART I

OPTIMISATION OF GENE DELIVERY TO DENDRITIC CELLS USING A PANEL OF HSV VECTORS

PART II

EFFECTS OF HSV VECTORS ON DENDRITIC CELL PHENOTYPE AND FUNCTION

PART I

OPTIMISATION OF GENE DELIVERY TO DENDRITIC CELLS

USING A PANEL OF HSV VECTORS

CHAPTER III

EFFECTS OF HSV VECTORS ON GENE DELIVERY TO DENDRITIC CELLS

CHAPTER IV

EFFECTS OF HSV VECTORS ON DENDRITIC CELL SURVIVAL

CHAPTER V

GROWTH CHARACTERISTICS OF HSV IN DENDRITIC CELLS AND PROTEOLYTIC PROCESSING OF THE DELIVERED ANTIGEN

CHAPTER III

EFFICIENCY OF HSV VECTORS IN GENE DELIVERY TO DENDRITIC CELLS

III.1. Introduction

Dendritic cells (DC) are the most potent antigen presenting cells, capable of stimulating very strong T-cell responses specific to exogenous or endogenous antigens. As mediators of immunity, DC have become increasingly important in cancer immunotherapy and are ideal targets for the delivery of genes coding for tumour-antigens or tumour-associated antigens. Various gene delivery methods have been developed for DC so far which include non-viral and viral strategies. Non-viral methods such as direct loading of synthetic peptides, RNA or DNA into DC have not shown good efficacy in terms of delivery and levels of expression of the desired antigen in DC. Viral methods include the use of vaccinia virus, adenovirus, adeno-associated virus, lentivirus and herpes simplex virus (HSV)-based vectors to transduce DC with the gene of interest. Although each of these virus-based vectors has its own advantage and disadvantage in terms of efficiency versus safety (described in the general introduction), this part of the thesis will focus on optimising gene delivery to DC using HSV vectors.

We and others have found that DC transduced with an essentially wild-type HSV-1 vector infect DC very efficiently (Coffin *et al.* 1998). This showed that HSV-1 can give efficient gene delivery at low MOI. However, wild-type HSV might be expected to affect cell viability post-infection and/or affect cell functioning at various levels in order to escape immune surveillance. Thus, the objective of this chapter is to identify HSV mutants giving an optimal balance between safety and efficiency of gene delivery to DC. For this purpose, various HSV-based vectors have been tested for gene delivery to DC (this chapter) and their effects also assessed on cell survival (chapter IV). In addition, the permissivity of DC for HSV replication was assessed by monitoring virus growth as well as levels of expression of immediate early (IE) proteins in DC post-infection (chapter V).

The vectors presented in this part of the thesis were previously constructed in our laboratory and fall into three categories depending on the level of disablement: four minimally disabled vectors containing only one deletion in a non-essential gene, five 'partially disabled' vectors containing multiple deletions

in non-essential genes and five replication incompetent vectors containing multiple deletions in essential and non-essential genes. All vectors contained cassettes in which *GFP* and/or *LacZ* reporter genes are driven by various promoters inserted at specific sites of the HSV-1 genome (details of the vector constructs are described in Materials and Methods and in table III.1).

Gene delivery was monitored as the percentage of GFP and/or β-gal expressing cells at 24 hour intervals, for four days after transduction of DC with these vectors and cell survival was assessed for the same time period by the trypan blue exclusion assay. A range of MOI was tested for the majority of the vectors (depending on the titer of stocks of virus available) in order to assess the dose related effects of virus on gene delivery and cell survival. For each category of vector, the gene delivery and cell survival results are presented comparatively at MOI=1 and then for each vector at the range of MOI tested. Viral growth curves were plotted following titration of virus at 24 hour intervals, for four days after transduction of DC with the vectors. These results are presented at MOI=1 for each category of vector and then for each vector at the range of MOI tested. Levels of expression of HSV IE proteins were monitored by collecting protein extracts at intervals for two days post-infection. Results are presented for three of the HSV IE proteins.

III.2. HSV-1 vectors deliver genes to DC efficiently

Minimally disabled vectors were used to show the absolute infectivity of DC with HSV-1 and to determine if the individual gene deletion could affect entry or gene delivery. The partially disabled vectors contain an inactivating mutation in vmw65 (the trans-activator of HSV IE proteins) which was anticipated to reduce toxicity with this category of vector. The fully disabled vectors contain at least one deletion in an essential gene and thus are replication incompetent. All the vectors presented in this chapter are described in table III.1 and the vector titer in each case is shown in table III.2. Experiments were repeated three times and the average values are presented in this chapter.

Virus Nomenclature	Genes deleted (d) or inactivated (i)	Promoter driving GFP	Promoter driving LacZ
17+/pR20.5/UL43	UL43 (i)	LATP2-CMV	LATP2-RSV
17+/pR20.5/US5	US5 (d)	LATP2-CMV	LATP2-RSV
17+/pR20.5/vhs	UL41 (i)	LATP2-CMV	LATP2-RSV
17+/UL43CMVGFP	UL43 (i)	CMV	NA
17+/vmw65/pR15	Vmw65 (i), UL41 (i)	NA	LAP1-MMLV-LTR
1764/pR20.9/UL43	ICP34.5 (d), vmw65 (i),UL43 (i)	LATP2-MMLV-LTR	LATP2-LAP1
1764/pR20.9/US5	ICP34.5 (d), vmw65 (i),US5 (d)	LATP2-MMLV-LTR	LATP2-LAP1
1764/pR15	ICP34.5 (d), vmw65 (i), UL41 (i)	NA	LAP1-MMLV-LTR
1764/pR15/UL43MSVGFP	ICP34.5 (d), vmw65 (i), UL41 (i), UL43 (i)	MSV	LAP1-MMLV-LTR
17+/27-/pR20	ICP27 (d)	NA	CMV
1764/27-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d), LATP2 (d)	LATP2-CMV	LATP2-RSV
1764/27-/4-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d), ICP4 (d), LATP2 (d)	LATP2-CMV	LATP2-RSV
1764/27-/4-/pR19GFP	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	LAP1-LATP2-CMV	NA
1764/27-/4-/pR19LacZ	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	NA	LAP1-LATP2-CMV

Table III.1: HSV-1-based vectors tested for gene delivery in dendritic cells (DC). Abbreviations: 17+: HSV-1 backbone wild-type (wt) strain. 1764: deletion in the ICP34.5 gene and inactivating mutation in vmw65. UL: unique long region. US: unique short region. ICP: infected cell polypeptide. Vhs: virion host shut-off. LAP1: latency-associated promoter 1. LATP2: latency-associated transcript promoter 2. CMV: cytomegalovirus IE promoter. RSV: rous sarcoma virus promoter. MMLV-LTR: moloney murine leukaemia virus long terminal repeat promoter. MSV: murine simian virus promoter. NA: not applicable.

Viruses	Titers (pfu/ml)
17+/pR20.5/UL43	$1x10^9$
17+/pR20.5/US5	$2x10^8$
17+/pR20.5/vhs	$2x10^8$
17+/UL43CMVGFP	$2x10^7$
17+/vmw65/pR15	$1x10^7$
1764/pR15	$5x10^6$
1764/pR20.9/UL43	$2x10^7$
1764/pR20.9/US5	$5x10^7$
1764/pR15/UL43MSVGFP	$2x10^8$
17+/27-/pR20	$1x10^7$
1764/27-/pR20.5/vhs	$1x10^6$
1764/27-/4-/pR20.5/vhs	$1x10^6$-$2x10^8$
1764/27-/4-/pR19GFP	$1x10^8$
1764/27-/4-/pR19LacZ	$5x10^8$

Table III.2: Virus titers of viruses used in DC in chapter III. Titers were obtained by calculating the number of plaque forming unit (pfu) of virus per ml following infection of BHKs or MAM49 cells at a series of viral stock dilution. Titers were obtained for GFP or β-galactosidase expressing plaques according to the specific virus stock grown.

III.2.1. Gene delivery using essentially wild type vectors

The minimally disabled vectors used in this section are based on the parent virus HSV-1 strain 17+, and differ from one another by one deletion in a non-essential gene. These genes are UL43 (encoding a membrane channel protein), US5 (encoding the viral glycoprotein gJ) or UL41 (encoding the tegument virion host shutoff protein, vhs, responsible for degrading mRNA)) (Carter *et al.* 1996), (Balan *et al.* 1994). A cassette (pR20.5) consisting of the LATP2 promoter linked in a bi-directional fashion to the CMV promoter driving green fluorescent protein *GFP* and to the RSV promoter driving *LacZ* is inserted into the UL43, US5 or UL41 gene respectively in each case.

DC were transduced with 17+/pR20.5/UL43 at MOI ranging from 0.1 to 10 (shown 24 hours post-infection in figure III.1). At this range of MOI, gene delivery increasing from 13% to 80% GFP or β-gal expression was observed. At MOI=1, an average of 43% GFP or β-gal positive cells was achieved. These results indicate that wild-type HSV infects DC even at low MOI and that at higher MOI very efficient gene delivery can be achieved. This suggests that HSV has evolved to naturally infect DC. In order to determine if the UL43 deletion affected this gene delivery, other wild type-vectors with a different gene deletion were then tested in DC.

It was found that in DC transduced with each of the vectors 17+/pR20.5/UL43, 17+/pR20.5/US5 and 17+/pR20.5/vhs, an average of 45% gene delivery was observed 24 hours post-infection at MOI=1. Thus, vectors containing the same cassette with the LATP2 promoter and with only one gene deletion or inactivation in UL43, US5 or UL41 (vhs) gave high efficiency gene delivery to DC at MOI=1. However, in most cases, gene delivery was reduced to less than half the original level by 72 hours post-infection (figures III.2 and III.4). No significant difference was observed between the vectors although the 17+/pR20.5/vhs virus in which vhs has been inactivated appeared to generally give better gene delivery to DC than the other vectors tested.

In order to investigate the hypothesis that high levels of GFP or β-gal expression may be associated with the LATP2 promoter in the transgene cassette, a vector containing only the CMV promoter driving *GFP* inserted in the unique Nsi site of the UL43 gene was tested in DC. DC transduced with this vector (17+/UL43CMVGFP) at MOI=1 still gave 40% gene delivery in terms of GFP expression. As previously noted, gene delivery was also reduced by half at 72 hours post-infection (figure III.2). Thus, gene delivery to DC using 17+/UL43CMVGFP gave similar results as the vectors presented above containing the pR20.5 cassette. This implies that the gene delivery recorded is not related to an enhancement provided by the LATP2 element. The observation that apparent gene delivery dropped after 48 hours can be explained either by a promoter effect in DC that reduces transgene expression over time and/or by a viral effect on cell viability (the latter possibility will be discussed in chapter IV).

Dose and time related effects of three of the minimally disabled vectors were compared in DC by assessing gene delivery daily, for four days following tranduction with 17+/pR20.5/UL43, 17+/pR20.5/US5 or 17+/UL43CMVGFP at a range of MOI. In DC transduced with 17+/pR20.5/UL43 at MOI=0.1, 13% GFP was observed at 24 hours and this value was only reduced by a third at 48 hours, and maintained up to 96 hours. At MOI=0.5, GFP or β-gal expression was high for 48 hours, and then dropped to a third of the initial level at 72 hours. However, at MOI=1, 5 and 10, the percentage GFP or β-gal positive cells was gradually lowered with time, reaching half the initial level, 72 hours post-infection (figure III.4).

Transduction of DC with 17+/pR20.5/US5 at MOI ranging from 0.1 to 10, gave increasingly higher gene delivery from 16% to 81%, 24 hours post-infection. Again as observed with 17+/pR20.5/UL43 at low MOI, cells were expressing GFP for four days, by which time gene delivery had only reduced by 25%. At MOI =0.1 and 0.5, gene delivery was decreased to half the original level, 72 hours post-infection. At higher MOI=1, 5 and 10, GFP expression was gradually lower with time, reaching less than 75% of the original level, at 72 hours (figure III.5).

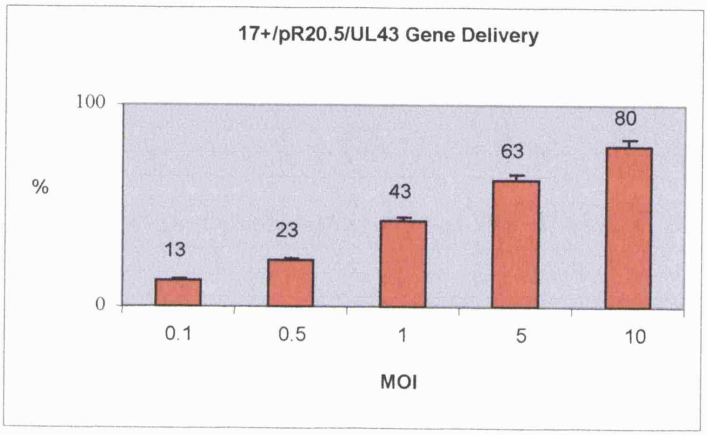


Figure III.1: Dose effect of HSV on dendritic cells (DC) using 17+/pR20.5/UL43 (UL43 inactivated), at a range of MOI. The graph shows the percentage GFP expressing cells, 24 hours post-infection.

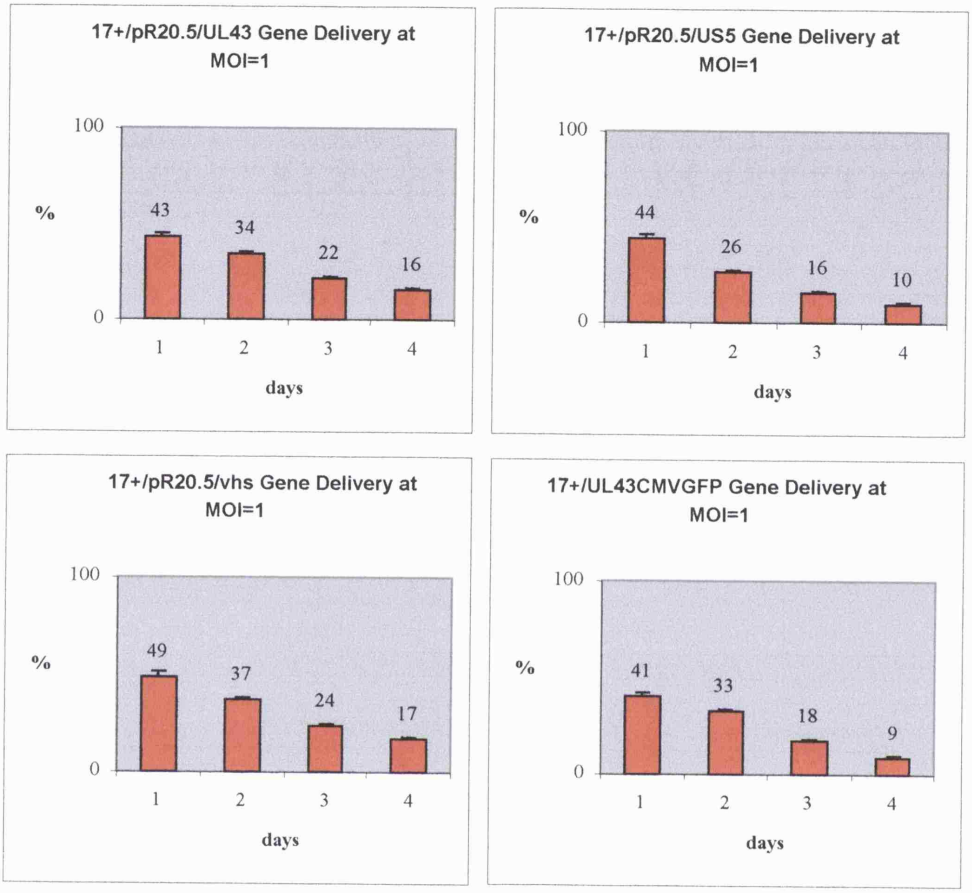


Figure III.2: Gene delivery to DC with the minimally disabled vectors at MOI=1. GFP or β-gal expressions were assessed every 24 hours, for four days with 17+/pR20.5/UL43 (UL43 inactivated), 17+/pR20.5/US5 (US5 deleted), 17+/pR20.5/vhs (UL41 inactivated) or 17+/UL43CMVGFP (UL43 inactivated). When using viruses containing both GFP and LacZ, levels of the corresponding proteins were identical.

Figure III.3: GFP expression in DC following transduction with 17+/pR20.5/UL43 (UL43 inactivated) at MOI=1, 24 hours post-infection.

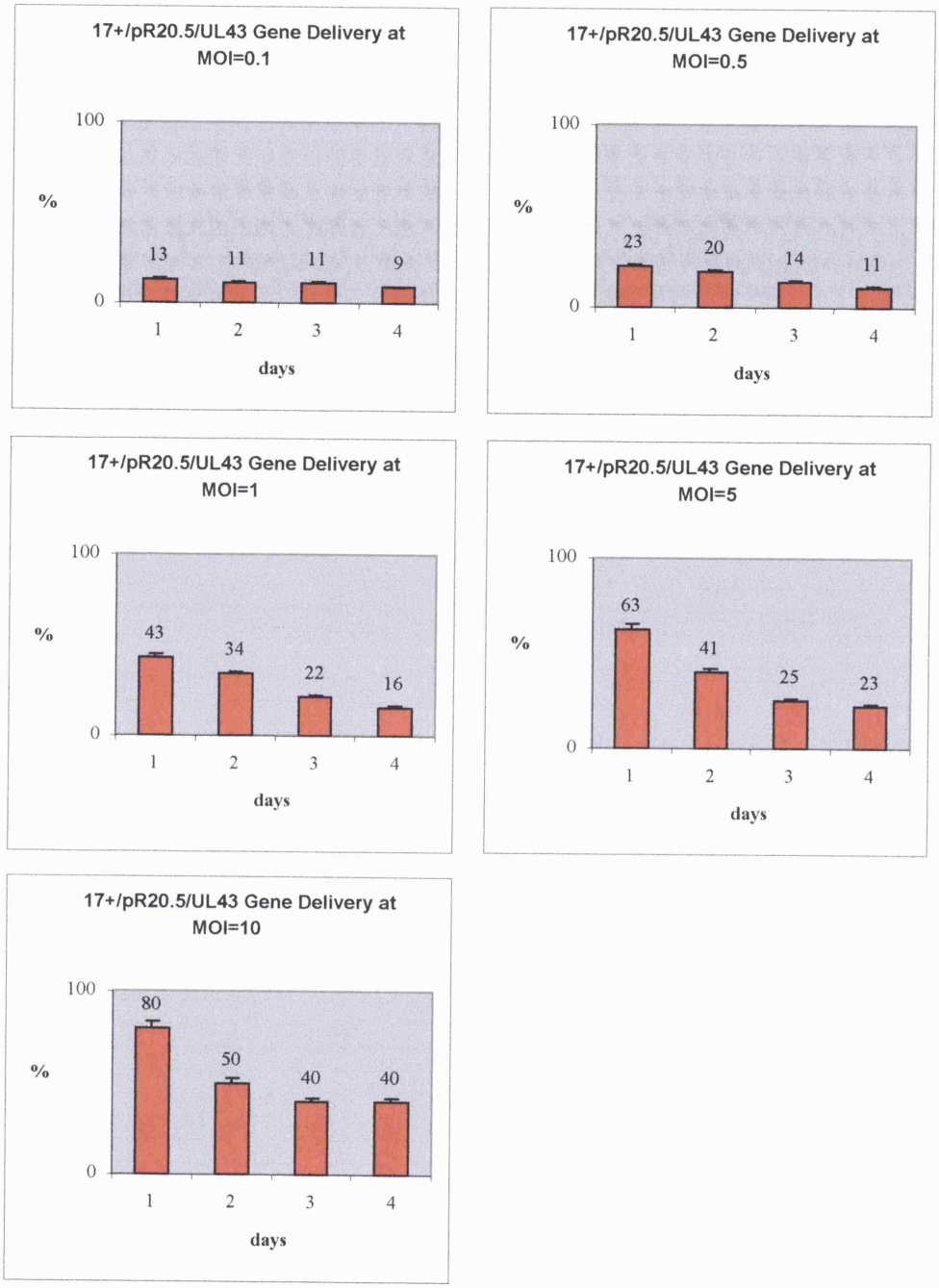


Figure III.4: Dose effect of 17+/pR20.5/UL43 (UL43 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP or β-gal expressing cells.

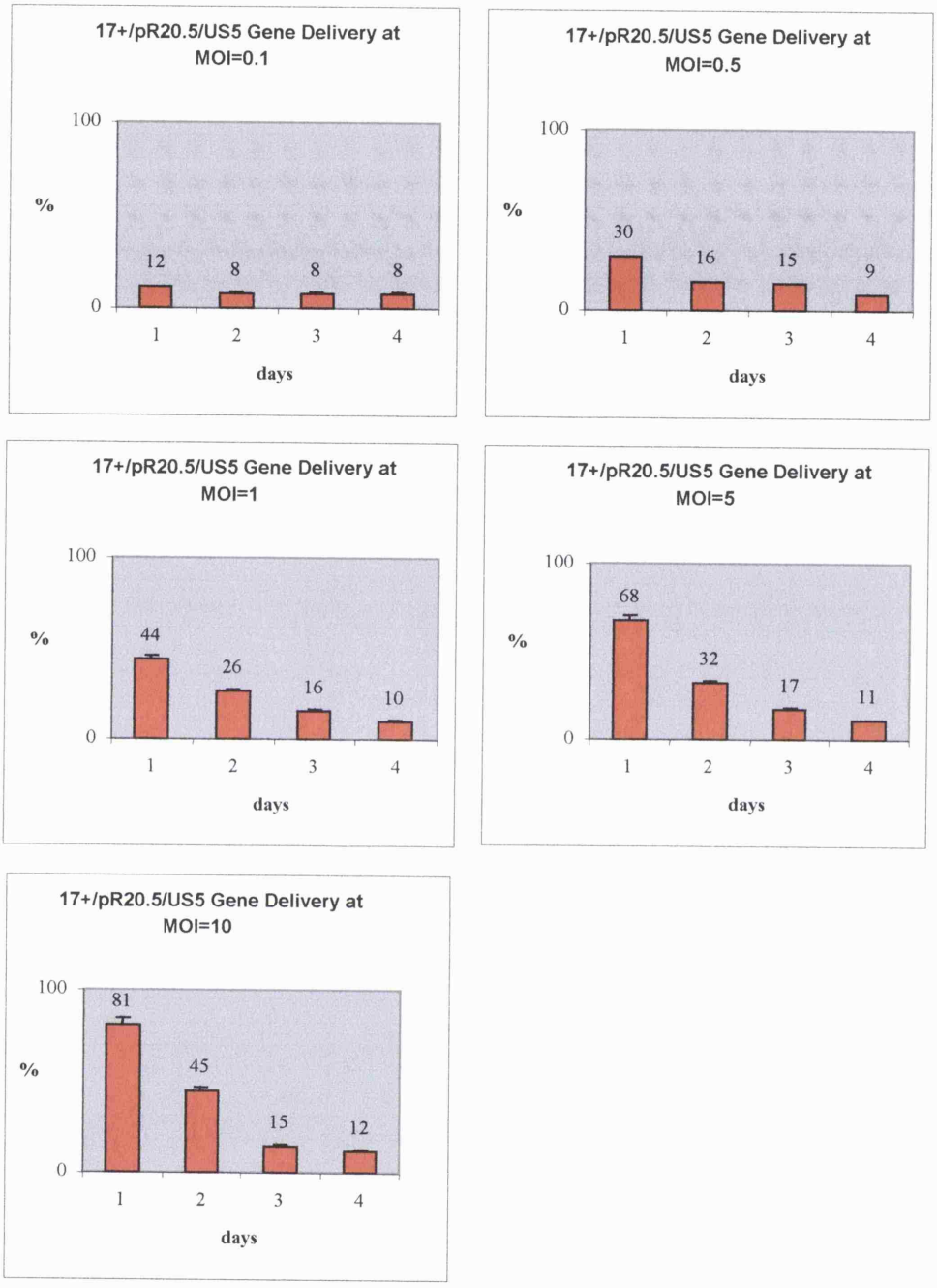


Figure III.5: Dose effect of 17+/pR20.5/US5 (US5 deleted) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP or β-gal expressing cells.

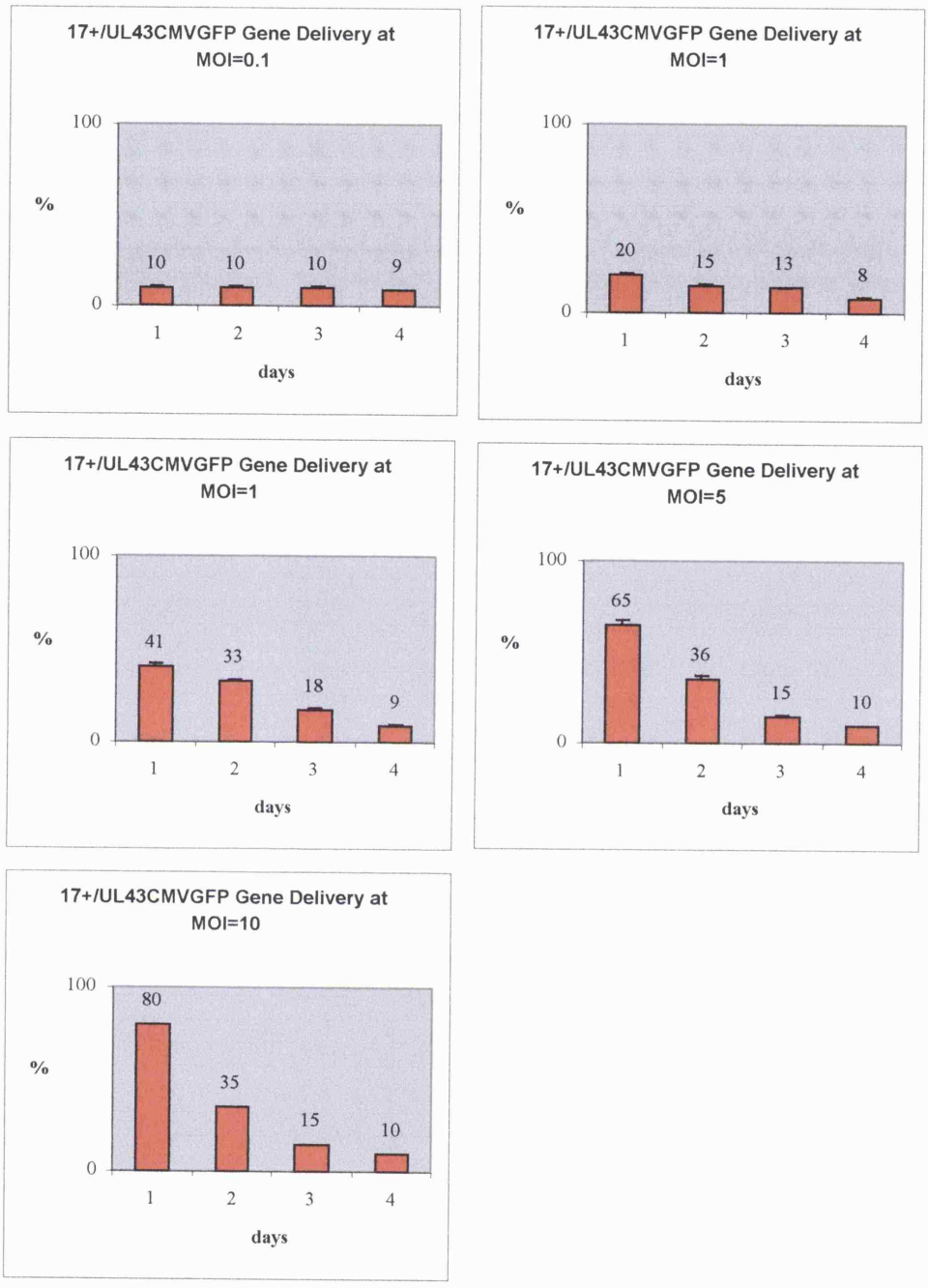


Figure III.6: Dose effect of 17+/UL43CMVGFP (UL43 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP expressing cells.

DC transduced with 17+/UL43CMVGFP at MOI ranging from 0.1 to 10, also gave increasingly higher gene delivery from 10% to 80%, 24 hours post infection. At MOI=0.1, 10% of GFP expressing cells was observed at 24 hours and this value was maintained for four days. At MOI=0.5 and 1, gene delivery was reduced by 50% at 72 hours. At higher MOI=5 and 10, gene delivery was reduced by 25% of the original level at 72 hours (figure III.6).

These results all show the same trend that the percentage GFP or β-gal expressing cells is dependent on the dose of wild-type virus used, as would be expected, although this effect is not directly proportional. Secondly, gene delivery is highest at 24 hours and then reduces with time at MOI>=1. However, at low MOI=0.1 and 0.5, levels of GFP or β-gal expressing cells (though relatively low) are not drastically decreased over four days. This suggests that lower doses of virus are affecting cell viability to a lesser extent than higher doses (discussed in chapter IV). Alternatively, slight viral growth in DC could be occuring leading to increased toxicity (discussed in chapter V).

III.2.2. Gene delivery using partially disabled vectors

Following the results with the minimally disabled vectors, a further disabled vector was tested in DC which contained an inactivating mutation in vmw65 and an insertion in vhs. vmw65 is a structural protein that cannot be completely removed as it is required for capsid assembly but its trans-activating capability can be inactivated by a 12 bp insertion into the region encoding the activation domain (Ace *et al.* 1989). This insertion removes the IE gene activation function of vmw65 but does not affect its structural function. Thus, 17+/vmw65/pR15 is partially disabled but can grow in culture by the addition of 3 mM hexamethylene bisacetamide (HMBA) which induce immediate early gene transcription. This vector 17+/vmw65/pR15 has a cassette (pR15) consisting of LAP1 linked to the MMLV-LTR promoter driving *LacZ* inserted into the UL41 gene (encoding vhs).

DC transduced with 17+/vmw65/pR15 at MOI=1 gave 43% β-gal expressing cells 24 hours post-infection (figure III.7). However, gene delivery was again reduced by more than a third by 72 hours. Thus, the inactivation in vmw65 together with vhs did not affect gene delivery compared to the previous vector 17+/pR20.5/vhs in which only vhs has been inactivated. Results with the partially disabled vector 17+/vmw65/pR15 suggest that further disabled vectors can be tested in DC and still retain the ability to deliver genes to DC efficiently.

Therefore, other partially disabled vectors were tested in DC with vmw65 inactivated and the non-essential gene γ34.5 (ICP34.5) deleted. ICP34.5 is a neurovirulence factor and thus ICP34.5 deleted vectors should generally provide a less virulent virus. A cassette (pR20.9) consisting of the LATP2 promoter linked to the MMLV-LTR promoter driving *GFP* and to the LAP1 promoter driving *LacZ* has been inserted in the UL43 or US5 gene respectively in viruses 1764/pR20.9/UL43 (ICP34.5 and UL43 deleted, vmw65 inactivated) or 1764/pR20.9/US5 (ICP34.5 deleted, vmw65 and UL43 inactivated). The pR15 cassette is inserted in the UL41 gene (encoding vhs) in virus 1764/pR15 (ICP34.5 deleted, vmw65 and UL41 inactivated).

DC transduced with 1764/pR20.9/UL43 and 1764/pR20.9/US5 at MOI=1 gave 70% GFP or β-gal positive cells in both cases, 24 hours post-infection, whereas transduction with 1764/pR15 resulted in only 33% gene delivery for β-gal (figures III.7 and III.8). In all cases, gene delivery was again significantly reduced (by up to 75%) compared to initial levels at 72 hours post-infection. Thus, the deletion/inactivation in ICP34.5 and vmw65 together with either the UL43 or US5 gene deletion gave very high gene delivery whereas this did not appear to be the case in combination with the vhs inactivation. Thus, even though the inactivation of vhs was shown to be important in the wild-type vector (17+/pR20.5/vhs) as well as in the partially disabled vector (vmw65/pR15), the further deletion of ICP34.5 in vector 1764/pR15 surprisingly gave the lowest gene delivery. This could not be explained at the time, but may be explicable in light of the results obtained later (see chapter IV).

Thus, another vector was tested in DC in order to test the hypothesis that high gene delivery can be achieved in DC with a vector disabled not only for ICP34.5 and vmw65 but also UL41 (vhs) and UL43 together (vector 1764/pR15/UL43MSVGFP). This partially disabled vector is based on 1764/pR15 with the insertion of a cassette containing the MSV promoter driving *GFP* in UL43. DC were transduced with this vector 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL43 and UL41 inactivated) at MOI=1 and gene delivery assessed as previously by monitoring GFP and β-gal levels post-infection.

Surprisingly, at least 81% of cells expressed GFP, 24 hours post-infection. GFP levels also remained significantly higher for 48 hours and were reduced by only half after 72 hours. However, at 24 hours only 13% β-gal positive cells were detected doubling to 24% at 48 hours (figures III.9 and III.10). Thus, the vector 1764/pR15/UL43MSVGFP gave the highest apparent gene delivery at MOI=1 when assessing GFP, even though β-gal expression levels were relatively low.

These results indicate that the combination of mutations in the 1764/pR15/UL43MSVGFP vector (ICP34.5 deleted, vmw65, UL43 and UL41 inactivated) gives the highest gene delivery to DC at least in terms of GFP expression. One explanation for the low level of β-gal expression detected 24 hours post-infection compared to the GFP level would be the different promoters used. However, the β-gal level at 24 hours is a third less than with vector 1764/pR15 which contains the same pR15 cassette in UL41 (vhs) but has the UL43 gene intact and the same 25% of β-gal positive cells is deleted with both vectors at 48 hours. Thus, it is possible that the reduced transgene expression from the pR15 cassette in the 1764/pR15/UL43MSVGFP vector is due to the presence of a factor encoded in the UL43 gene that affects LAP1/MMLV-LTR promoter activity. An alternative possibility is that transduced DC proteolytically process the delivered β-gal with the more disabled virus, as this is less toxic, which does not occur with the less disabled virus. This is explored in further experiments in which the processing ability of DC is

blocked by proteasome inhibitors and the β-gal (X-gal staining) and GFP (fluorescence) levels assessed. *LacZ* and *GFP* RNA levels are also compared by slot blots (see chapter V).

Dose and time related effects of the five partially disabled vectors were assessed in DC by transducing with 17+/vmw65/pR15, 1764/pR20.9/UL43, 1764/pR20.9/US5, 1764/pR15/UL43MSVGFP and 1764/pR15, at a range of MOI. At MOI ranging from 0.1 to 5, the percentage β-gal positive cells gradually increased from 23% to 58% with 17+/vmw65/pR15 and from 15% to 48% with 1764/pR15. Expression was the highest 24 hours post-infection. At the range of MOI tested, gene delivery was on average significantly reduced at 72 hours, and almost no β-gal was detected at 96 hours (figures III.11 and III.12).

Transduction with 1764/pR20.9/UL43 and 1764/pR20.9/US5 showed a similar trend. At MOI ranging from 0.1 to 10, gene delivery on average increased from 37% to 92%, and was the highest 24 hours post-infection. Again, as observed above, gene delivery on average was decreased by 80% of the original level, at 72 hours (figures III.13 and III.14).

In DC transduced with 1764/pR15/UL43MSVGFP at MOI ranging from 0.1 to 5, there were differences between apparent GFP and β-gal expression. While the apparent GFP level increased from 10% to 80%, and was the highest 24 hours post infection, the β-gal level only increased from 3% to 12%, at 24 hours and was higher at 48 hours (from 10% to 24%). Unlike with the previous viruses, gene delivery also remained high for 48 hours. However, it was reduced by more than half the original level at 72 hours (figure III.15).

Overall, these results show the same trend that the percentage GFP or β-gal expressing cells is dependent on the dose of the partially disabled virus used, as expected, even though this is not directly proportional to the MOI. Secondly, gene delivery is the highest at 24 hours and then reduces with time. This might be

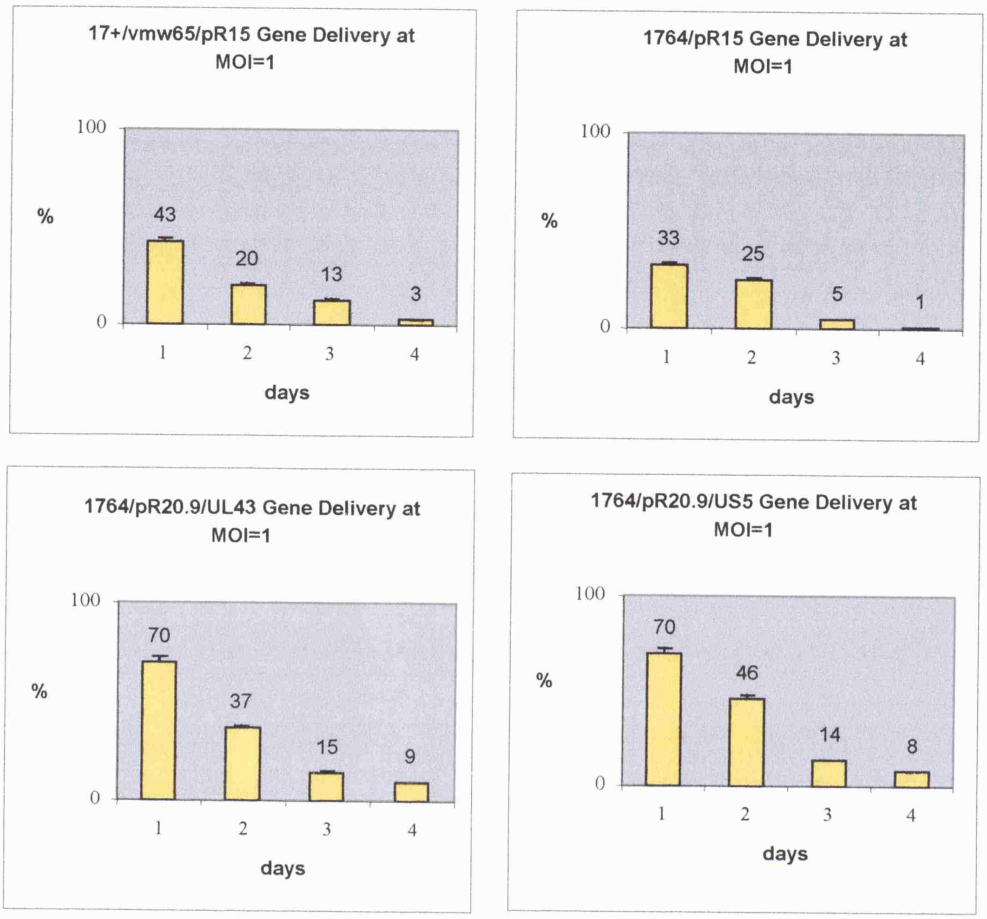


Figure III.7: Gene delivery to DC with partially disabled vectors at MOI=1. GFP and β-gal expressions were detected every 24 hours, for four days with 17+/vmw65/vhs (vmw65 and UL41 inactivated), 1764/pR15 (ICP34.5 deleted, vmw65 and UL41 inactivated), 1764/pR20.9/UL43 (ICP34.5 deleted, vmw65 and UL43 inactivated) or 1764/pR20.9/US5 (ICP34.5 and US5 deleted, vmw65 inactivated). When using viruses containing both *GFP* and *LacZ*, the percentage of cells expressing the corresponding proteins was identical.

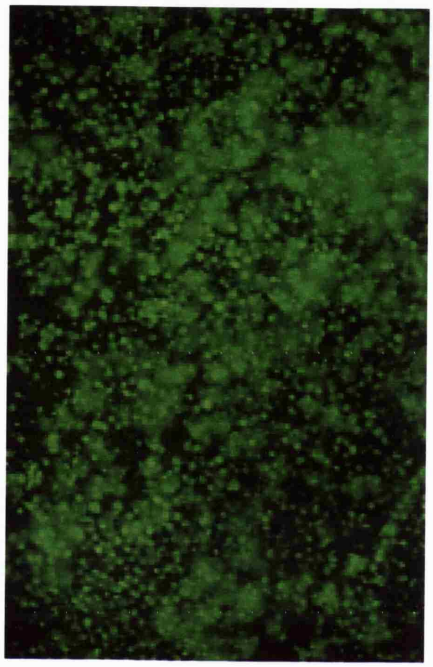


Figure III.8: GFP expression in DC following transduction with 1764/pR20.9/UL43 (ICP34.5 deleted, vmw65 and UL43 inactivated) at MOI=1, 24 hours post-infection.

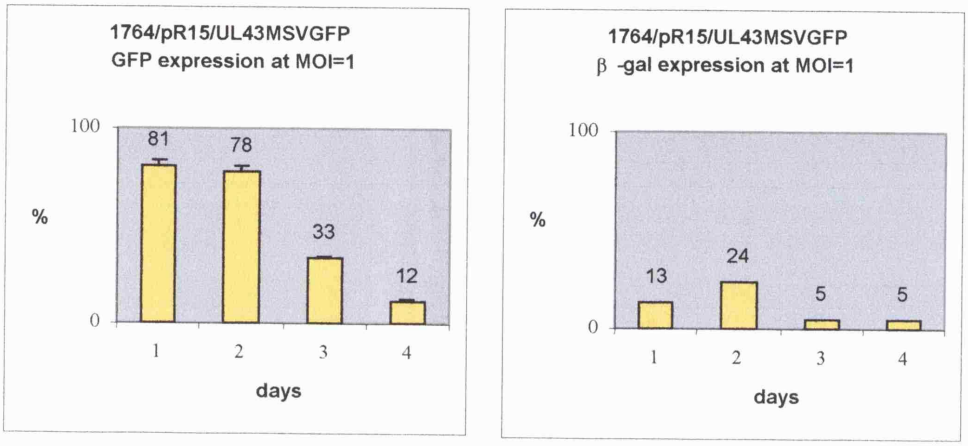


Figure III.9: Differential GFP and β-gal gene delivery to DC with the partially disabled vector 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL41 and UL43 inactivated). GFP and β-gal expression were detected every 24 hours, for four days after transduction of DC with 1764/pR15/UL43MSVGFP, at MOI=1.

Figure III.10: GFP expression in DC following transduction with vector 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL41 and UL43 inactivated) at MOI=1, 24 hours post-infection.

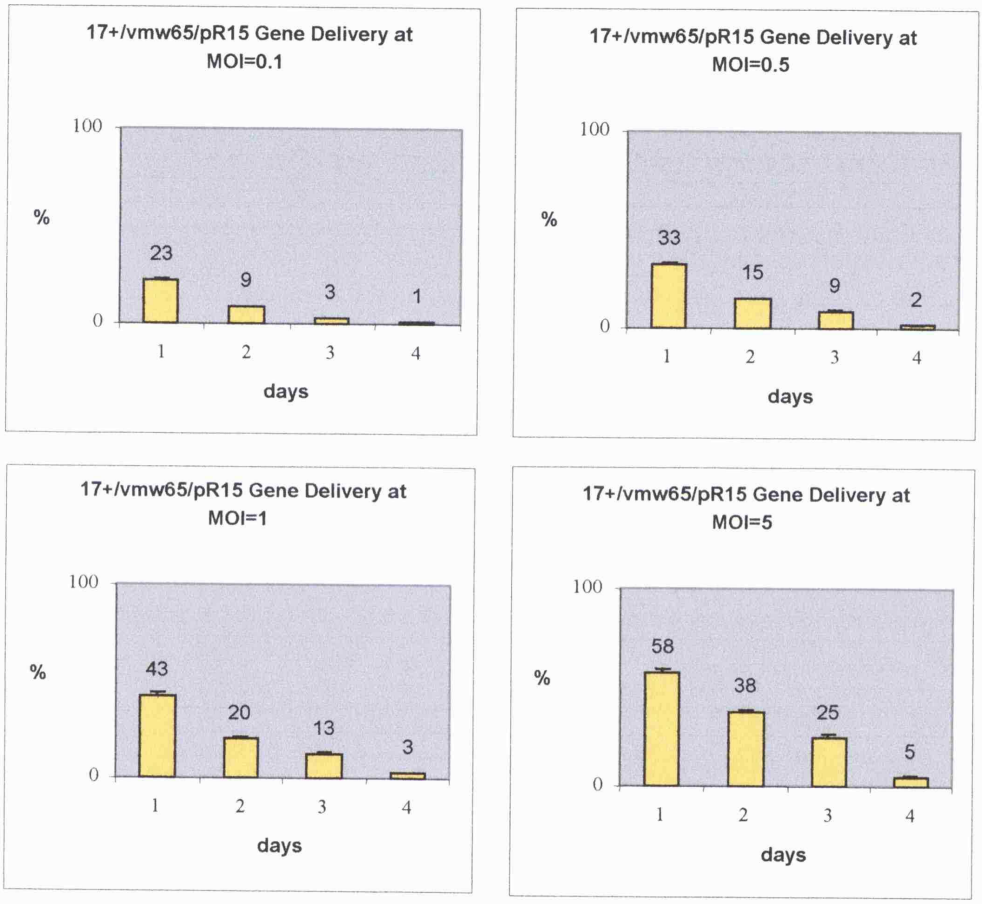


Figure III.11: Dose effect of 17+/vmw65/pR15 (vmw65 and UL41 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage β-gal expressing cells.

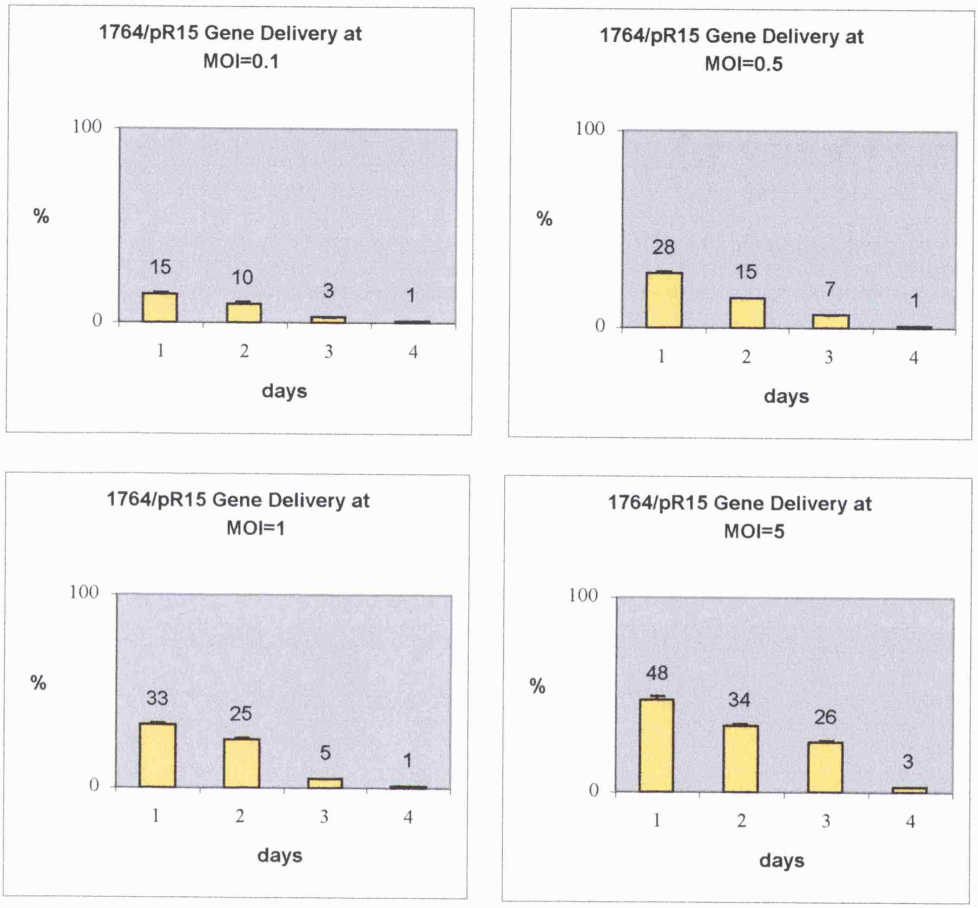
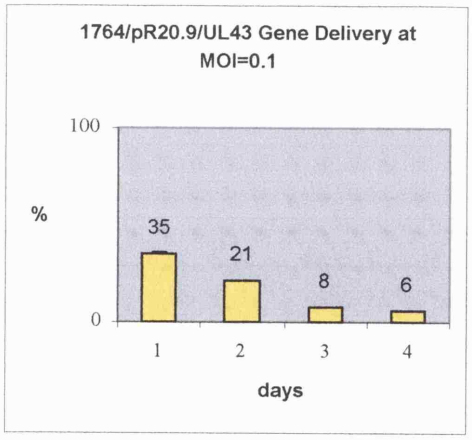
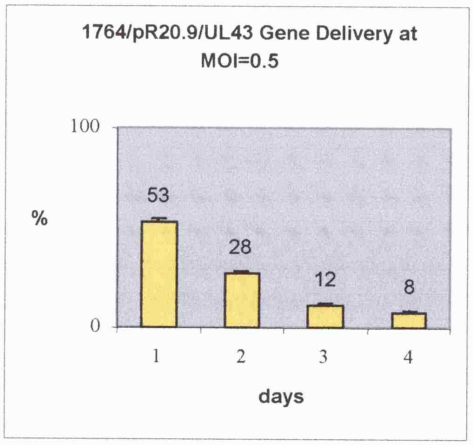
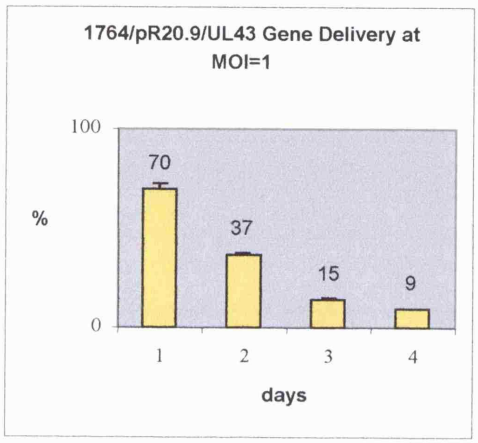
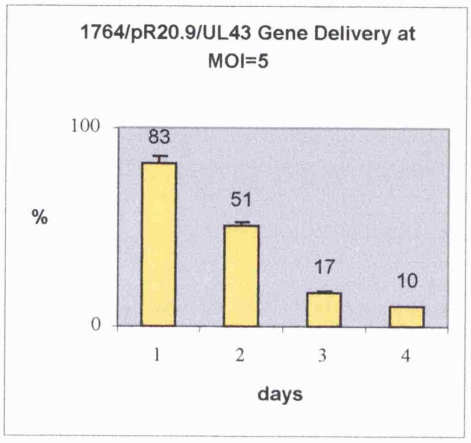


Figure III.12: Dose effect of 1764/pR15 (ICP34.5 deleted, vmw65 and UL41 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage β-gal expressing cells.









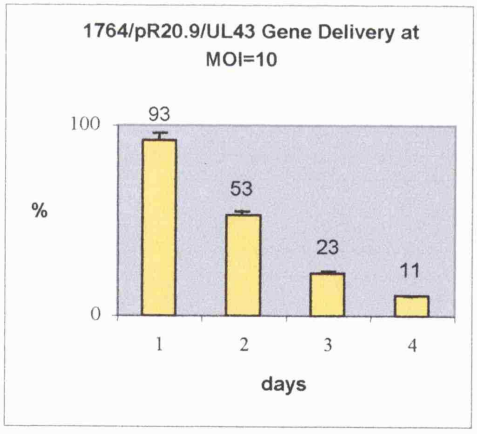


Figure III.13: Dose effect of 1764/pR20.9/UL43 (ICP34.5 deleted, vmw65 and UL43 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP or β-gal expressing cells.

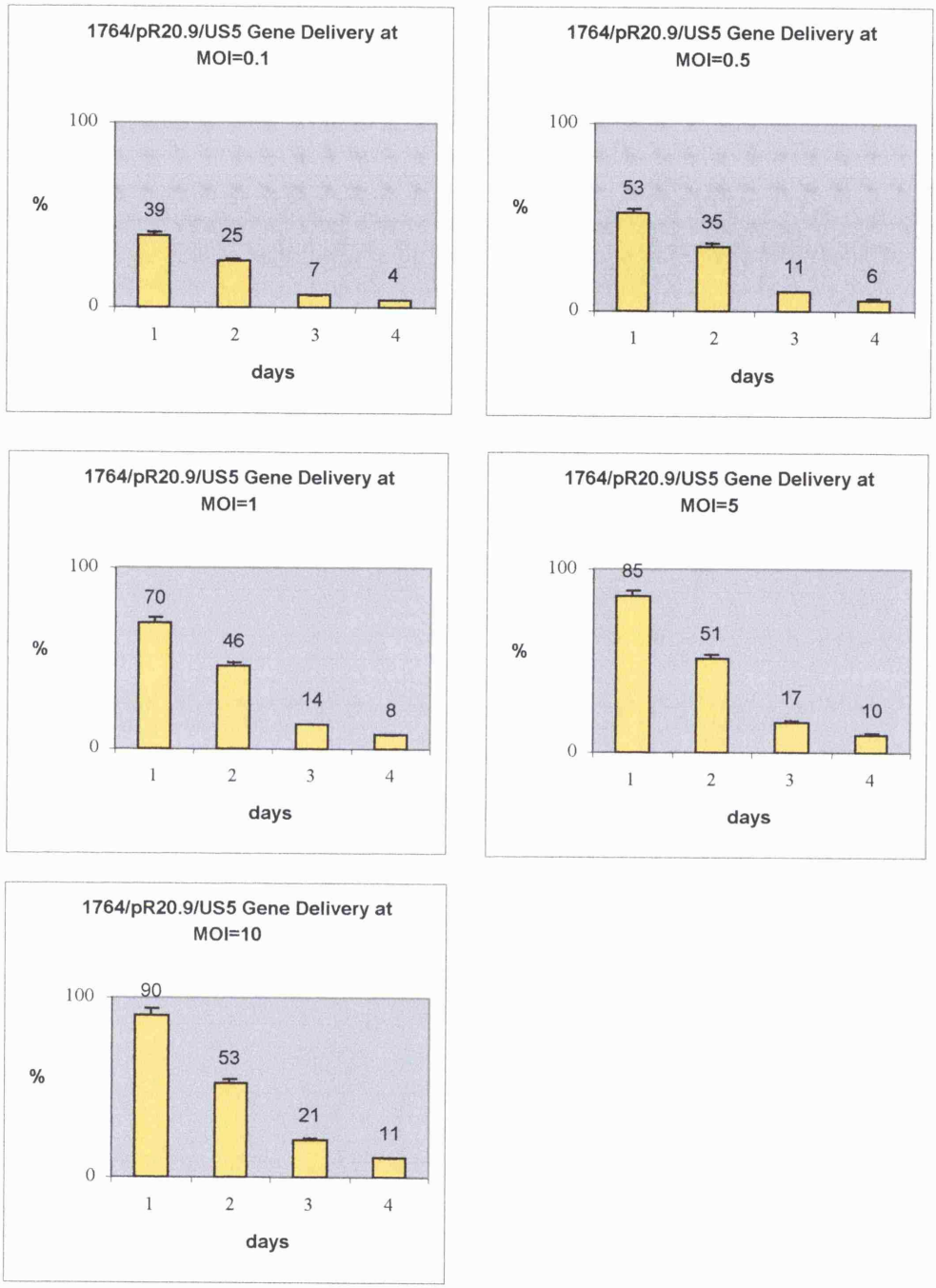


Figure III.14: Dose effect of 1764/pR20.9/US5 (ICP34.5 and US5 deleted, vmw65 inactivated) at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP or β-gal expressing cells.

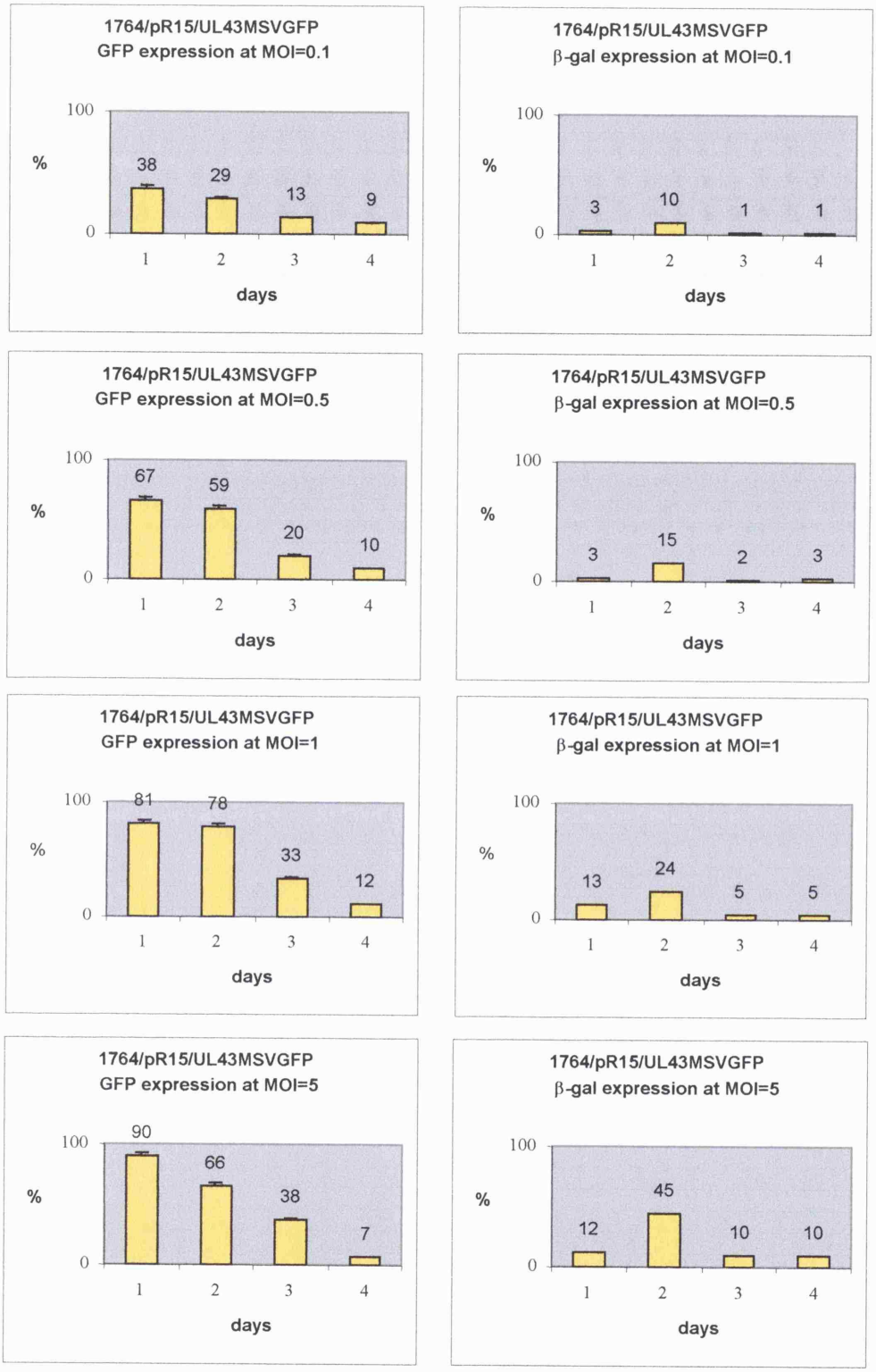


Figure III.15: Dose effect of 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL41 and UL43 inactivated) on DC at a range of MOI. Gene delivery was assessed every 24 hours, for four days as the percentage GFP (left side panel) and β-gal (right side panel) expressing cells.

explained by the activity of the promoters inserted in various loci of the HSV genome not allowing continued transgene expression over time. However, as one of the viruses (1764/pR15/UL43MSVGFP) gave higher level and more persistent expression of GFP (although low level of β-gal) an alternative explanation is that toxicity to DC occurs with the less disabled viruses. A particularly large differential expression effect of GFP and β-gal was also noted with this virus which may suggest that efficient antigen processing is occuring, and which is further explored later in this thesis.

III.2.3. Gene delivery using replication incompetent vectors

The fully disabled replication incompetent vectors used here are also based on HSV-1 strain 17+. These viruses have one or more deletions in essential genes together with in some cases deletions in non-essential genes. Five such vectors are used in this section. The base vector is deleted for the ICP27-encoding essential gene into which a cassette (pR20) consisting of the CMV promoter driving *LacZ* has been inserted. DC transduced with this vector (17+/27-/pR20) at an MOI of 1 gave an average of only 3% β-gal expression 24 hours post-infection (figure III.16), and at 72 hours, no β-gal could be detected in the cells.

Thus, this replication incompetent vector gave very low apparent gene delivery compared to the other vectors tested so far. As the CMV promoter was shown to be active in DC in the previous sections it appears that the ICP27 deletion itself is responsible for this low level of apparent gene delivery in DC at MOI=1. This result may be thought of as similar to, although more extreme than, the result found with 1764/pR15/UL43MSVGFP which gave high GFP expression but low β-gal expression which was hypothesised as being due to a reduced toxicity to DC compared to previous vectors tested. Thus the ICP27 deleted virus may be of even further reduced toxicity compared to 1764/pR15/UL43MSVGFP, allowing even more efficient antigen processing to occur. This is explored further later in this thesis. Following these observations, further replication incompetent vectors were tested in

DC in order to further test the phenotype in DC associated with the removal of various genes.

The 1764/27-/pR20.5/vhs vector is further disabled as compared to 17+/27-/pR20 by the removal of the non-essential gene encoding ICP34.5, the deletion of the LATP2 region and with an inactivating mutation in vmw65 and in UL41 (vhs). The pR20.5 cassette (LATP2 linked to CMV and RSV driving *GFP* and *LacZ* respectively) has been inserted in the unique NruI site of the UL41 gene (encoding vhs). DC transduced with 1764/27-/pR20.5/vhs (ICP34.5, ICP27 and LATP2 deleted, vmw65 and UL41 inactivated) at MOI=1, gave 15% GFP and 15% β-gal expression at 24 hours, and 3% GFP and 3% β-gal expression at 72 hours (figure III.16).

Thus, at MOI=1, higher gene delivery was achieved compared to the 17+/27-/pR20 vector. Therefore, the further disablement of the virus by the ICP27 deletion increased gene delivery five fold. However, this apparent gene delivery is still less than half that obtained with a similarly disabled vector but with ICP27 intact (1764/pR15) where approximately 30% gene delivery was recorded. This suggests that when ICP27 is deleted a similar effect occurs as with the UL43 deletion from 1764/pR15 – i.e. apparent gene delivery reduces (in this case for both β-gal and GFP). Each of three theories as to why this may occur (promoter silencing, lack of virus entry, or proteolytic processing of the GFP/β-gal) are explored later in this thesis.

However, it was apparent that at higher MOI 's (MOI of 0.1, 0.5, 1, 5, 10, 20 and 40 tested), increasing apparent gene delivery as recorded by GFP or β-gal positive cells at 24 hours occurred (5%, 10%, 15%, 25%, 40%, 45% and 50% respectively) (figure III.17). Therefore, high gene delivery as indicated by X-gal staining and GFP fluorescence (51%) can be reached with 1764/27-/pR20.5/vhs if high MOI are used. However, this is still considerably lower than with the partially disabled vectors at MOI=1, particularly 1764/pR15/UL43MSVGFP where 82% GFP fluorescence at MOI=1 could be achieved.

As well as the above viruses, an even further disabled vector was also tested in DC. This vector has the further deletion in the essential gene encoding ICP4 as compared to 1764/27-/pR20.5/vhs (ICP34.5, ICP27, ICP4 and LATP2 deleted, vmw65 and UL41 inactivated). DC transduced with this vector (1764/27-/4-/pR20.5/vhs) at MOI=1, gave 3% GFP expression and 30% β-gal expression 24 hours post-infection (figures III.18). At 72 hours, the apparent levels of GFP and β-gal were respectively reduced to 1% and 7%. This compares to 15% and 15% expression at 24 hours, and 3% and 3% at 72 hours respectively with 1764/27-/pR20.5/vhs.

This indicates that the differences recorded between GFP and β-gal levels with these vectors are not due to a differential activity of the promoters in the pR20.5 cassette (RSV/LATP2/CMV) in DC but are associated with other factors associated with the infection of DC with differentially disabled viruses. Similar differences in the levels of expression of GFP and β-gal were previously shown with 1764/pR15/UL43MSVGFP, but in this case high GFP levels (81%) and low β-gal levels (13%) were observed. Thus, minimally disabled vectors give relatively high level gene delivery (~ 30% at MOI=1) which is equal between GFP and β-gal. More disabled vectors give higher apparent GFP delivery, but lower β-gal, and replication incompetent vectors give relatively low apparent gene delivery of both GFP and β-gal. It is possible that this differential and reducing apparent gene delivery is due to antigen processing in DC. This again suggests that the levels of transgene expression are in reality greater than the apparent levels recorded and this is explored further later.

In order to investigate this phenomenon further, two other replication incompetent vectors were tested in DC. Both vectors are equally disabled (ICP34.5, ICP27, ICP4 deleted, vmw65 inactivated) and contain the same promoter driving transgene expression in the pR19 cassette (CMV) inserted in the LAT region of the virus, but express either *GFP* or *LacZ*. Transduction of DC with 1764/27-/4-/pR19GFP and 1764/27-/4-/pR19LacZ at MOI=1, respectively gave 5% GFP and 17% β-gal positive cells, 24 hours post-infection (figure III.20). Thus even when

driven by the same promoter, differential expression levels are observed suggestive of differential processing occuring in the cells.

Transduction of DC with 1764/27-/4-/pR19GFP or LacZ gave the same level of GFP but a slightly lower level of β-gal as with 1764/27-/4-/pR20.5/vhs. This may be explained by the fact that the further removal of vhs as previously noticed with the wild-type and partially disabled vectors is important for high efficiency gene delivery at least in terms of β-gal expression in this context.

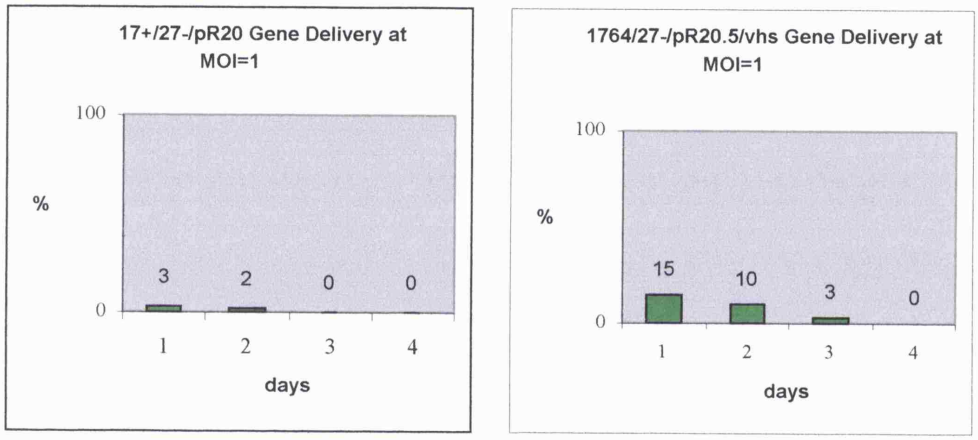


Figure III.16: Gene delivery to DC with replication incompetent vectors at MOI=1. GFP and β-gal expressions were monitored every 24 hours, for four days with 17+/27-/pR20 (ICP27 deleted) or 1764/27-/pR20.5/vhs (ICP34.5, ICP27 and LATP2 deleted, vmw65 and UL41 inactivated). The graph shows the percentage β-gal and GFP expression after transduction of DC with respectively 17+/27-/pR20 and 1764/27-/pR20.5/vhs, at the indicated time points.

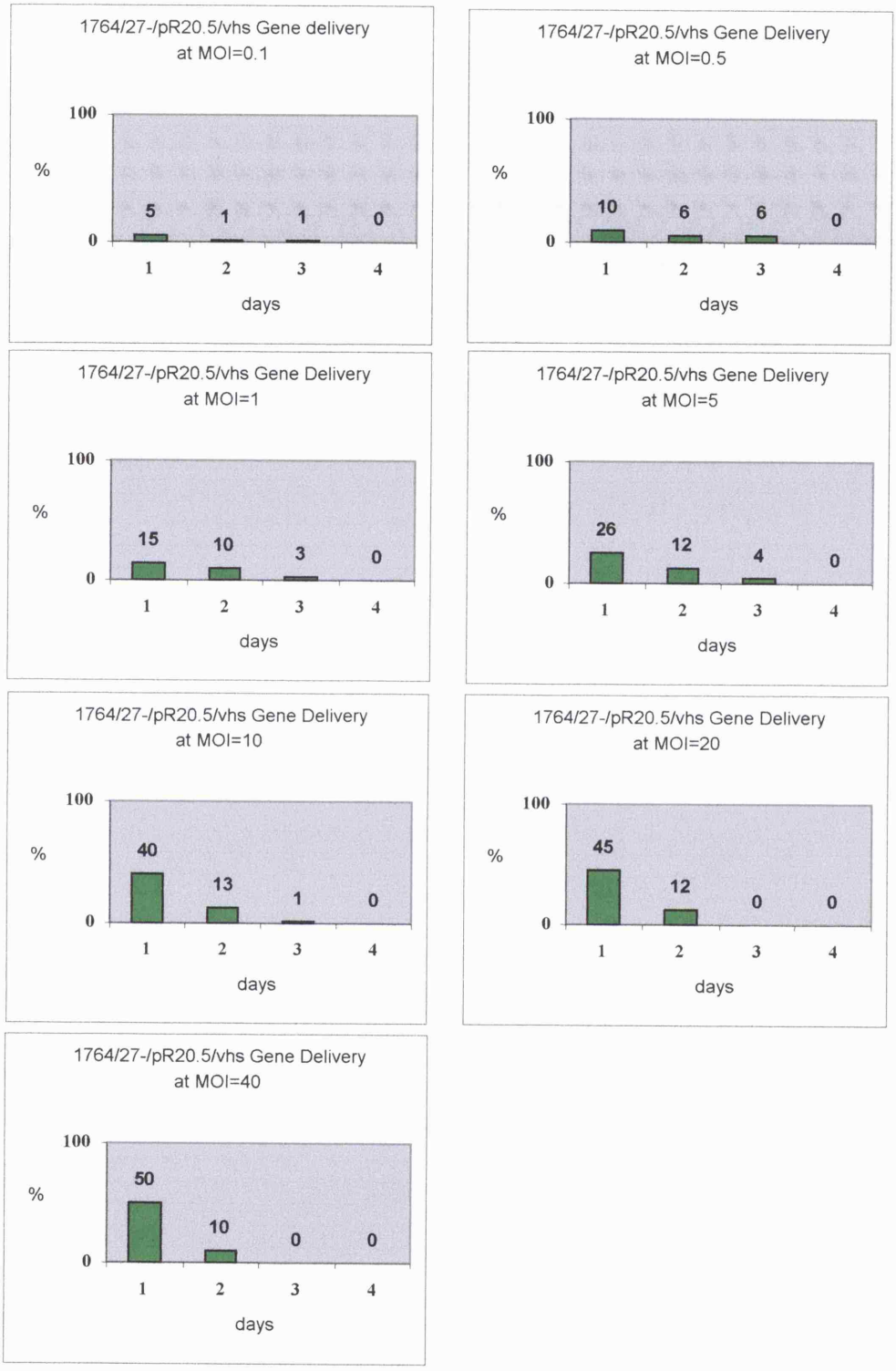


Figure III.17: Gene delivery to DC with 1764/27-/pR20.5/vhs at a range of MOI. GFP expression was detected, 24 hours post-infection.

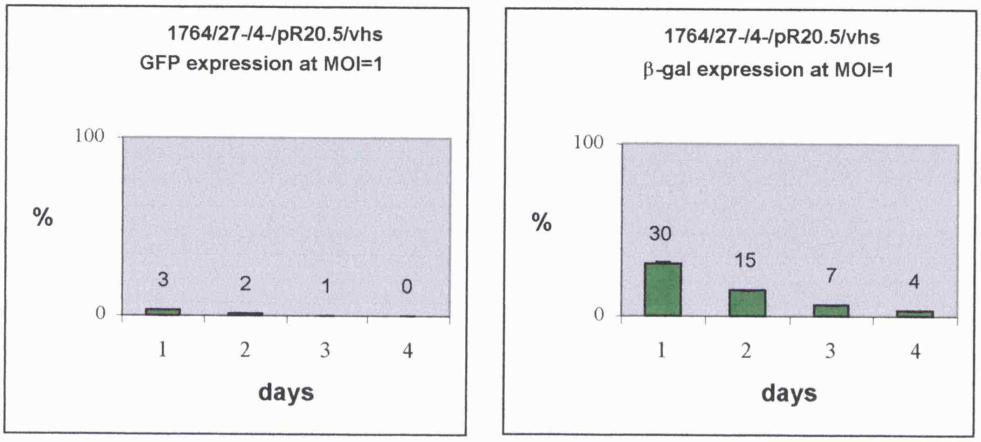


Figure III.18: Gene delivery to DC with the replication incompetent vector 1764/27-/4-/pR20.5/vhs (ICP34.5, ICP27, ICP4 and LATP2 deleted, and vmw65 and UL41 inactivated). GFP and β-gal expressions were monitored every 24 hours, for four days. The graphs show the percentage GFP and β-gal after transduction of DC with 1764/27-/4-/pR20.5/vhs at MOI=1.

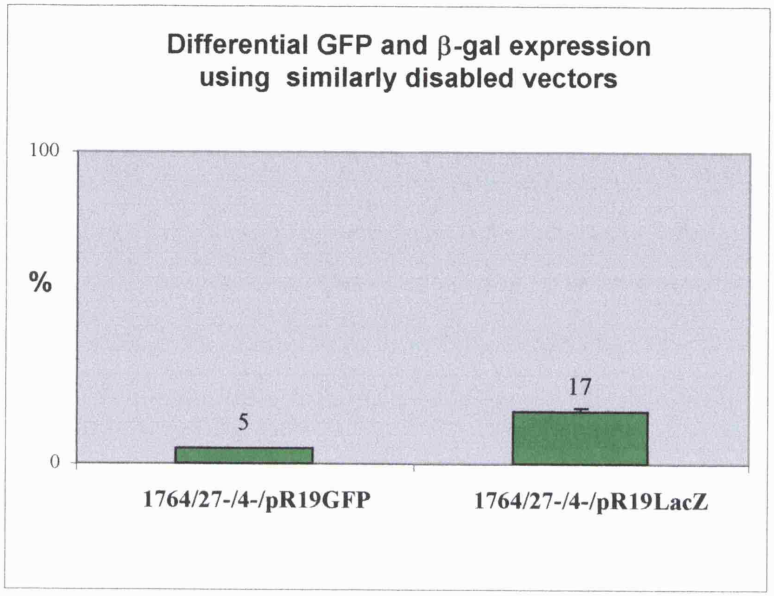


Figure III.19: Gene delivery to DC with 1764/27-/4-/pR19GFP and 1764/27-/4-/pR19LacZ. Both vectors have ICP27, ICP4 and ICP34.5 deleted, and vmw65 inactivated. Vectors differ by the reporter gene contained within the transgene cassette both driven by the CMV promoter. GFP and β-gal expression were assessed 24 hours, at MOI=1.

III.3. Discussion

A panel of HSV-based vectors were tested in DC at a range of MOI and over a period of four days in order to assess gene delivery to DC. These results are summarised in table III.3 and figure III.21 and are discussed below in terms of:

1) The effects of individual or combined gene deletion or inactivation on gene delivery.
2) The effects of promoters in relation to the insertion of the transgene cassette in different loci of the HSV genome and their activity with time.
3) The effects of virus dose with time on gene delivery.

Gene delivery was high with the single deletion of either UL43 or US5. UL41 (vhs) gene inactivation alone gave slightly better results still. The inactivation of vmw65 in combination with UL41 (vhs) gave similar levels of gene delivery as compared to less disabled vectors but the further deletion of ICP34.5 rather than vmw65 reduced gene delivery. However, in vectors with ICP34.5 deleted, vmw65 inactivated, and UL43 or US5 deleted, gene delivery was nearly twice that compared to vectors with only UL43 or US5 inactivated. Thus, the ICP34.5/vmw65 (1764) gene deletion/inactivation gives better gene delivery when combined with the UL43 or US5 deletion but not with the UL41 (vhs) inactivation. However, as discussed later this lower apparent delivery may have resulted from processing of β-gal. Furthermore, when ICP34.5/vmw65 deletion/inactivation was combined with the UL41 (vhs) and UL43 gene inactivation, the highest gene delivery was observed, at least in terms of GFP expression. Surprisingly, apparent β-gal levels were 60-70% lower than the GFP level and 50% lower than the apparent β-gal levels from a similarly disabled vector in which UL43 was intact.

The single deletion in the essential gene encoding ICP27 gave very low apparent gene delivery to DC. The further deletion of ICP34.5, and further inactivation of vmw65 and UL41 (vhs), gave higher GFP or β-gal expression. This

confirms that the ICP34.5/vmw65 deletion/inactivation increases apparent gene delivery as noticed with the partially disabled vectors. A further disabled vector, in which ICP4 was also deleted, showed the highest apparent gene delivery in terms of β-gal expression for the replication incompetent vectors. However, the apparent GFP level was only 10% that of β-gal and was identical to transgene expression from a vector with ICP27 deleted alone and the CMV promoter driving the expression of GFP.

Vectors of a similar level of disablement (ICP34.5/vmw65 deleted/inactivated, ICP27 and ICP4 deleted) but containing the pR19 cassette (LAP1/LATP2) in the LAT region instead of the pR20.5 cassette in the UL41 locus showed very similar results in terms of differential apparent transgene expression in DC when expressing either GFP or β-gal.

The differences seen in the levels of GFP or β-gal detected after transduction of DC with 1764/pR15UL43MSVGFP, 1764/27-/4-/pR20.5/vhs, 1764/27-/4-/pR19GFP or LacZ show that these differences are not due to differential promoter activity in the pR15, pR20.5 or pR19 cassette, but that levels of each transgene detected is mainly dependent on the particular deletions from the HSV vector used. Thus, a reasonable hypothesis would be that as vectors which are less toxic to DC are used, DC transduced with these vectors retain more of the ability to process antigens and differences in the ability to process GFP or β-gal results in the differences in detectable levels of the transgene observed.

Levels of detectable gene expression were increased with higher doses of virus. However, the increase was not directly proportional to the MOI. It can be seen that at MOI=1 with 1764/pR15/UL43MSVGFP at least 80% of cells are infected as assessed by GFP expression, and as it would seem unlikely that removing particular genes would affect their infectability, it seems more likely that with viruses which give low detectable levels of GFP and/or β-gal at MOI=1 (hypothesised to be due to antigen processing), the increase in apparent gene delivery at higher MOI is due to

increasing toxicity and thus increasing inhibition of antigen processing, resulting in higher detectable gene expression levels. The dose effect in relation to time showed that gene delivery was the highest at 24 hours and then decreased with time in the following three days. However, with the wild-type vectors but not with the partially disabled vectors at lower MOI, gene delivery was maintained which suggests that very low doses of wild-type virus are not toxic to the cells. This may suggest that cells do not detect the virus at least when UL43 or US5 is deleted, thus DC do not undergo apoptosis/necrosis. It also suggests that with the wild-type vectors as compared to more disabled vectors, because high titers can be obtained more easily, cleaner stocks of virus may beneficially affect the maintenance of gene delivery with time. In addition, the results obtained with the wild-type vectors at low MOI might suggest that some residual virus growth is occurring in DC with these viruses. This is tested later in this thesis (chapter V).

So far, the best vectors for gene delivery to DC are the partially disabled vector 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL41 and UL43 inactivated) and the replication incompetent vector 1764/27-/4-/pR20.5/vhs (ICP34.5, ICP27, ICP4 and LATP2 deleted and vmw65 and UL41 inactivated). In order to conclude which vector gives the best balance of safety and gene delivery to DC, further experiments are required to assess the effect of these viruses on cell viability (chapter IV), to monitor viral growth and HSV IE protein expression (chapter V) and to understand better if differences noticed in gene delivery are due to a promoter effect and/or to differential processing of the delivered antigen (GFP/β-gal) by DC (chapter V).

Virus Nomenclature	Genes deleted (d) or inactivated (i)	Promoter driving GFP	Promoter driving LacZ	% GFP (MOI=1) 24h - 72h	% β-gal (MOI=1) 24h, 72h
17+/pR20.5/UL43	UL43 (i)	LATP2-CMV	LATP2-RSV	**43 - 22**	**43 - 22**
17+/pR20.5/US5	US5 (d)	LATP2-CMV	LATP2-RSV	**44 - 16**	**44 - 16**
17+/pR20.5/vhs	UL41 (i)	LATP2-CMV	LATP2-RSV	**49 - 24**	**49 - 24**
17+/UL43CMVGFP	UL43 (i)	CMV	NA	**40 - 18**	**NA**
17+/vmw65/pR15	vmw65 (i), UL41 (i)	NA	LAP1-MMLV-LTR	**NA**	**43 - 13**
1764/pR20.9/UL43	ICP34.5 (d), vmw65 (i), UL43 (d)	LATP2-MMLV-LTR	LATP2-LAP1	**70 - 15**	**70 - 15**
1764/pR20.9/US5	ICP34.5 (d), vmw65 (i), US5 (d)	LATP2-MMLV-LTR	LATP2-LAP1	**70 - 14**	**70 - 14**
1764/pR15	ICP34.5 (d), vmw65 (i), UL41 (i)	NA	LAP1-MMLV-LTR	**NA**	**33 - 5**
1764/pR15/ UL43MSVGFP	ICP34.5 (d), vmw65 (i), UL41 (i), UL43 (I)	MSV	LAP1-MMLV-LTR	81 – 27	13 - 5
17+/27-/pR20	ICP27 (d)	NA	CMV	**NA**	**3 - 0**
1764/27-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d)	LATP2-CMV	LATP2-RSV	**15 - 3**	**15- 3**
1764/27-/4-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d), ICP4 (d)	LATP2-CMV	LATP2-RSV	3 - 1	30 - 7
1764/27-/4-/pR19GFP	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	LAP1-LATP2-CMV	NA	**5**	**NA**
1764/27-/4-/pR19LacZ	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	NA	LAP1-LATP2-CMV	**NA**	**17**

Table III.3: Summary of the gene delivery results using different vectors. Abbreviations: 17+: HSV-1 wt strain. 1764: deletion in the ICP34.5 gene and inactivating mutation in vmw65. UL: unique long region. US: unique short region. ICP: infected cell polypeptide. vhs: virion host shut-off. LAP1: latency-associated promoter. LATP2: latency-associated transcript promoter 2. CMV: cytomegalovirus IE promoter. RSV: rous sarcoma virus promoter. MMLV-LTR: moloney murine leukaemia virus long terminal repeat promoter. MSV: murine simian virus promoter. NA: not applicable

CHAPTER IV

EFFECTS OF HSV VECTORS ON DENDRITIC CELL SURVIVAL

IV.1. Introduction

In chapter III, a panel of vectors were used in order to assess their efficiency for gene delivery to DC. Previous work has shown that HSV-1 induces apoptosis in a number of cell types and that inhibition of DC function is noted following infection with wild-type HSV (Kruse *et al.* 2000). In addition, some work has been performed showing that HSV does induce limited apoptosis in a proportion of dendritic cells. However, this seems at a delayed time following infection (Immunology Department, UCL, unpublished results). Thus, the objective of this chapter was to assess the effects of various HSV-1 vectors on cell survival.

The viability of cells was measured by trypan blue exclusion daily, for four days after transduction of DC with the various vectors used in chapter III. In this assay, live cells pump out the blue dye and are white under the microscope whereas dead cells cannot do it and thus are blue. A range of MOI was used for the majority of these vectors in order to assess dose-related effects on cell survival. Experiments were repeated three times and the average values for cell survival are presented in this chapter.

IV.2. HSV-1 mediated effects on cell survival

Non-transduced mock-infected cells were followed as a control for the assessment of cell survival. 24 hours after transduction, 100% of non-transduced cells were viable in culture. This percentage decreased on average to 94% by four days. The average percentage cell survival of non-infected cells over four days is presented together with the cell survival using the various vectors.

IV.2.1 Cell survival using minimally disabled HSV-1 vectors

Cell survival was assessed 24 hours after transduction of DC with 17+/pR20.5/UL43 at MOI ranging from 0.1 to 10. At increasing MOI, on average cell

survival decreased from 94% down to 80% (figure IV.1). At MOI=1, 88% of cells were alive 24 hours post-infection. These results indicate that minimally disabled HSV (17+/pR20.5/UL43) is not highly toxic to DC even at higher MOI at least as assessed by the trypan blue exclusion assay. In order to determine the importance of the UL43 deletion for cell survival, the effect of other minimally disabled vectors with a different gene inactivated were also tested.

After transduction with 17+/pR20.5/UL43, 17+/pR20.5/US5, 17+/pR20.5/vhs or 17+/UL43CMVGFP cell survival was 88%, 94%, 93% and 94% respectively 24 hours post-infection and was decreased to 75%, 77%, 79% and 73% respectively at 72 hours (figure IV.2). Thus, vector 17+/pR20.5/vhs gave slightly better results than 17+/pR20.5/UL43 or 17+/pR20.5/US5. This shows that minimally disabled vectors all give high cell survival rates in DC and that the inactivation in UL41 may slightly reduce toxicity further.

Dose and time related effects of three of these vectors were compared in DC by assessing cell survival every day, for four days following transduction with 17+/pR20.5/UL43, 17+/pR20.5/US5 or 17+/UL43CMVGFP.

In DC transduced with 17+/pR20.5/UL43 at MOI ranging from 0.1 to 10, cell survival decreased from 94% to 80%, 24 hours post-infection. At 72 hours compared to 24 hours, cell survival was further reduced by only a further 2-4% at MOI=0.1 and 0.5 after taking into account the reduction in the control cells, and by a further 10-20% at MOI=1 and 5. However, at MOI=10, up to a 50% further reduction in cell survival was observed at 72 hours post-infection (figure IV.3). Results with other vectors were very similar with no significant differences observed (figures IV.4 and IV.5).

Thus minimally disabled HSV is relatively non-toxic to DC, as assessed by cell survival, although cell death does increase with time and increasing MOI.

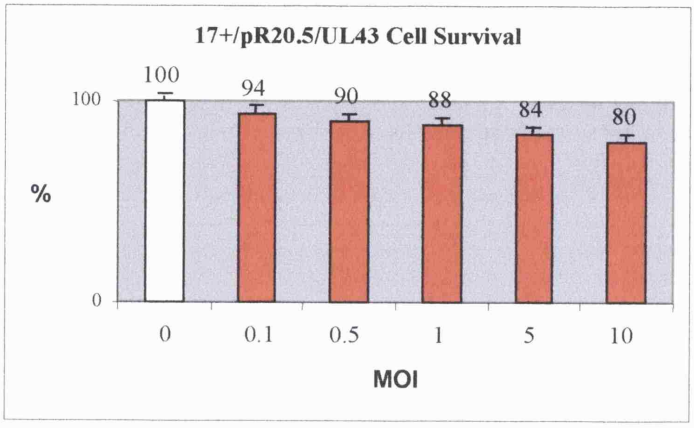


Figure IV.1: Dose effect of HSV on dendritic cell survival using 17+/pR20.5/UL43 at a range of MOI. Cell survival was assessed by the trypan blue exclusion assay after transduction of DC at a range of MOI, with minimally disabled HSV. The graph shows the percentage cell survival, 24 hours post-infection for mock infected cells (control) and after transduction with 17+/pR20.5/UL43 (UL43 inactivated).

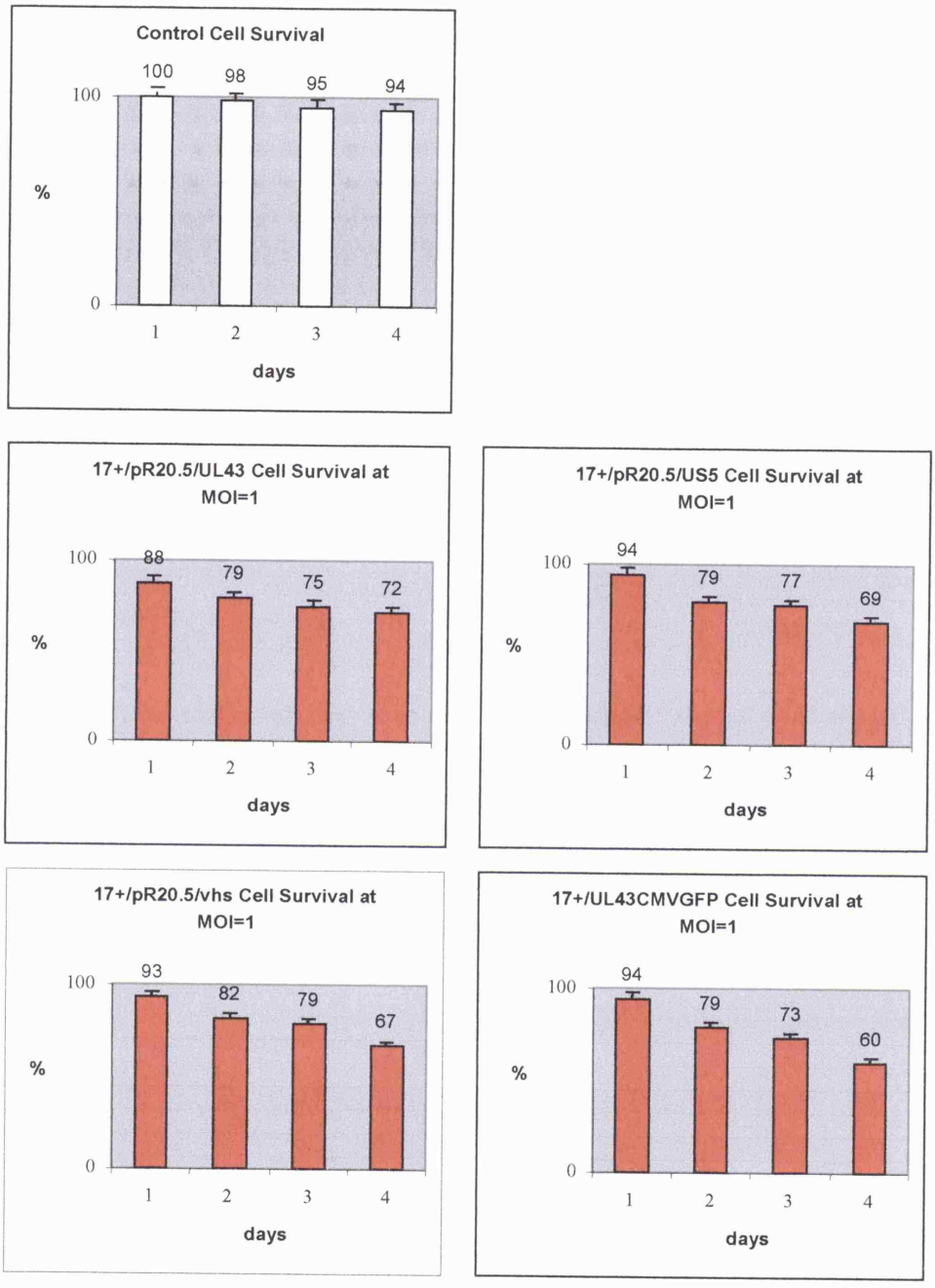


Figure IV.2: Cell survival assessed by the trypan blue exclusion assay after transduction of DC with minimally disabled vectors at MOI=1. The graphs show the percentage cell survival over time for mock infected cells (control) and after transduction with the indicated vectors.

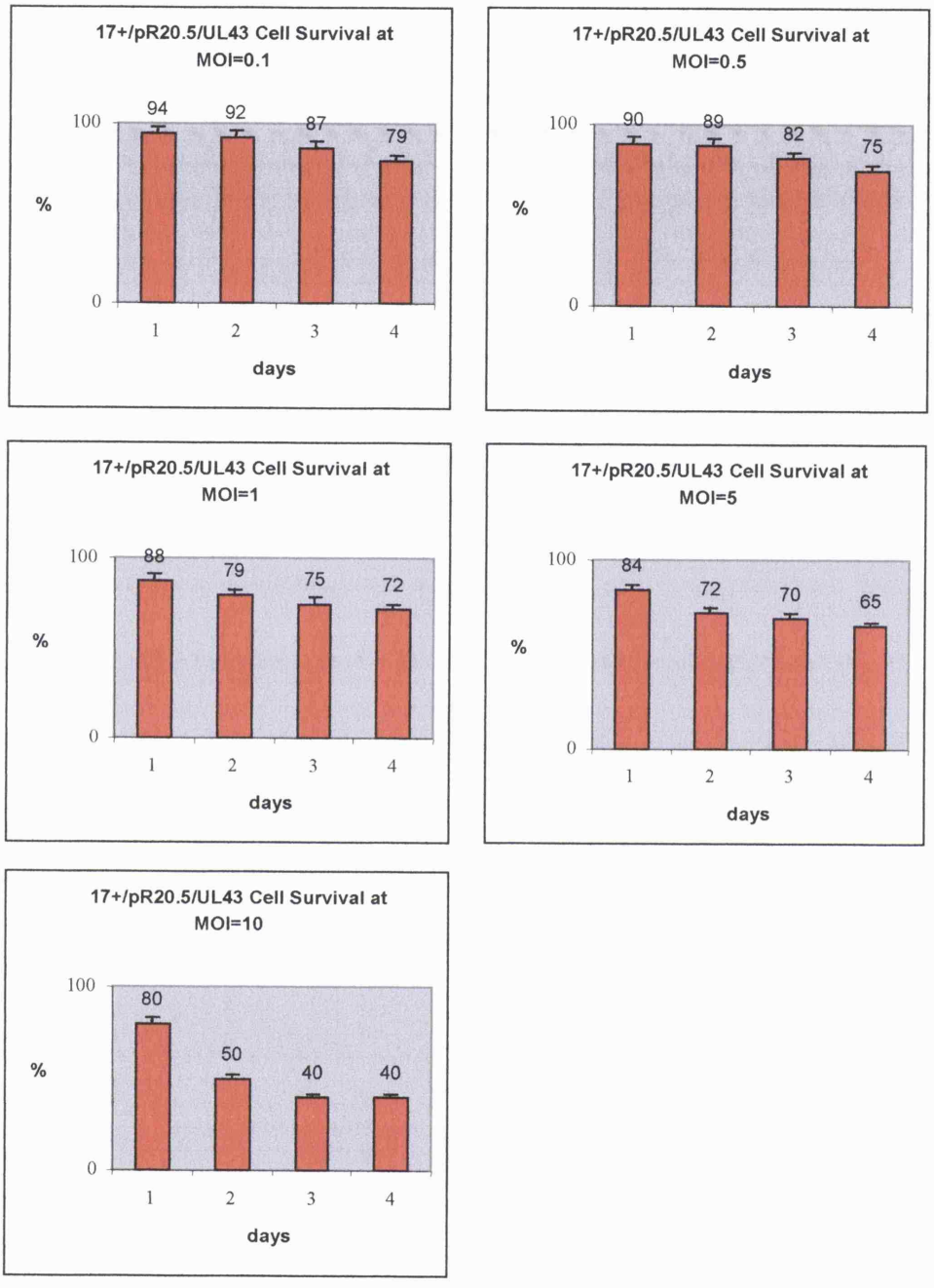


Figure IV.3: Dose effect of 17+/pR20.5/UL43 (UL43 inactivated) at a range of MOI

.

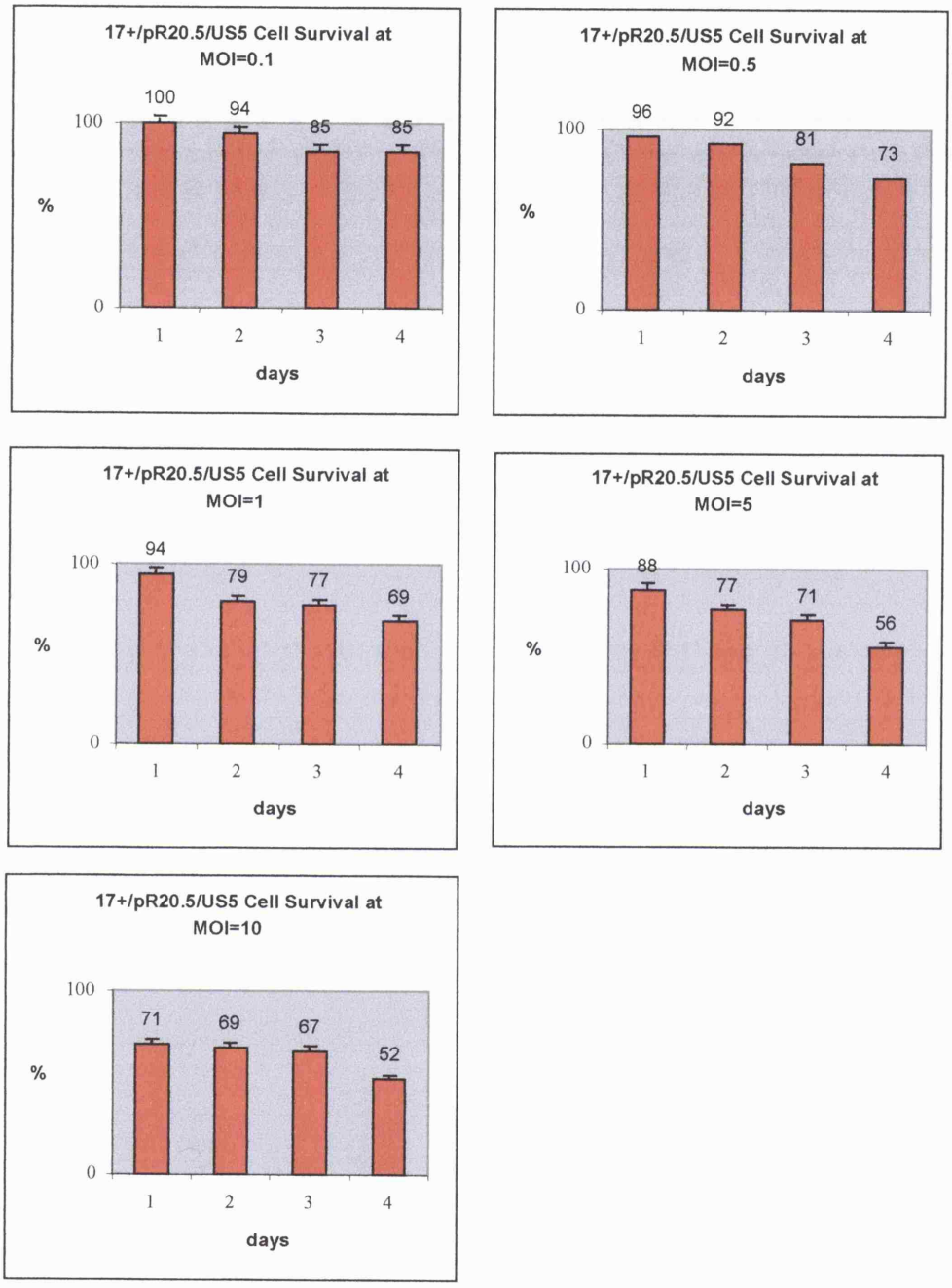


Figure IV.4: Dose effect of 17+/pR20.5/US5 (US5 deleted) at a range of MOI.

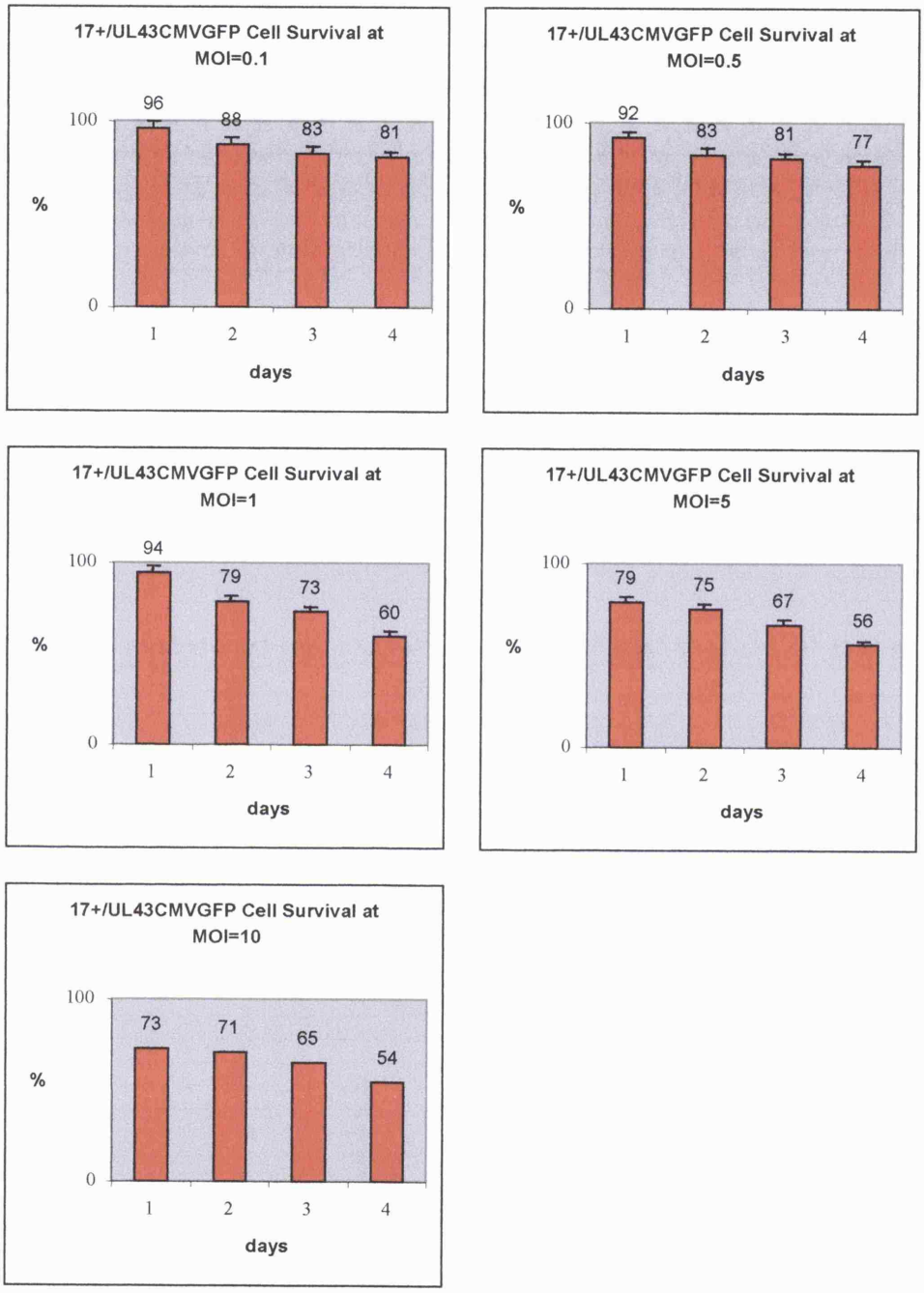


Figure IV.5: Dose effect of 17+/UL43CMVGFP (UL43 inactivated) at a range of MOI.

IV.2.2 Cell survival using partially disabled HSV-1 vectors

In this section, DC were transduced with five partially disabled vectors 17+/vmw65/pR15, 1764/pR20.9/UL43, 1764/pR20.9/US5, 1764/pR15, and 1764/pR15/ UL3MSVGFP in order to assess their effect on cell survival. Surprisingly, the partially disabled vectors generally gave slightly lower cell survival in DC compared to the minimally disabled vectors tested in the last section, although1764/pR15/UL43MSVGFP gave comparably good survival (figures IV.6, IV.7, IV.8, IV.9, IV.10, IV.11).

These results show a similar trend as with the minimally disabled viruses that cell survival is reduced with increasing MOI although this is not directly proportional. As noticed for the previous vectors, there is a consistent reduction of cell survival with time which is more marked at higher MOI. The highest percentage cell survival was obtained with 1764/pR15/UL43MSVFGP for all MOI tested. This indicates as previously found that both the inactivation in UL41 (vhs) and UL43 give high cell survival. Cell survival was slightly higher with 1764/pR20.9/UL43 compared to 1764/pR20.9/US5 at most MOI tested except MOI=0.1 and 10. Cell survival was the lowest with 17+/vmw65/pR15 at all MOI tested.

In this section, cell survival was assessed after transduction of DC with five partially disabled vectors. The partially disabled vectors gave relatively low cell survival in DC compared to minimally disabled vectors. The reason for this is not clear or obvious. However, these vectors generally give lower titers following growth than minimally disabled vectors, thus a greater volume of the virus preparation must be added to the cells to give similar MOI which may increase toxicity to cells due to carry over of more cell debris (virus titers expressed in pfu/ml are listed in table III.2). However, as cell debris from virally infected cells has separately been shown not to be toxic to DC (data not shown) and cell survival was consistent between different stocks of the same virus, even this explanation seems unlikely. As the particle/pfu ratios for partially disabled vectors are higher than for minimally disabled

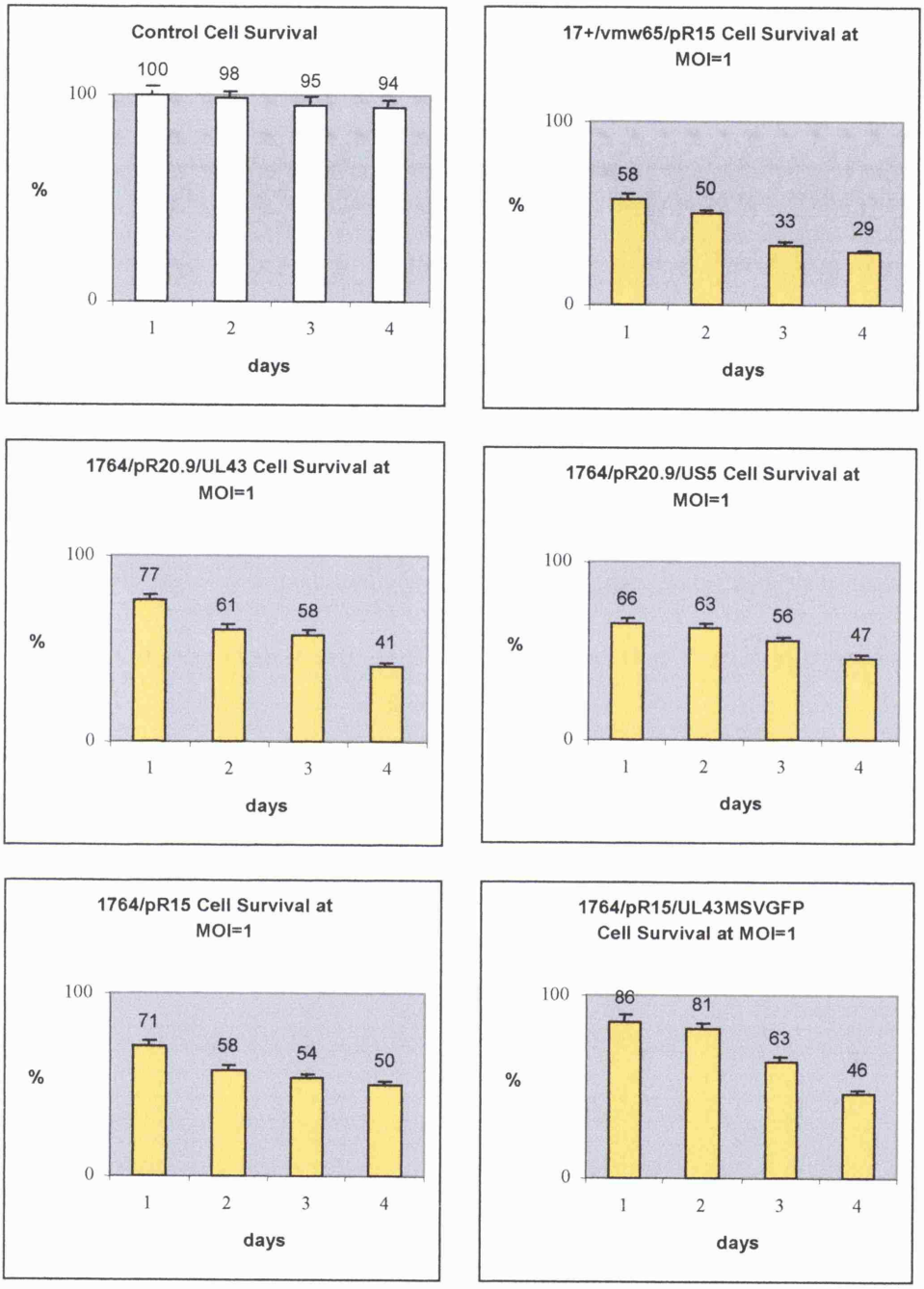


Figure IV.6: Cell survival assessed by the trypan blue exclusion assay after transduction of DC with the partially disabled vectors at MOI=1.

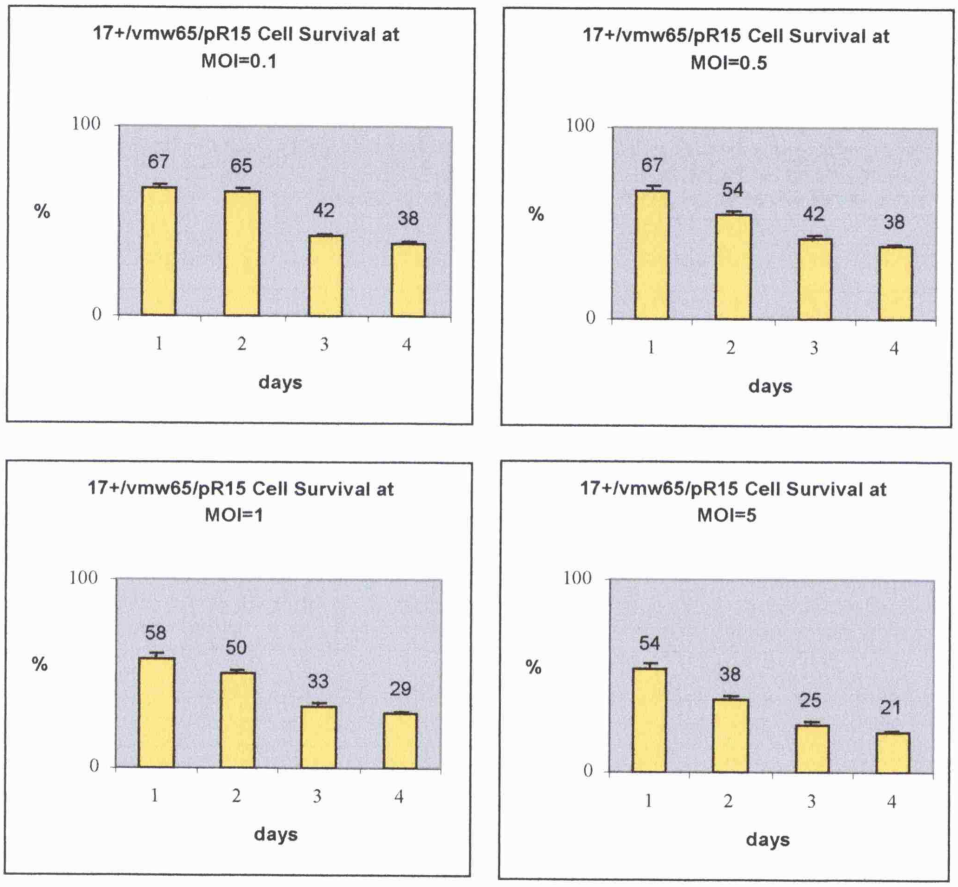


Figure IV.7: Dose effect of partially disabled HSV on DC survival using 17+/vmw65/pR15 (vmw65 and UL41 inactivated) at a range of MOI.

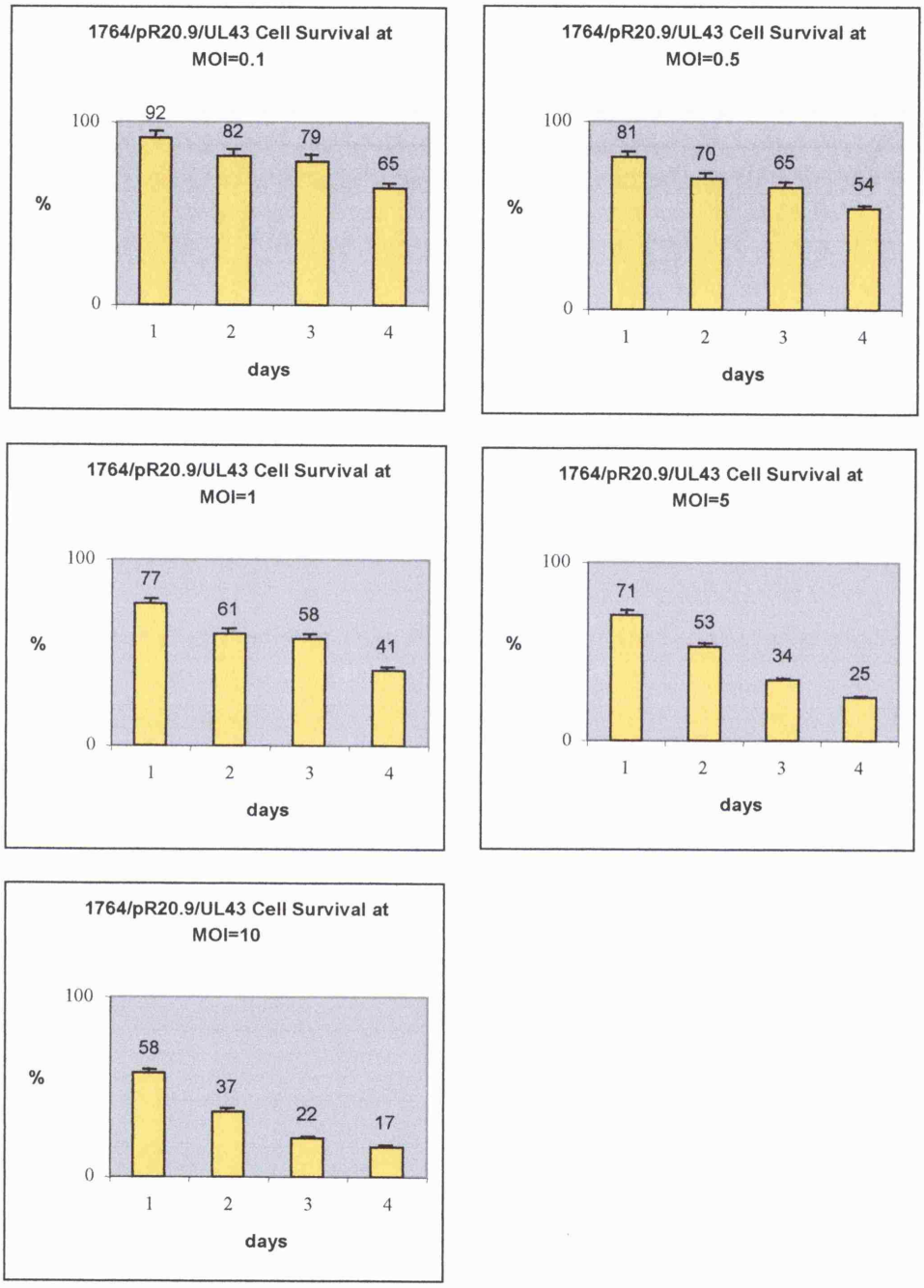


Figure IV.8: Dose effect of 1764/pR20.9/UL43 (ICP34.5 deleted, vmw65 and UL43 inactivated) at a range of MOI.

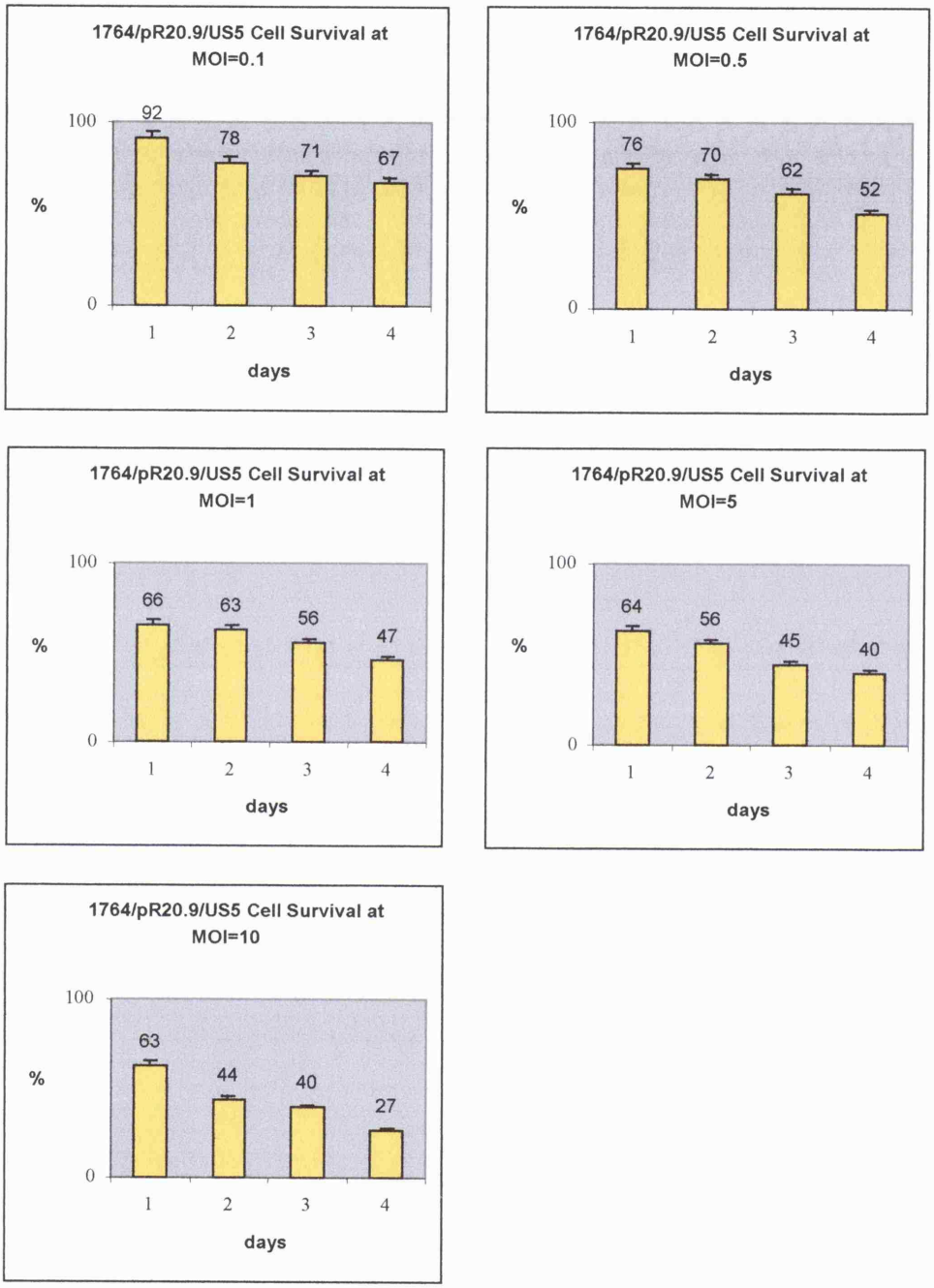


Figure IV.9: Dose effect of 1764/pR20.9/US5 (ICP34.5 and US5 deleted, vmw65 inactivated) at a range of MOI.

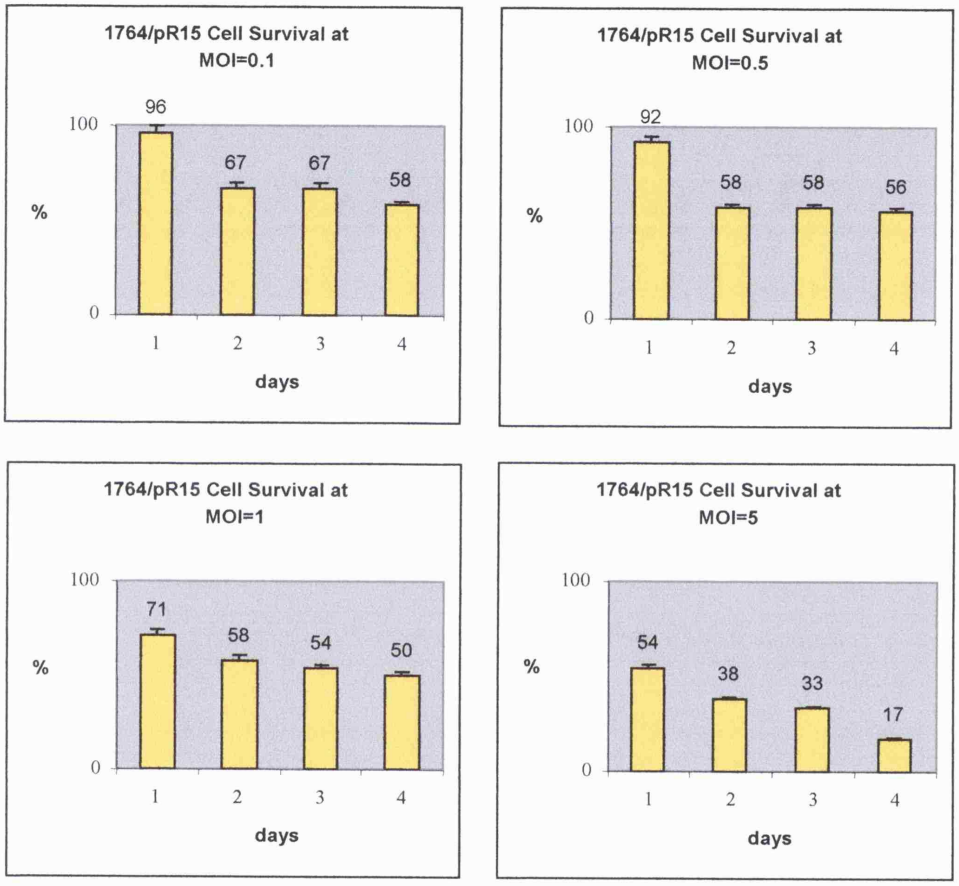


Figure IV.10: Dose effect of 1764/pR15 (ICP34.5 deleted, vmw65 and UL41 inactivated) at a range of MOI.

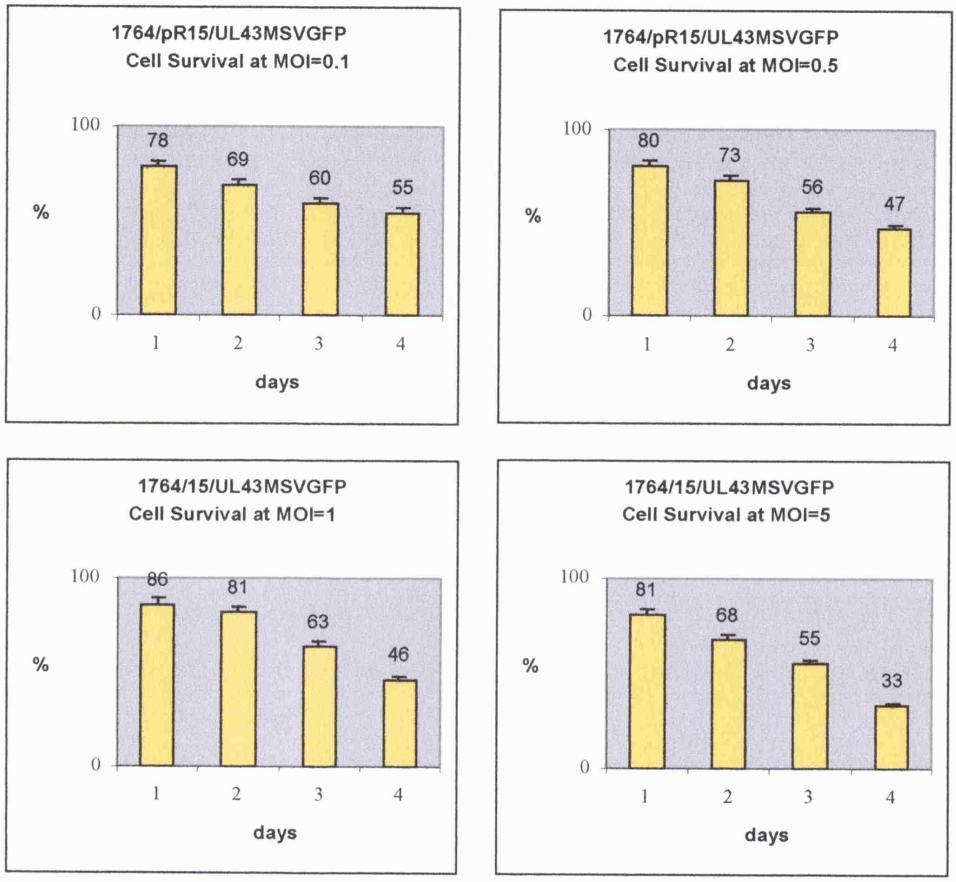


Figure IV.11: Dose effect of 1764/pR15/UL43MSVGFP (ICP34.5 deleted, vmw65, UL43 and UL41 inactivated) at a range of MOI.

vectors, another explanation is that more viral particles are present at a given MOI after transduction of DC with the partially disabled vectors compared to the minimally disabled vectors. This factor could contribute to cell survival effects in DC using vectors with different levels of disablement and titers.

IV.2.3 Cell survival using replication incompetent HSV-1 vectors

In this section, DC were transduced with three replication incompetent vectors 17+/27-/pR20, 1764/27-/pR20.5/vhs and 1764/27-/4-/pR20.5/vhs in order to assess their effect on cell survival. The replication incompetent vectors generally gave very high cell survival in DC, higher than the less disabled vectors and this decreased to a lesser extent over time (figures IV.12 and IV.13).

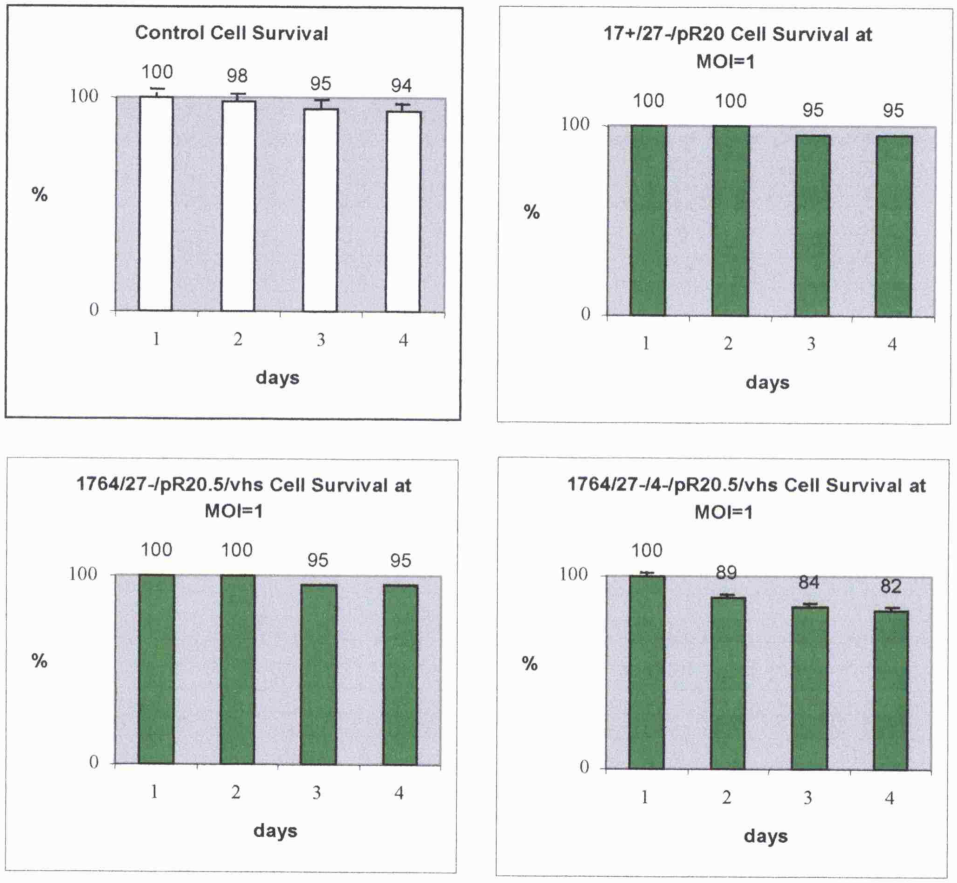


Figure IV.12: Cell survival assessed by the trypan blue exclusion assay after transduction of DC with the replication incompetent vectors at MOI=1.

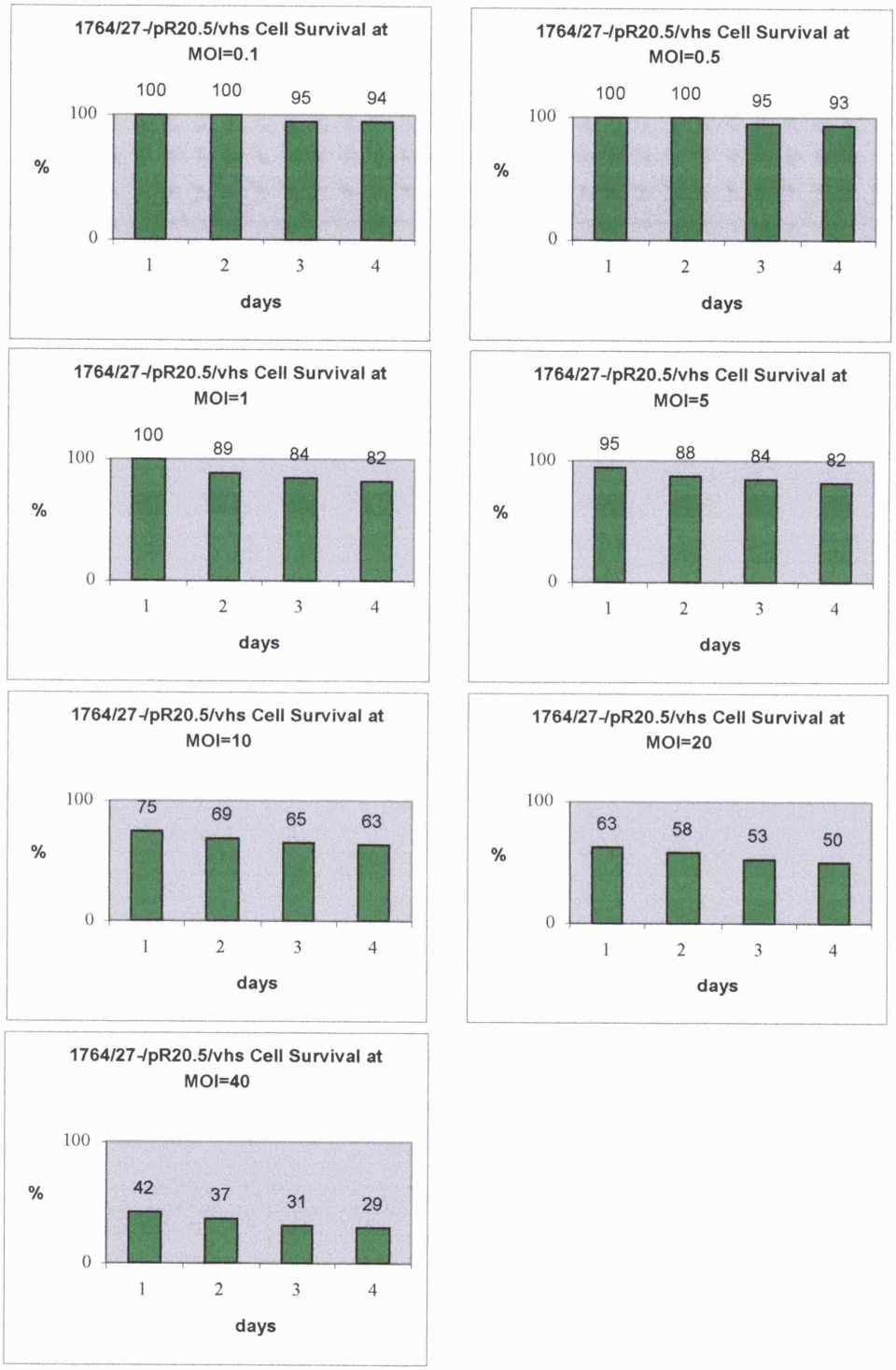


Figure IV.13: Dose effect of 1764/27-/pR20.5/vhs (ICP34.5, ICP27 and LATP2 deleted, vmw65 and UL41 inactivated) at a range of MOI.

IV.3. Discussion

In this chapter, cell survival was assessed following transduction of DC with four wild-type vectors, five partially disabled vectors and five replication incompetent vectors. These results are summarised in table IV.1. Figure IV.14 shows levels of GFP and β-gal detected and cell survival with these viruses at MOI=1. These will be discussed below in terms of:

1) The effect of gene deletion on cell survival at MOI=1 and other doses
2) The optimal vectors to be used for gene delivery to DC after comparing the gene delivery and cell survival results.

Cell survival with the minimally disabled vectors was generally high at MOI=1, 24 hours post-transduction. The US5 or UL41 single gene deletion/inactivation resulted in slightly higher cell survival than the UL43 deletion, although this is probably not significant. Surprisingly, the partially disabled vectors gave lower cell survival values. The partially disabled vectors with vmw65 and UL41 inactivated gave the lowest survival of all the viruses for reasons which have not been explained. The replication incompetent vectors were essentially non-toxic to DC as indicated by cell survival at MOI=1 although at higher doses some toxicity was observed.

Figure IV.14 summarises the percentage GFP and β-gal expression observed and the cell survival following transduction of DC at MOI=1 with the viruses tested so far. Minimally disabled vectors gave efficient gene delivery to DC and relatively low toxicity to cells. This suggests that wild-type HSV naturally infects DC as part of its life cycle as it would seem unlikely such high gene delivery rates would be achieved otherwise. However, partially disabled vectors gave two types of result. The first set of viruses gave similar levels of gene delivery as the minimally disabled vectors, while the second result was with viruses which gave extremely high gene delivery with in one case differential gene expression of GFP and β-gal. This virus

has ICP34.5 and UL43 deleted, and vmw65 and vhs inactivated, and also gave very good cell survival.Thus, this virus even though not entirely replication-incompetent would be a potential candidate for gene delivery to DC. It was hypothesised that DC transduced with this vector process delivered proteins more effectively than other partially disabled vectors which will be discussed later (chapter V). The results for the replication incompetent vectors showed that apparent gene delivery was the lowest in DC while all cells remained alive. Thus, these vectors were reduced in toxicity due to the deletion of genes coding for IE proteins known to be toxic to cells which may have further increased the antigen processing capability of the DC. 1764/27-/4-/pR20.5/vhs gave the highest gene delivery results with 99% live cells at 24 hours suggesting this vector may be the best candidate vector for gene delivery to DC as it is also replication incompetent.

Thus two vectors giving the best results for gene delivery in DC so far are 1764/pR15/UL43MSVGFP and 1764/27-/4-/pR20.5/vhs.

Virus Nomenclature	Genes deleted (d) or inactivated (i)	% cell survival (MOI=1) 24h - 72h
Control cells	NA	**100 – 95**
17+/pR20.5/UL43	UL43 (i)	**88 – 75**
17+/pR20.5/US5	US5 (d)	**94 – 77**
17+/pR20.5/vhs	UL41 (i)	**93 – 79**
17+/UL43CMVGFP	UL43 (i)	**94 – 73**
17+/vmw65/pR15	Vmw65 (i), UL41 (i)	**58 – 33**
1764/pR20.9/UL43	ICP34.5 (d), UL41 (i), UL43 (d)	**77 – 58**
1764/pR20.9/US5	ICP34.5 (d), vmw65 (i), US5 (d)	**66 – 56**
1764/pR15	ICP34.5 (d), vmw65 (i), UL41 (i)	**71 – 54**
1764/pR15/UL43MSVGFP	ICP34.5 (d), vmw65 (i), UL41 (i), UL43 (i)	86 – 63
17+/27-/pR20	ICP27 (d)	**100 – 95**
1764/27-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d), LATP2 (d)	**100 - 84**
1764/27-/4-/pR20.5/vhs	ICP34.5 (d), vmw65 (i), UL41 (i), ICP27 (d), ICP4 (d), LATP2 (d)	99 – 95
1764/27-/4-/pR19GFP	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	**100**
1764/27-/4-/pR19LacZ	ICP34.5 (d), vmw65 (i), ICP27 (d), ICP4 (d)	**100**

Table IV.1: Summary of cell survival results obtained using HSV vectors at MOI=1, 24 hours post-infection. The values shown for mock-infected DC (control cells) represent the average of all experiments. Abbreviations: 17+: HSV-1 wt strain. 1764: deletion in the ICP34.5 gene and inactivating mutation in vmw65. UL: unique long region. US: unique short region. ICP: infected cell polypeptide. vhs: virion host shut-off.

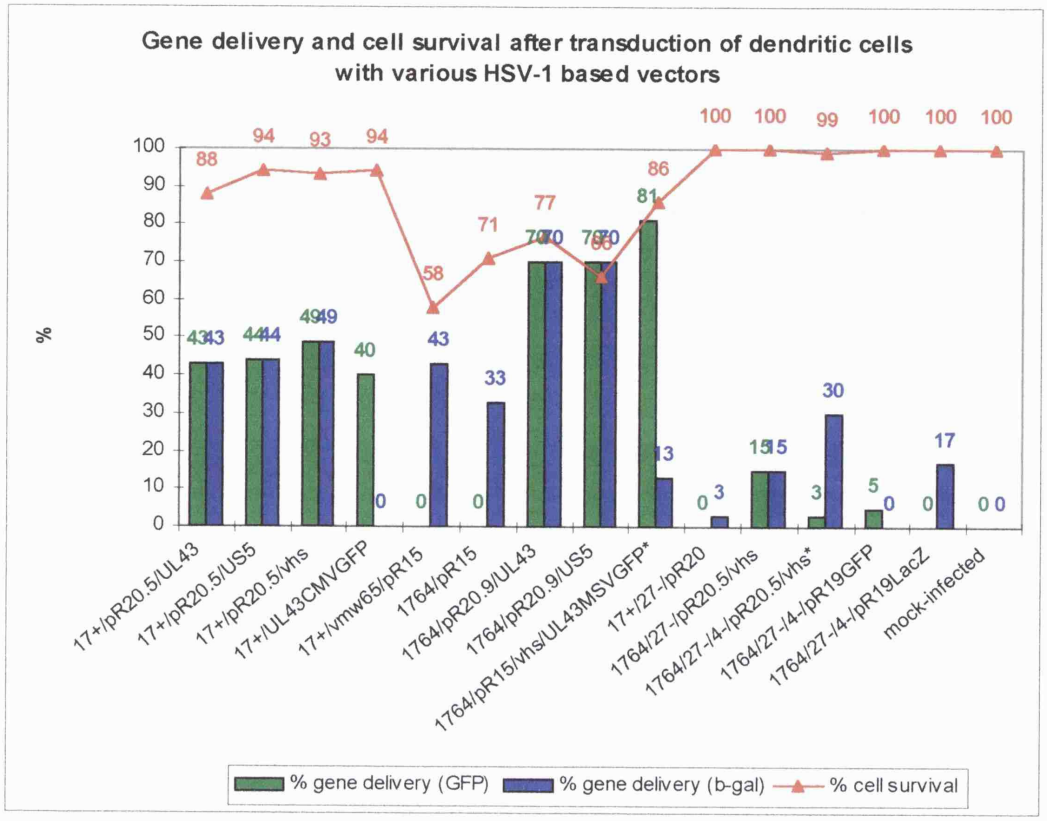


Figure IV.14: Summary of gene delivery and cell survival results, 24 hours after transduction of DC with various HSV based vectors (described below) at MOI=1. *For these viruses, differences were detected between the apparent levels of GFP and β-gal.

Gene deleted or Inactivated (x) / Virus	ICP34.5	vmw65	ICP27	ICP4	UL43	US5	UL41	LATP2
17+/pR20.5/UL43					x			
17+/pR20.5/US5						x		
17+/pR20.5/vhs							x	
17+/UL43CMVGFP					x			
17+/vmw65/pR15		x					x	
1764/pR15	x	x					x	
1764/pR20.9/UL43	x	x			x			
1764/pR20.9/US5	x	x				x		
1764/pR15/UL43MSVGFP	x	x			x		x	
17+/27-/pR20			X					
1764/27-/pR20.5/vhs	x	x	X				x	x
1764/27-/4-/pR20.5/vhs	x	x	X	x			x	x
1764/27-/4-/pR19GFP	x	x	X	x				x
1764/27-/4-/pR19LacZ	x	x	X	x				x

CHAPTER V

GROWTH CHARACTERISTICS OF HSV IN DENDRITIC CELLS AND PROTEOLYTIC PROCESSING OF THE DELIVERED ANTIGEN

V.1. Introduction

In chapter III and IV, a range of HSV-1 vectors were used in order to test their effect on gene delivery and cell survival. In the first section of this chapter, the growth characteristics of HSV vectors in DC are studied. For this purpose, viral growth was monitored over time following transduction with various HSV vectors tested previously in order to check whether infectious virus was generated in infected cells. The expression of immediate early (IE) proteins by HSV in DC was also monitored at intervals for two days post-infection.

In addition, as previous work in chapter III had suggested that DC transduced with some of the vectors (1764/pR15/UL43MSVGFP, 1764/27-/4-/pR19 and 1764/27-/4-/pR20.5/vhs particularly) may proteolytically process the delivered GFP and β-gal protein, this hypothesis was further explored at the end of this chapter.

V.2. Growth characteristics of HSV in DC

Growth curves were plotted for the minimally disabled and partially disabled vectors at a range of MOI.

V.2.1. Growth of the minimally disabled vectors in DC

Growth curves for 17+/pR20.5/UL43, 17+/pR20.5/US5 and 17+/UL43MSV GFP were generated at MOI ranging from 0.1 to 10 (figures V.1, V.2 and V.3). At low MOI=0.1 and 0.5, there was a decrease in viral titer from the time of transduction up to 24 hours post-infection with these vectors. By 72 hours, the wild type vectors had slight growth which was reduced by 96 hours. Surprisingly, this phenomenon was not observed at higher MOI. Indeed, at MOI=1, 5 and 10, there was a decrease in viral titer after transduction up to 96 hours for each vector tested. These results indicate that DC were slightly permissive for viral growth at low MOI for 17+/pR20.5/UL43, 17+/pR20.5/US5 and 17+/UL43CMVGFP but not at higher MOI.

Thus, the level of HSV growth which DC can support does not reach titers above input at high MOI, whereas low doses of virus allow the slight growth which is observed.

At MOI=0.1, slightly more growth was observed for 17+/pR20.5/US5 in DC compared to 17+/pR20.5/UL43 and 17+/UL43CMVGFP (figure V.4). At MOI=1, no significant differences were seen between the various growth curves (figure V.5).

V.2.2. Growth curves for the partially disabled vectors

Growth curves for 1764/pR20.9/UL43, 1764/pR20.9/US5, 1764/pR15 and 1764/pR15/UL43MSVGFP were also generated at a range of MOI (respectively, figures V.6, V.7, V.8 and V.9). At MOI=0.1 and 0.5, the viral titer decreased after transduction of DC up to 96 hours. At MOI=1, 5 and 10, no growth of these viruses was detected in DC. At MOI=0.1 or 1, no growth was observed with the partially disabled vectors (figures V.10 and V.11). These results show that DC are not permissive for the partially disabled vectors.

The results presented in this section showed that DC transduced with wild-type HSV vectors but not the partially disabled vectors allowed slight viral growth. DC are thus essentially non-permissive for the growth of HSV, even though DC are infected very efficiently. As it would seem unlikely that HSV would have evolved the ability to enter DC without some purpose (e.g. prevention of induction of cellular immune responses to HSV by DC) it would seem likely that DC are in some way affected by HSV infection in a manner not involving lytic replication. This, along with a study of HSV gene expression in DC, will be addressed later in this thesis where it is shown that specific HSV functions prevent the activation of DC following HSV infection.

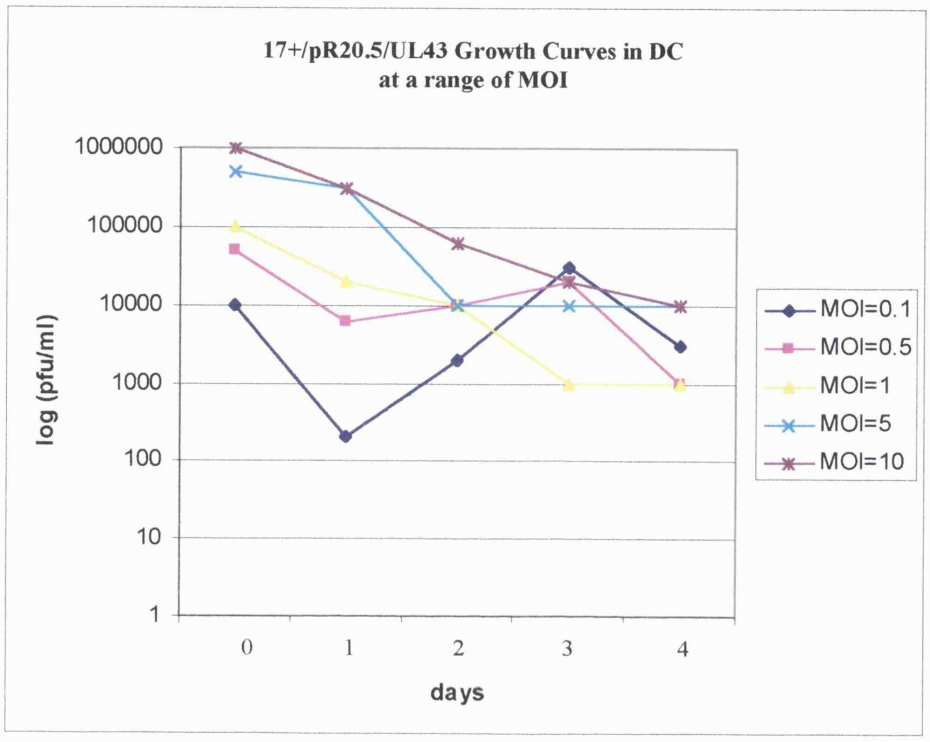


Figure V.1: Growth curves for 17+/pR20.5/UL43 in DC at a range of MOI.

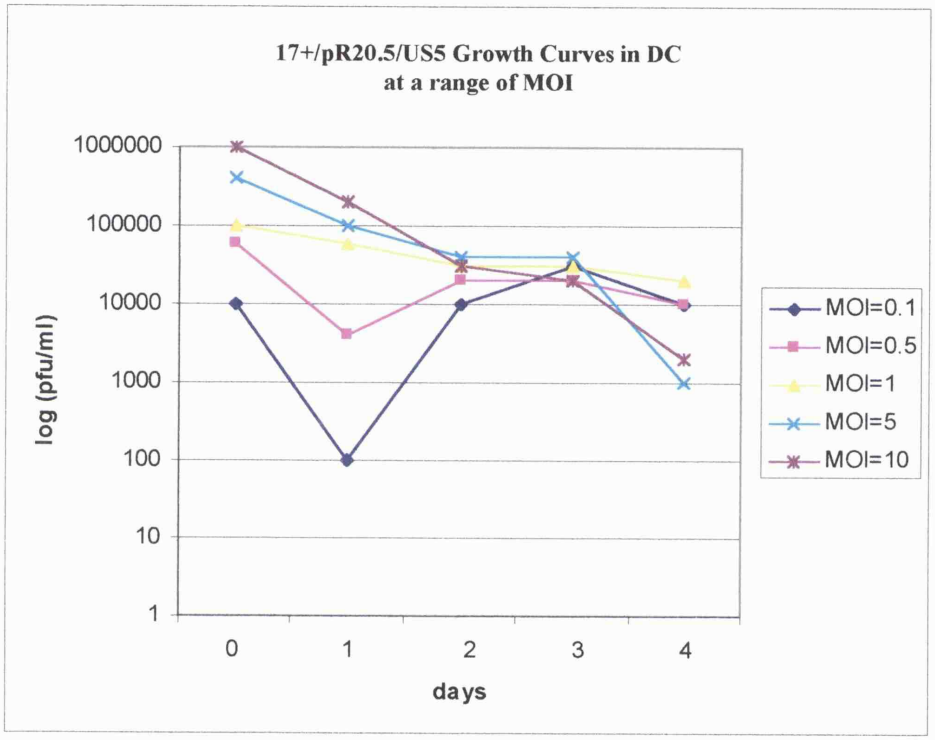


Figure V.2: Growth curves for 17+/pR20.5/US5 in DC at a range of MOI.

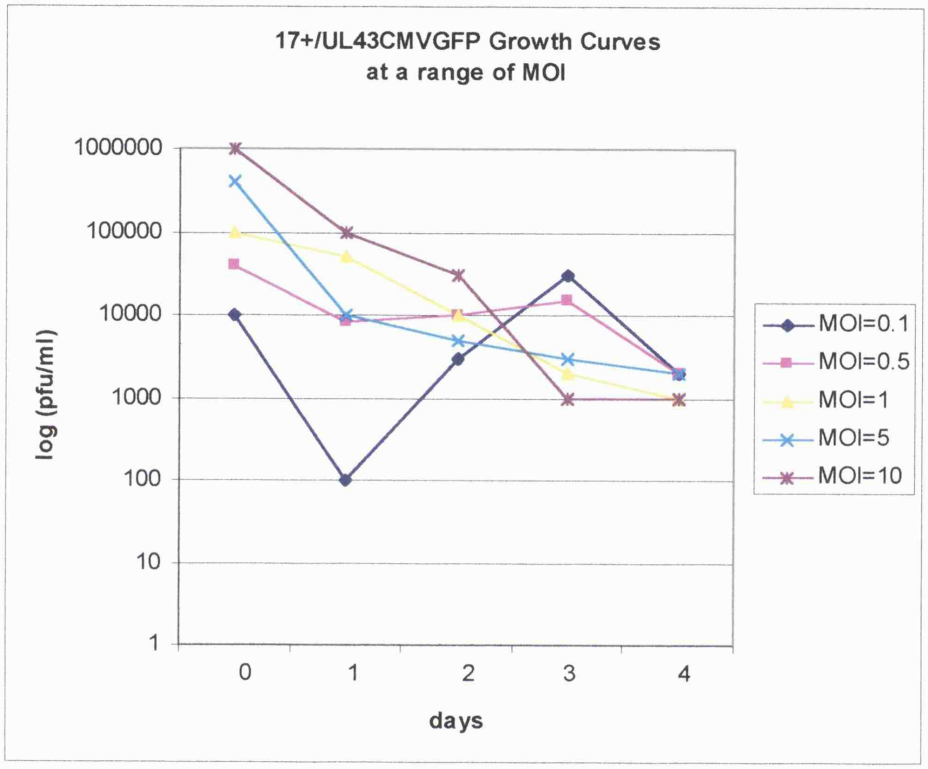


Figure V.3: Growth curves for 17+/UL43CMVGFP in DC at a range of MOI.

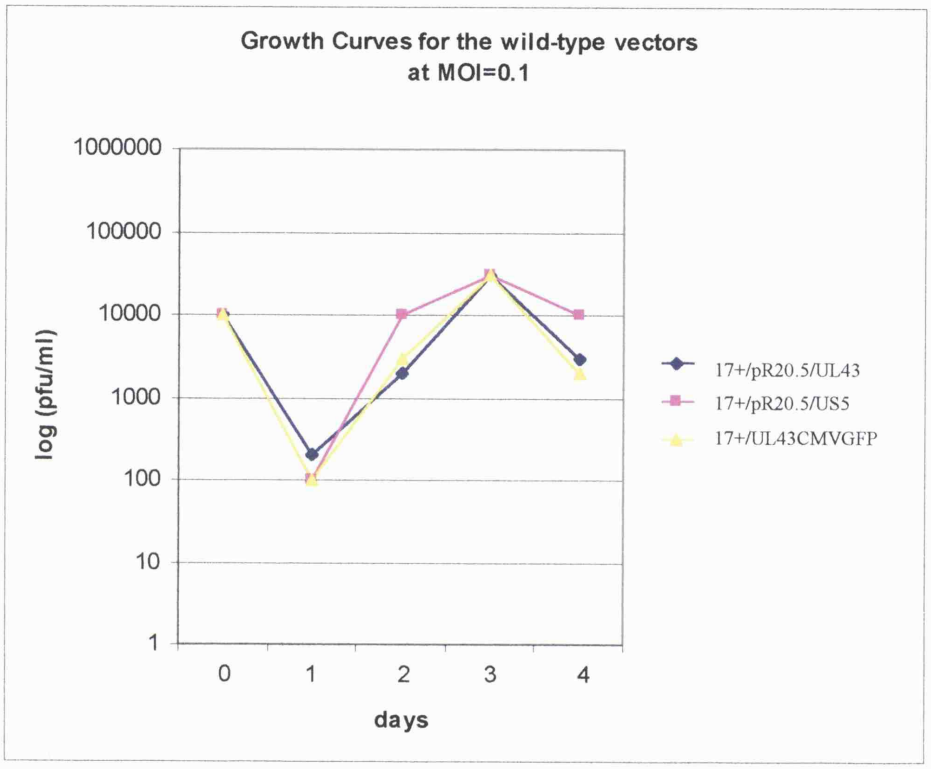


Figure V.4: Growth curves for the minimally disabled vectors in DC at MOI=0.1.

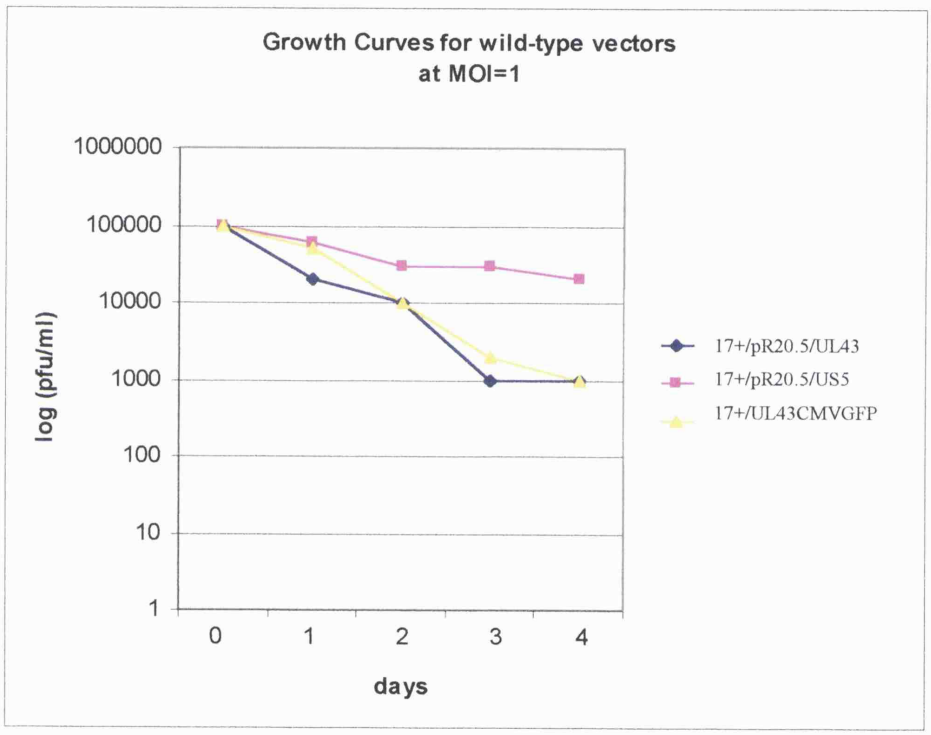


Figure V.5: Growth curves for minimally disabled vectors in DC at MOI=1.

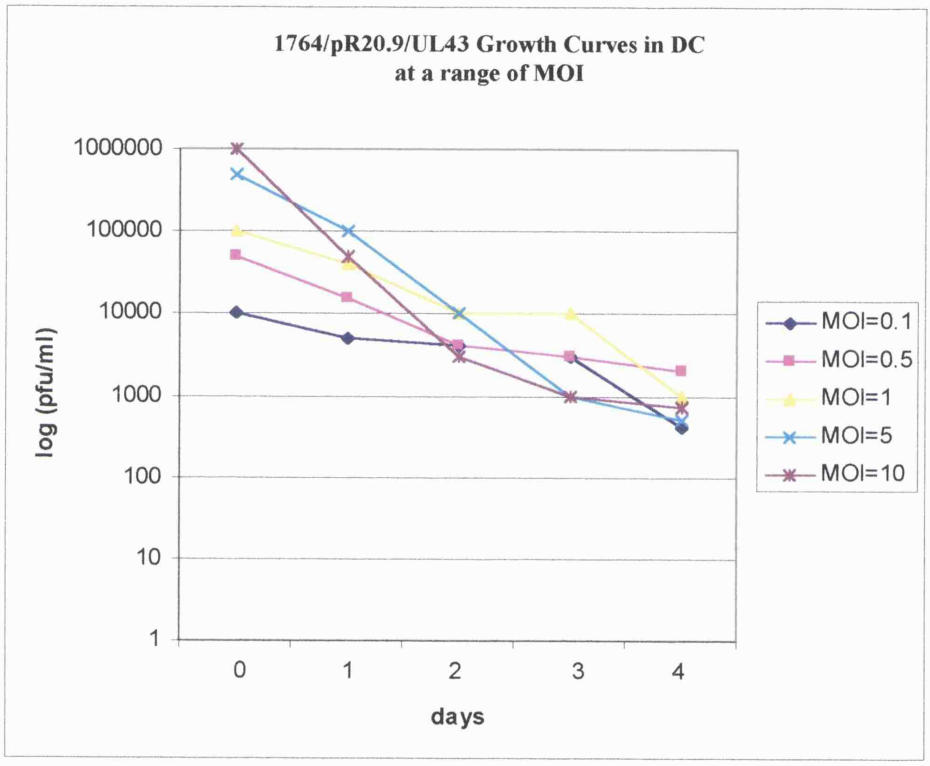


Figure V.6: Growth curves for 1764/pR20.9/UL43 in DC at a range of MOI.

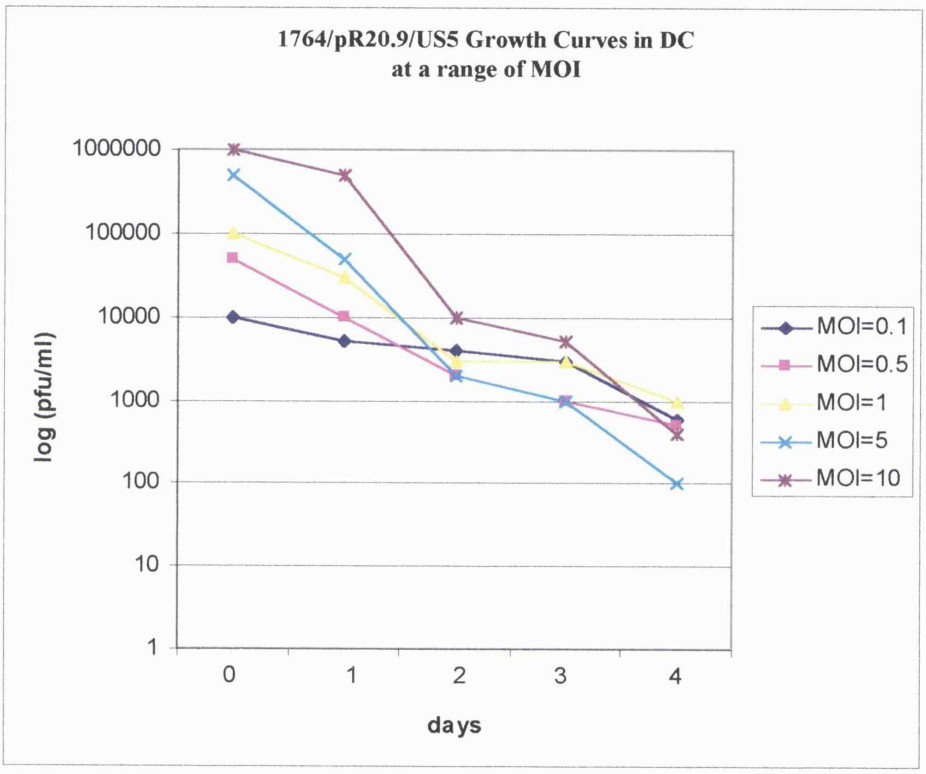


Figure V.7: Growth curves for 1764/pR20.9/US5 in DC at a range of MOI.

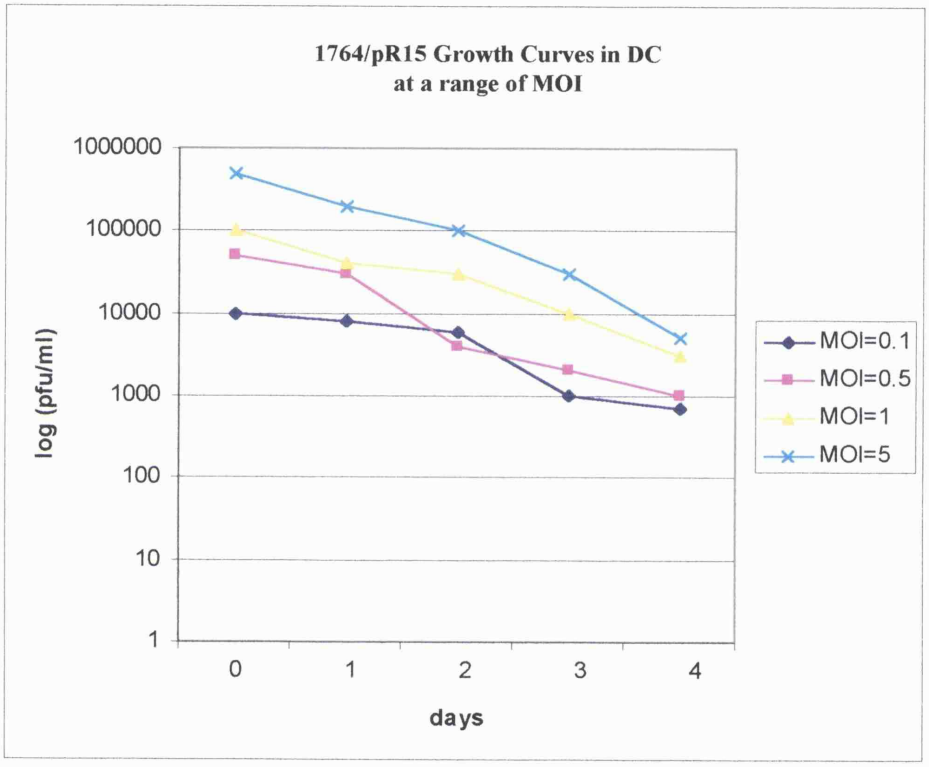


Figure V.8: Growth curves for 1764/pR15 in DC at a range of MOI.

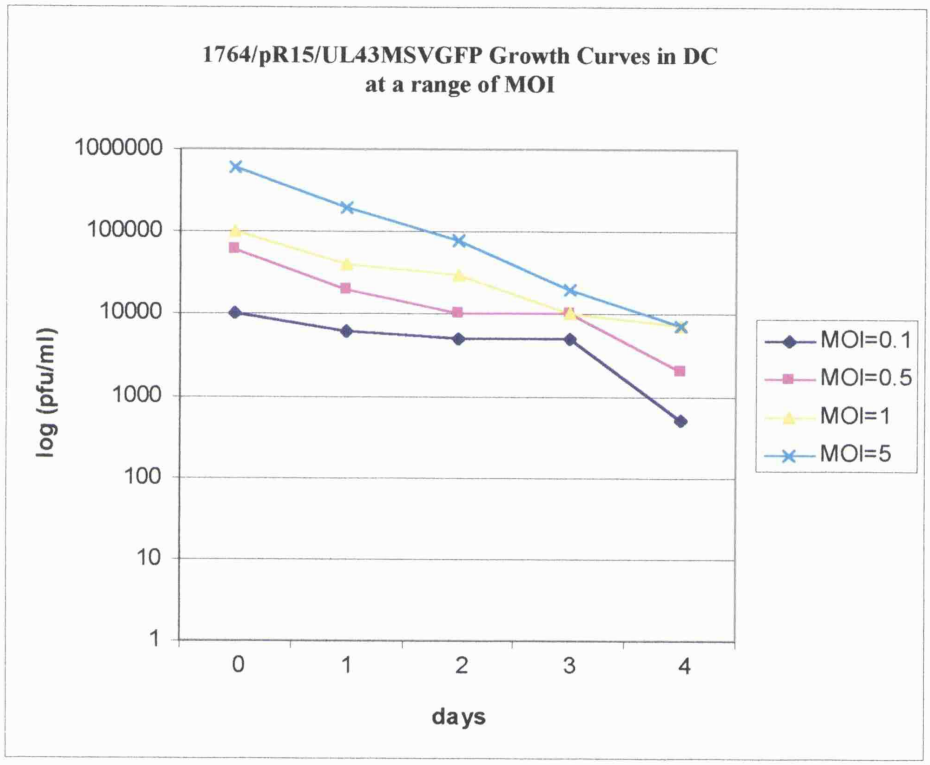


Figure V.9: Growth curves for 1764/pR15/UL43MSVGFP in DC at a range of MOI.

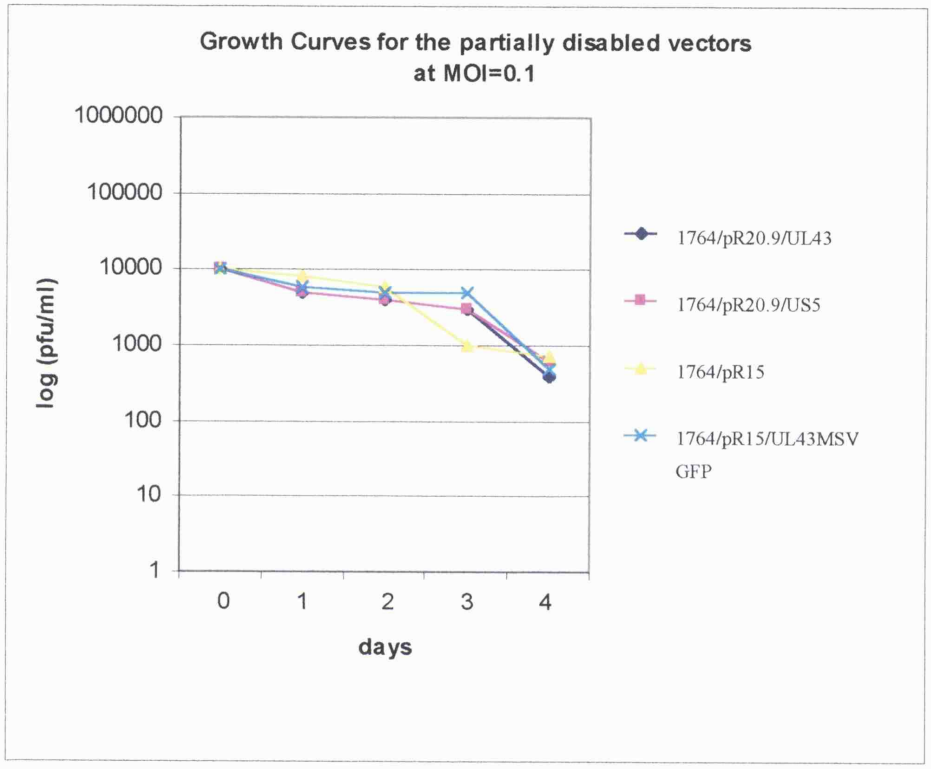


Figure V.10: Growth curves for the partially disabled vectors in DC at MOI=0.1.

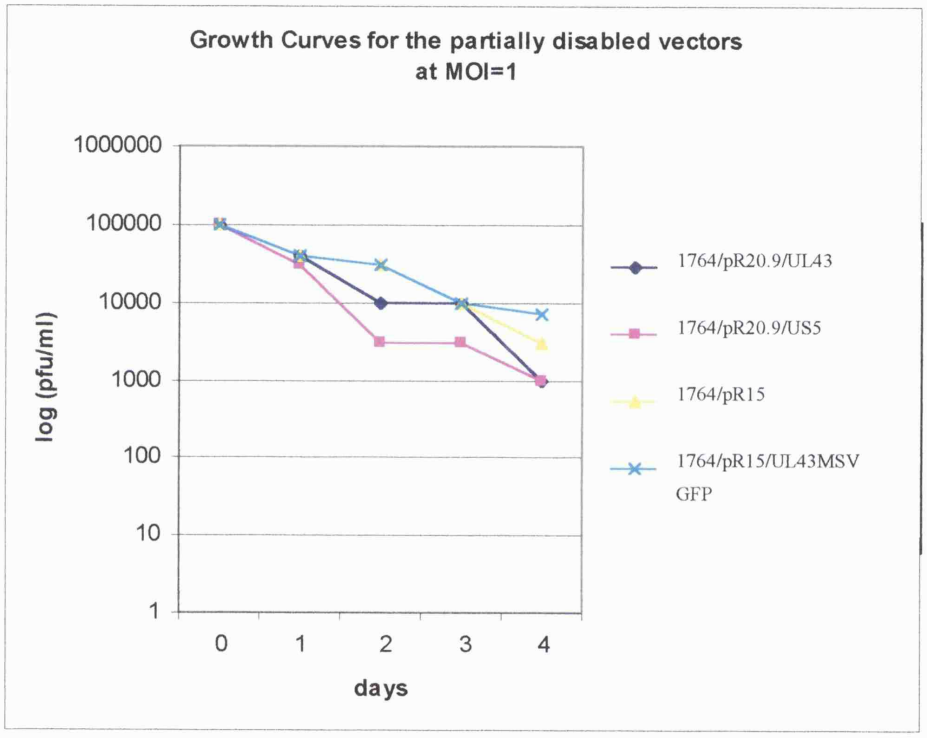


Figure V.11: Growth curves for the partially disabled vectors in DC at MOI=1.

V.3. Expression of immediate early proteins by HSV in DC

Even though very little viral growth was detected after transduction of DC with the wild type vectors and none with the partially disabled vectors, further experiments were conducted to assess the level of expression of the HSV-1 immediate early (IE) proteins in DC. The products of all the α genes with the exception of ICP47 (ICP22, ICP0, ICP4 and ICP27), have important regulatory effects on HSV-1 gene expression. The expression of the ICP22, ICP0 and ICP47 proteins were followed over a period of 48 hours after transduction of DC with the wild type and the partially disabled vectors at MOI=1. As ICP47 has been shown to interfere with antigen presentation, it was potentially important to check the levels of expression of this protein in DC (Goldsmith *et al.* 1998;Hill *et al.* 1995). The expression of these IE proteins was monitored by western blot after collecting protein extracts at 2, 4, 8, 12, 16, 24 and 48 hours after transduction of DC. Controls included protein extracts from non-transduced DC and from BHK cells (highly permissive cells for HSV-1 growth) infected with the wild-type vector. For each experiment, protein loading was monitored by Coomassie blue staining.

V.3.1. Expression of ICP22 and ICP0

The time course expression of ICP22 (80 kDa) was monitored after transduction of DC with vectors 17+/pR20.5/UL43 and 1764/pR15/UL43MSVGFP (figure V.12). No ICP22 expression was detected up to 12 hours post-infection time after which low levels of ICP22 were detected in DC transduced with both vectors. Protein extracts from transduced BHK cells showed ICP22 as early as 2 hours post-infection as expected.

The time course expression of ICP0 (110 kDa) was obtained after transduction of DC with vectors 17+/pR20.5/UL43 and 1764/pR15/UL43MSVGFP (figure V.12). No ICP0 expression was detected up to 16 hours post-infection; at 24 hours and 48

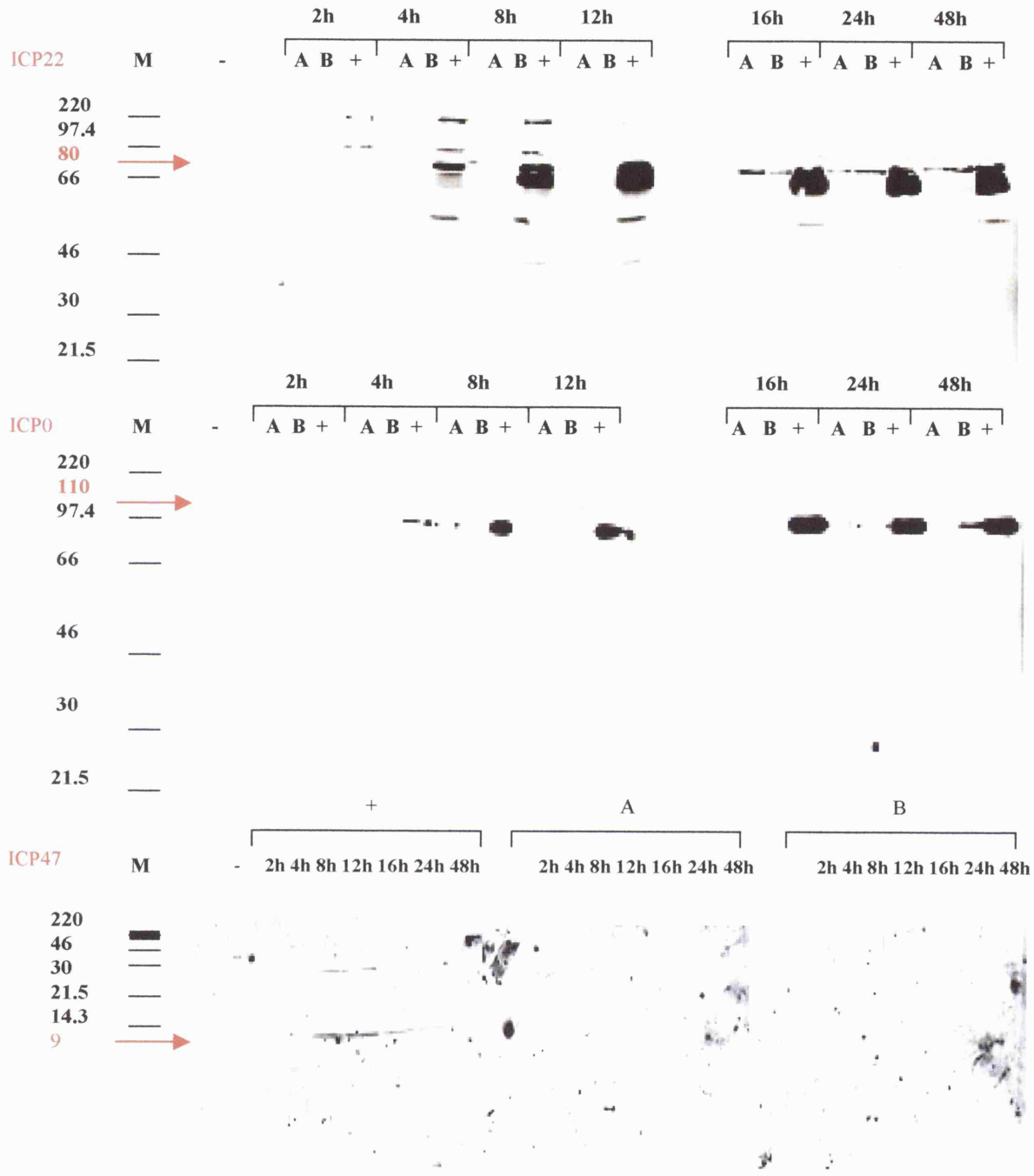


Figure V.12: Western blots showing the expression of ICP22, ICP0 and ICP47 in BHKs and DC – 10% or 15% acrylamide gel showing the time course expression of ICP22 (80 kDa), ICP0 (110 kDa) and ICP47 (9 kDa) in DC, 2, 4, 8, 12, 16, 24 and 48 hours, following transduction with 17+/pR20.5/UL43 (A) and 1764/pR15/UL43MSVGFP (B), at MOI=1. Positive control (+) = time course extracts following infection of BHKs with 17+/pR20.5/UL43, at MOI=1. Negative control (-) = mock infected DC. Molecular weight M = Rainbow mix (kDa).

hours, very low ICP0 levels were detected with both vectors. Protein extracts from transduced BHK cells showed ICP0 to be expressed by 4 hours post-infection.

V.3.2. Expression of ICP47

The time course expression of the immediate early protein ICP47 (9 kDa) was followed after transduction of DC with vectors 17+/pR20.5/UL43 and 1764/pR15/UL43MSVGFP (figure V.12). No ICP47 was detected in DC transduced with these vectors at any time. Protein extracts from transduced BHK cells showed ICP47 to be expressed by 4 hours post-infection.

V.4 Discussion

In this section, it was shown that DC transduced with the virus 17+/pR20.5/UL43 containing one deletion in the UL43 gene expressed the ICP22 protein from the virus 12 hours post-infection. This implied that the ICP22 promoter was transactivated by vmw65 as virus 17+/pR20.5/UL43 contains intact vmw65. The ICP0 protein was also expressed by 16 hours post-infection.

Surprisingly, the partially disabled vector 1764/pR15/UL43MSVGFP which contains an inactivating mutation in the gene encoding vmw65 gave similar results to the 17+/pR20.5/UL43 vector. This might suggest that even with the 17+/pR20.5/UL43 vector vmw65 transactivated IE gene expression is not occuring, which requires cellular transcription factors which may well not be present in DC. The expression levels observed are possibly due to the background 'un-tansactivated' expression levels from the ICP0 and ICP22 promoters. This explanation may be the case as the *in1814* mutation in vmw65 present in 1764/pR15/vhs/UL43MSVGFP has been shown to efficiently abolish the transactivating activity of vmw65 and there was no difference in IE gene expression levels with and without the mutation. As ICP47 inhibits antigen presentation, it is useful in an HSV vector that this is not significantly expressed in DC.

V.5. Proteolytic processing of delivered proteins in DC following transduction

Based on previous work from chapter III that had suggested that DC transduced with some of the vectors may proteolytically process the delivered GFP and β-gal, further studies were undertaken to test this hypothesis.

As professional antigen presenting cells, DC process endogenous or exogenous antigens into small peptides which are then transported to the surface of the cell bound to MHC-class I or MHC-class II molecules respectively for presentation to CD8+ cytotoxic T cells or CD4+ helper T cells. Thus, DC are well equipped to capture and process antigens and a number of molecules involved in these processes have been identified. Thus, DC are, for example, rich in proteasomes which are large, multisubunit protease complex involved in processing endogenous antigens (Bogyo M. et al., 1997). As antigens delivered to DC through the HSV virus follow an endogenous pathway, it was thought that blocking the proteasome activity may increase the level of protein detected in DC and further indicate whether such processing might explain the differential GFP/β-gal expression patterns previously observed.

Thus in this section, proteasome inhibitors were used in order to detect if differences between GFP and β-gal expression could still be detected following transduction with various of the vectors including the partially disabled vector 1764/pR15/UL43MSVGFP and the fully disabled vector 1764/27-/4-/pR20.5/vhs, which gave significant differences in terms of protein expression detected.

In addition to blocking antigen processing in DC, experiments were performed to check the *GFP* and *LacZ* RNA levels after transduction of DC with various of the vectors.

V.5.1. Blocking antigen processing in DC

The use of the proteasome inhibitor MG132 was first optimised as it was not clear which would be the best conditions for use in DC. For this purpose, virus 1764/pR15/UL43MSVGFP was used to transduce DC as previously and the inhibitor was added in each of the following conditions using a concentration of 10 μM for 10^5 cells as has been previously reported to be appropriate (Bogyo *et al.* 1997):

- half an hour prior to transduction
- half an hour prior to transduction and post-transduction (while adding media to the infected DC)
- as above, but with the addition of the inhibitor during transduction
- post-transduction only

The percentage of DC expressing GFP and β-gal 24 hours post-transduction in these conditions is shown in figure V.13. Following transduction in this case, 60% GFP and 10% β-gal was obtained without any inhibitor. This value is slightly lower than usual, possibly as the virus stock had been defrosted several times.

Surprisingly, following treatment with the inhibitor no GFP level was detectable under the fluorescent microscope with any of the conditions. This suggested that the inhibitor blocked GFP expression or alternatively the processing of GFP which is required for it to take up its fluorescent form (Stripecke *et al.* 1999). This latter would seem the most likely in this case. For β-gal expression, treatment of cells prior to or post transduction did not affect the apparent protein level (10%). However the addition of inhibitor both before and after

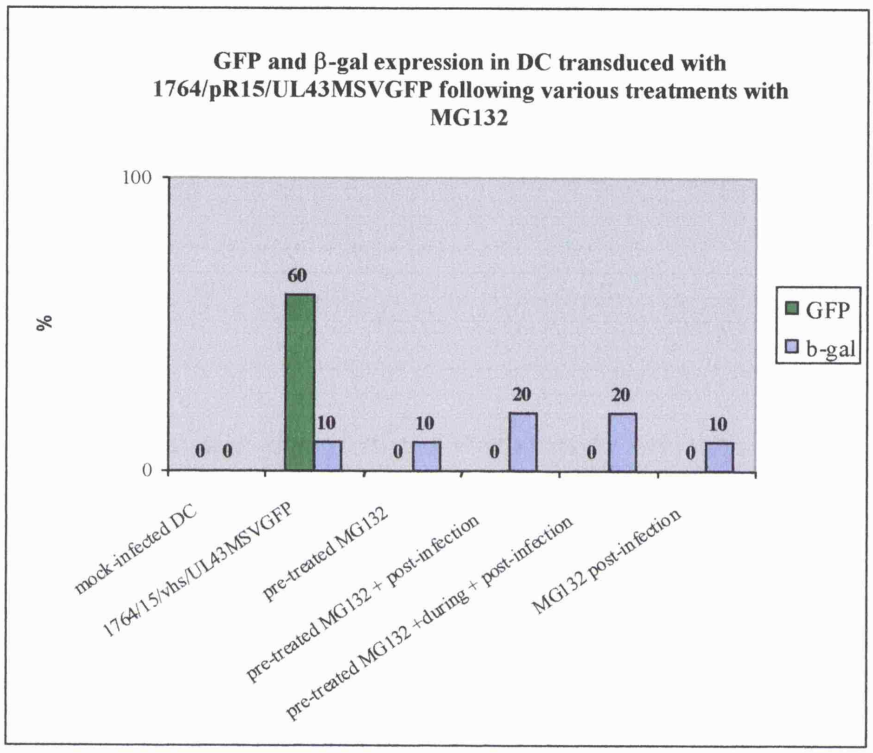


Figure V.13: Optimisation experiment showing GFP and β-gal expression in DC transduced with 1764/pR15/UL43MSVGFP following treatment with the proteasome inhibitor MG132 under various conditions.

transduction, or before, during and post-transduction gave twice the expression recorded without the inhibitor (20%). This experiment showed firstly that it appears possible to at least partially block antigen processing in DC using the MG132 inhibitor and as a consequence to observe more cells staining positive for β-gal compared to the level obtained following transduction without any inhibitor. Secondly, from the various combinations tested, the optimal conditions for use of MG132 inhibitor in DC were identified which were used for subsequent experiments. In addition, cell survival following treatment with MG132 was monitored in order to check if the inhibitor was toxic to cells. The results showed that 100% of DC were alive after treatment with the inhibitor as assessed by the trypan blue exclusion assay (data not shown).

Following these optimisation results, DC were either treated or not treated with the proteasome inhibitor MG132, and transduced with vectors 17+/pR20.5/UL43, 1764/pR15/UL43MSVGFP or 1764/27-/4-/pR20.5/vhs, at MOI=1. Controls included mock infected DC and mock infected DC treated with MG132. Experiments were repeated three times and the average value is presented here. The average value for the percentage β-gal expressing cells was slightly lower than usual (compared to those listed in table III.3), possibly due to the repeated use and defrosting of the virus stock. As noted previously, the treatment of transduced DC with MG132 completely inhibited GFP expression 24 hours post-infection (figure V.14). However, after transduction of DC with the HSV-1 vectors tested, an average of nearly double the number of cells was observed in which β-gal expression could be detected by X-gal staining in DC treated with the proteasome inhibitor compared to non-treated transduced cells (figure V.15).

These results indicated that DC transduced with HSV vectors tested retained the ability to process antigens as apparent β-gal expression levels are increased following treatment with MG132. This suggests that the differential GFP/β-gal detectable with some of the viruses may indeed be due to antigen processing by the

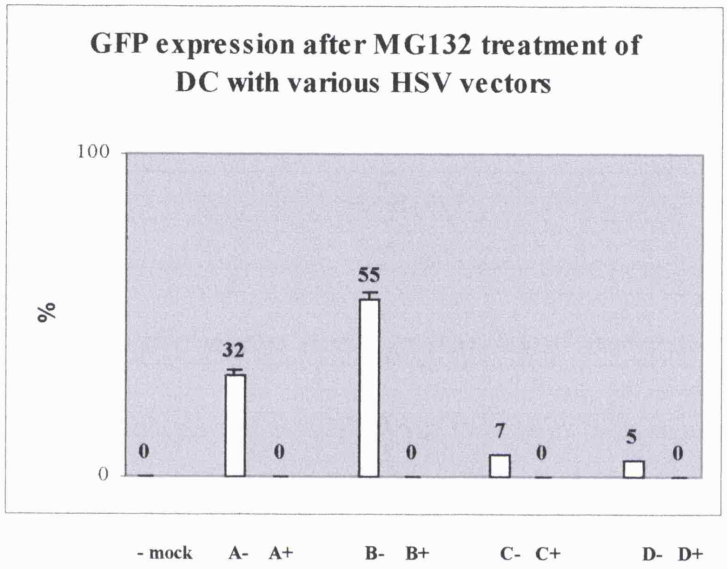


Figure V.14: DC were either treated with the proteasome inhibitor MG132 (+) or left untreated (-) before and immediately after transduction with (A)17+/pR20.5/UL43, (B)1764/pR15/UL43MSVGFP, (C)1764/27-/4-/pR20.5/vhs and (D)1764/27-/4-/pR19GFP (at MOI=1). The figure shows GFP expression at 24 hours. Negative controls (-) = mock infected DC and mock infected DC treated with MG132.

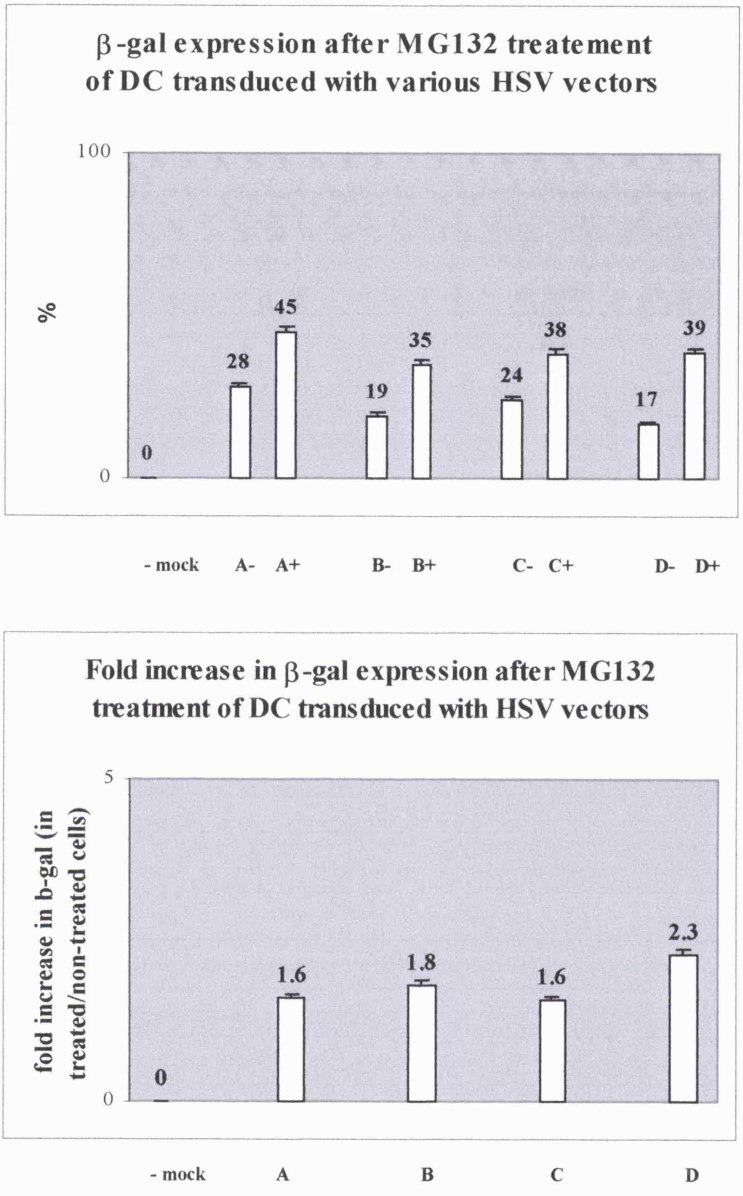


Figure V.15: β-gal expression after MG132 treatment of DC transduced with various HSV vectors. DC were either treated with the proteasome inhibitor (+) or left untreated (-) before and immediately after transduction with (A)17+/pR20.5/UL43, (B)1764/pR15/UL43MSVGFP, (C)1764/27-/4-/pR20.5/vhs and (D)1764/27-/4-/pR19LacZ, at MOI=1. The figure shows respectively β-gal expression and the fold increase 24 hours after treatment of transduced DC with MG132 compared to untreated transduced cells. Negative controls (-) = mock infected DC and mock infected DC treated with MG132.

transduced cells which is only apparent when these vectors are appropriately disabled. Further confirmation that actual gene expression levels of GFP and β-gal were similar even though the protein level was not (as shown by fluorescence or X-gal stain), was made by assessing *GFP* and *LacZ* RNA levels below.

V.5.2. Determination of *GFP* and *LacZ* RNA levels

In this section, a slot blot was performed in order to monitor the *GFP* and *LacZ* RNA levels following transduction with viruses that showed significant differences in the levels of apparent protein expression. If antigen processing was the reason for this discrepancy more equal amounts of *GFP* and *LacZ* RNA would expected to be present than suggested by GFP fluorescence and X-gal staining. However, if *GFP* and *LacZ* RNA levels were markedly different and matched the ratio of the corresponding proteins, then differences seen between the apparent level of proteins could not easily be attributed to proteolytic processing as the previous data appears to suggest. Viruses tested here were the following:

- 1764/pR15/UL43MSVGFP (ICP34.5 deleted and vmw65, UL41 and UL41 inactivated); giving 81% GFP and 12% β-gal apparent expression levels (at MOI=1, at 24 hours) by fluorescence and X-gal staining respectively
- 1764/27-/4-/pR20.5/vhs (ICP27, ICP4, LATP2 and ICP34.5 deleted, and vmw65 and UL41 inactivated); giving 3% GFP and 30% β-gal apparent expression levels (at MOI=1, at 24 hours) by fluorescence and X-gal staining respectively

DC were transduced with viruses 1764/pR15/UL43MSVGFP and 1764/27-/4-/pR20.5/vhs at MOI=1. RNA was extracted from transduced cells, 24 hours post-infection in order to estimate the presence of *GFP* and *LacZ* RNA by slot blot (figure V.16). The negative control was RNA from mock infected DC and the positive controls were RNA extracted from BHK cells transfected with plasmids containing GFP (pcDNA3GFP), LacZ (pCH110LacZ) or actin genes (pSK+actin).

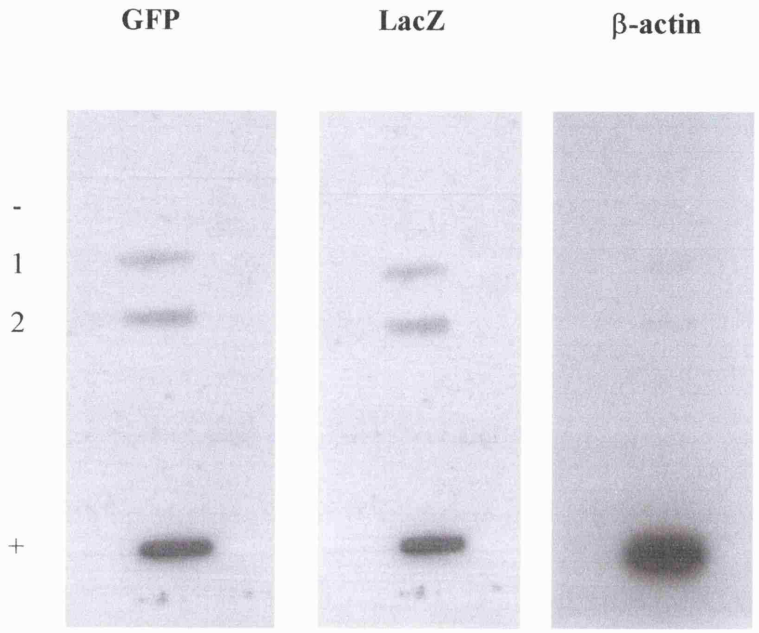


Figure V.16: Slot blots showing *GFP* and *LacZ* RNA levels 24 hours after tranduction of DC with (1) 1764/pR15/vhs/UL43MSVGFP or (2) 1764/27-/4-/pR20.5/vhs at MOI=1. Positive control (+) = RNA extracted from BHK cells transfected with plasmids containing GFP (pcDNA3GFP), LacZ (pCH110LacZ) or actin genes (pSK+actin). Negative control (-) = RNA from mock infected DC.

The negative control did not show any signal for *GFP* or *LacZ*. The β-actin probe showed that the RNA loading was equal for the samples transduced with 1764/pR15/UL43MSVGFP and 1764/27-/4-/pR20.5/vhs whereas less RNA was loaded in the negative control.

Values corresponding to the intensity of each band were equalised by densitometry after taking in account the background. Following transduction with virus 1764/pR15/UL43MSVGFP, *GFP* RNA levels were slightly higher than *LacZ* RNA levels (ratio of 1.5) after taking in account the background. Thus, very little difference was noted in this case where great differences were observed at the protein level post-transduction (ratio of GFP protein level to β-gal protein level of 6). Furthermore, very similar results were also seen post-transduction with virus 1764/27-/4-/pR20.5/vhs (ratio of *GFP* RNA levels to *LacZ* RNA levels of 1.2 after taking in account the background) which suggested that gene delivery was greater than the apparent percentage of cells expressing the protein detected post-transduction (ratio of GFP protein level to β-gal protein level of 0.2).

These results, although not conclusive, supported the hypothesis that DC transduced with HSV-1 proteolytically process delivered antigens, this being a likely explanation for the differential GFP and β-gal observed with some of the vectors. This implies that protein expression was generally higher than the levels observed by GFP fluorescence and X-gal staining.

PART II

EFFECTS OF HSV VECTORS ON DENDRITIC CELL PHENOTYPE AND FUNCTION

CHAPTER VI

EFFECTS OF HSV VECTORS ON DENDRITIC CELL PHENOTYPE AND ACTIVATION STATE

CHAPTER VII

STIMULATION OF ANTIGEN SPECIFIC T CELL PROLIFERATION BY HSV TRANSDUCED DENDRITIC CELLS

CHAPTER VIII

CONSTRUCTION OF A REPLICATION INCOMPETENT HSV VECTOR EXPRESSING THE MUC-1 TUMOR ANTIGEN IN DENDRITIC CELLS

CHAPTER VI[1]

EFFECTS OF HSV VECTORS ON DENDRITIC CELL PHENOTYPE AND ACTIVATION STATE

[1] Work in this chapter was performed jointly with Dr. Luci MacCormac and Dr. Steve Cleverley who performed the FACS analysis presented.

VI.1. Introduction

The work described in part I of the results section shows that DC can be infected with HSV very efficiently. This suggests that the wild type HSV may have evolved to naturally infect DC in peripheral tissues and possibly inhibit their ability to stimulate anti-HSV cellular immune responses. Thus, the observation that DC are efficiently transduced with HSV-1 suggests that:

1. HSV naturally infect DC as part of its life cycle. This suggests that DC express receptors that allow HSV entry
2. HSV may use this means to inactivate DC and escape from immune surveillance, thus enabling the virus to become latent and not be cleared during periods of reactivation.

The second part of the results section deals with the effects of HSV on DC phenotype and activation state (chapter VI) and their ability to express a delivered antigen in DC and stimulate T-cell responses specific to the delivered antigen (chapters VII and VIII).

For our purpose, the ideal vector is one that infects DC efficiently without toxicity and without affecting DC phenotype and function. Thus, the objective of this chapter was to analyse the phenotype of DC after transduction with HSV vectors. The immune evasive functions of five HSV proteins have been described so far although none of these have been shown to affect DC functions (described in the general introduction). Preliminary experiments showed that disabled HSV vectors with the UL41 gene inactivated (encoding the tegument virion host shutoff protein, vhs, responsible for degrading mRNA) allowed the up-regulation of the CD86 surface marker to similar levels as with the LPS control whereas other vectors did not. CD86 is a T-cell co-stimulator expressed on DC and that appears to be crucial in primary immune response (Lenschow *et al.* 1996). These findings generated the hypothesis that the vhs protein within the HSV virion is important for the inactivation of DC and

thus aids the virus to avoid cellular immune responses. Thus, the effect of various vectors were assessed on DC phenotype in this chapter.

The four viruses tested are outlined below:

- 17+: wild-type (wt) HSV-1 virus
- 17+/pR20.5/vhs: minimally disabled vector with the UL41 gene inactivated (encoding the vhs protein)
- 1764/27-/4-: replication incompetent vector with ICP27, ICP4 and ICP34.5 deleted and vmw65 inactivated
- 1764/27-/4-/pR20.5/vhs: replication incompetent vector with ICP27, ICP4 and ICP34.5 deleted and vmw65 and UL41 inactivated (encoding the vhs protein)

DC surface markers tested and presented in this chapter were the following:

- HLA-DR (human leucocyte antigen): MHC-class II type molecule specifically expressed by antigen presenting cells (e.g. DC) and that present exogenously derived antigen to CD4+ cells (Rudensky 1995). This molecule in presence of peptides is up-regulated during activation in order to provide primary antigen specific signal for T cells (Littman 1996)
- CD40: molecule expressed by antigen presenting cells (e.g. DC) and B cells and up-regulated during activation. CD40 in DC binds CD40 ligand (CD40L) on T cells and drives IL-12 production by T cells (van Kooten and Banchereau 1996)
- CD83: one of the best markers for mature DC and up-regulated during maturation however, the precise function of this surface molecule is unknown (Zhou and Tedder 1995)
- CD86: molecule expressed on resting peripheral blood monocytes and on DC and up-regulated during activation in order to provide co-stimulatory signals for T cells activated through the TCR. CD86 is expressed earlier than CD80 which is another T-cell co-stimulator and appears to dominate in primary

immune response (Lenschow *et al.* 1996). CD80 and CD86 bind CD28 on T cells and this deliver co-stimulatory signal.

The viral titer of each virus was previously determined. DC were purified from peripheral blood as described in Materials and Methods and transduced with the four vectors at an MOI=1 or mock-infected. DC phenotype was analysed by FACS. Mock-infected DC expressed HLA-DQ, HLA-DR, MHC Class I and CD1a surface markers and not CD3, CD4, CD19 or CD14 markers implying a relatively pure DC population (not contaminated with T, B lymphocytes or monocytes – less than 2% of cells were contaminants). 24 hours after transduction with the HSV-1 vectors, the phenotype of the DC was analysed by FACS for the expression of HLA-DR, CD40, CD83 and CD86.

IgG-1 was used as a negative control for the FACS analysis experiments (figure VI.1). Other controls included the use of LPS (lypopolysaccharide) to activate DC. LPS treated DC should strongly express the tested activation markers. FACS analysis was performed on DC transduced or mock-infected with the vectors with and without LPS. The first set of experiments was performed with the disabled vectors and then with the wild-type vectors. Experiments were repeated twice and one representative FACS analysis of DC populations is presented in this chapter.

VI.2. Replication incompetent HSV-1 vectors prevent activation of DC unless vhs is deleted

Vectors 1764/27-/4-/pR20.5/vhs and 1764/27-/4- were used in this section. 1764/27-/4- is an equivalent vector to 1764/27-/4-/pR20.5/vhs that does not contain an expression cassette and has the UL41 gene intact. The phenotype of DC transduced with both vectors was analysed by FACS for the expression of HLA-DR, CD40, CD83 and CD86, 24 hours after transduction with and without LPS.

VI.2.1. HLA-DR

HLA-DR is expressed on all peripheral blood mononuclear cells but high HLA-DR expression is characteristic of DC and even higher levels of expression are observed during DC maturation. The FACS analysis results for HLA-DR are shown in figure VI.2. In the absence of LPS, mock-infected DC and DC transduced with vector 1764/27-/4- were positive for HLA-DR and cells transduced with 1764/27-/4-/pR20.5/vhs expressed higher levels of the marker. In the presence of LPS, a significant shift of cell population was observed for mock-infected cells and with DC transduced with 1764/27-/4- compared to the samples without LPS, and this shift was even more marked with cells transduced with 1764/27-/4-/pR20.5/vhs. Firstly, these results showed that DC transduced with the disabled vector express HLA-DR at similar levels to non-infected DC and secondly that DC transduced with the disabled vector with the UL41 gene inactivated (and thus with vhs inactivated) express higher levels of HLA-DR.

VI.2.2. CD40

The FACS analysis results for CD40 are shown in figure VI.3. In the absence of LPS, mock-infected DC and DC transduced with vector 1764/27-/4- strongly expressed CD40 and cells transduced with 1764/27-/4-/pR20.5/vhs up-regulate CD40 further. In the presence of LPS, a small shift of cell population was observed for mock-infected cells and with DC transduced with 1764/27-/4- compared to the samples without LPS, and this shift was slightly more in cells transduced with 1764/27-/4-/pR20.5/vhs. Thus, these results indicate that DC transduced with the disabled vector express high levels of CD40 similarly to mock-infected DC and secondly that DC transduced with the disabled vector with the UL41 gene inactivated express slightly higher levels of CD40.

VI.2.3. CD83

The FACS analysis results for CD83 are shown in figure VI.4. In the absence of LPS, mock-infected DC and DC transduced with vector 1764/27-/4- expressed low levels of CD83 whereas cells transduced with 1764/27-/4-/pR20.5/vhs expressed intermediate levels of the marker. In the presence of LPS, a split in cell population was observed for mock-infected cells and a shift was noted with DC transduced with 1764/27-/4- compared to the samples without LPS. This shift was more marked with cells transduced with 1764/27-/4-/pR20.5/vhs compared to the samples without LPS. Firstly, these results showed that DC transduced with the disabled vector express low levels of CD83 similarly to non-infected DC. Also, these results suggest that even though little change is seen following transduction of DC with the disabled vector, DC transduced with the UL41 gene inactivated express higher levels of CD83 compared to the equivalent vector with UL41 (vhs) intact, particularly following treatment with LPS.

VI.2.4. CD86

The FACS analysis results for CD86 are shown in figure VI.5. In the absence of LPS, mock-infected DC expressed low levels of CD86, DC transduced with vector 1764/27-/4- expressed an intermediate level of the marker whereas cells transduced with 1764/27-/4-/pR20.5/vhs expressed significantly higher levels of CD86. In the presence of LPS, a significant shift of cell population was observed for mock-infected cells and a slight increase of CD86 was observed with DC transduced with 1764/27-/4- compared to the samples without LPS. The level of CD86 expression was again significantly increased with cells transduced with 1764/27-/4-/pR20.5/vhs compared to mock-infected DC or DC transduced with an equivalent vector with UL41 (vhs) intact. These results showed that DC transduced with the disabled vector express low levels of CD86 similarly to mock-infected DC. Also, these results show that DC transduced with the disabled vector with the UL41 gene inactivated express high levels of CD86.

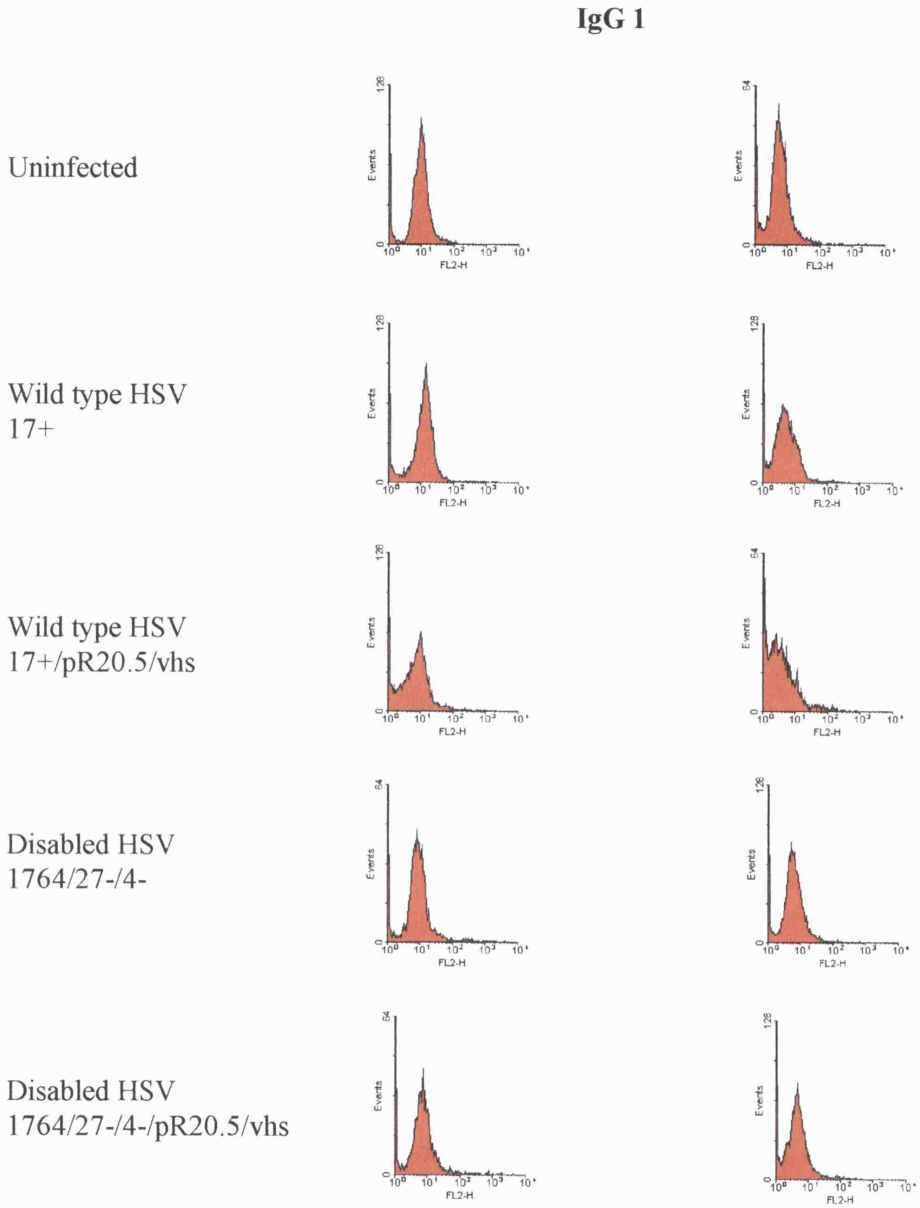


Figure VI.1: FACS analysis of DC population stained for IgG1 as a negative control following transduction with vectors 17+, 17+/pR20.5/vhs, 1764/27-/4-, 1764/27-/4-/pR20.5/vhs. Cells were either treated with lypopolysacchariden(+LPS) or not (-LPS). FL2-H represents arbitrary fluorescent intensity.

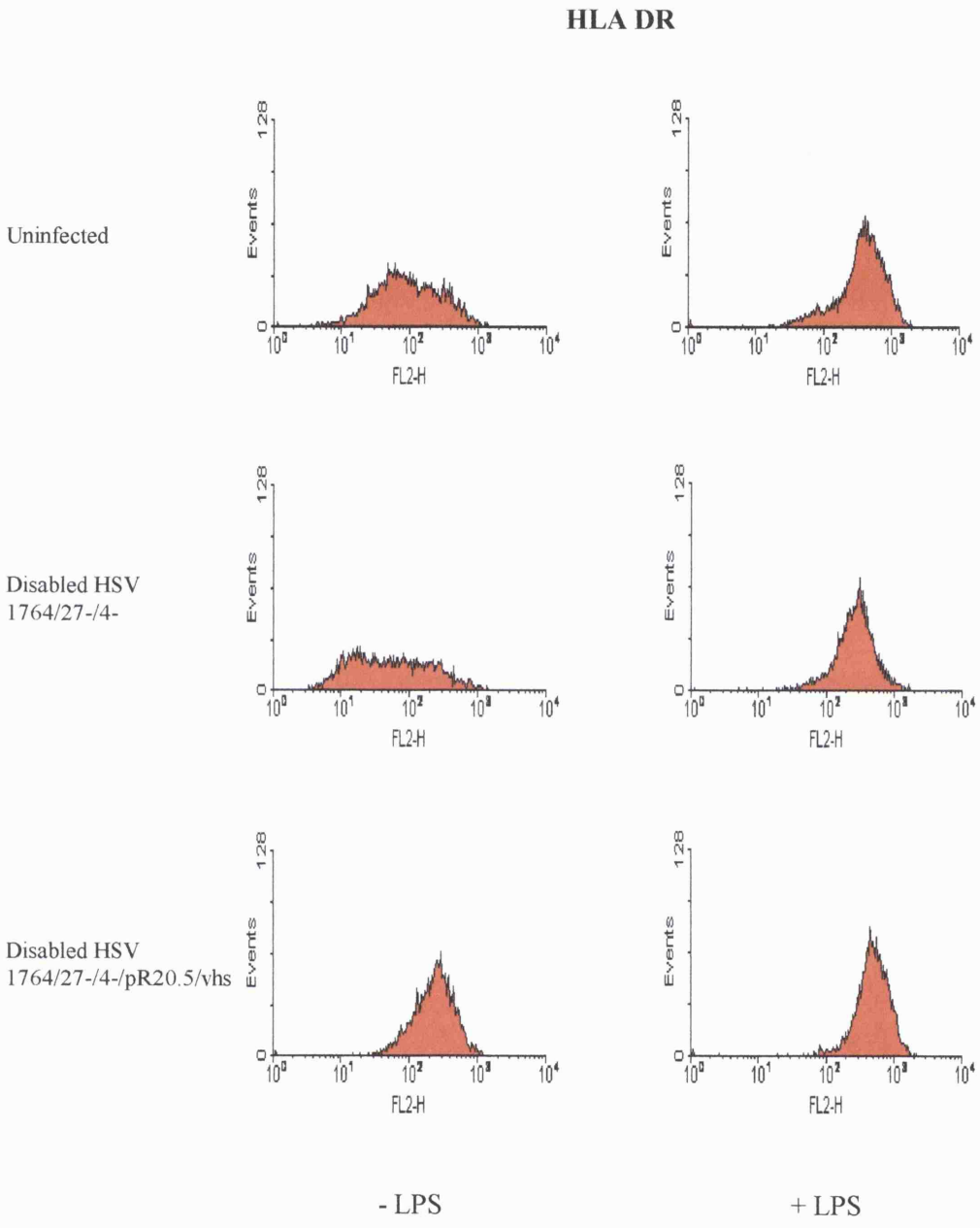


Figure VI.2: FACS analysis of DC population stained for HLA-DR following transduction with vectors 1764/27-/4- and 1764/27-/4-/pR20.5/vhs. Cells were either treated with lypo- polysaccharide (+LPS) or not (-LPS). FL2-H represents arbitrary fluorescent intensity.

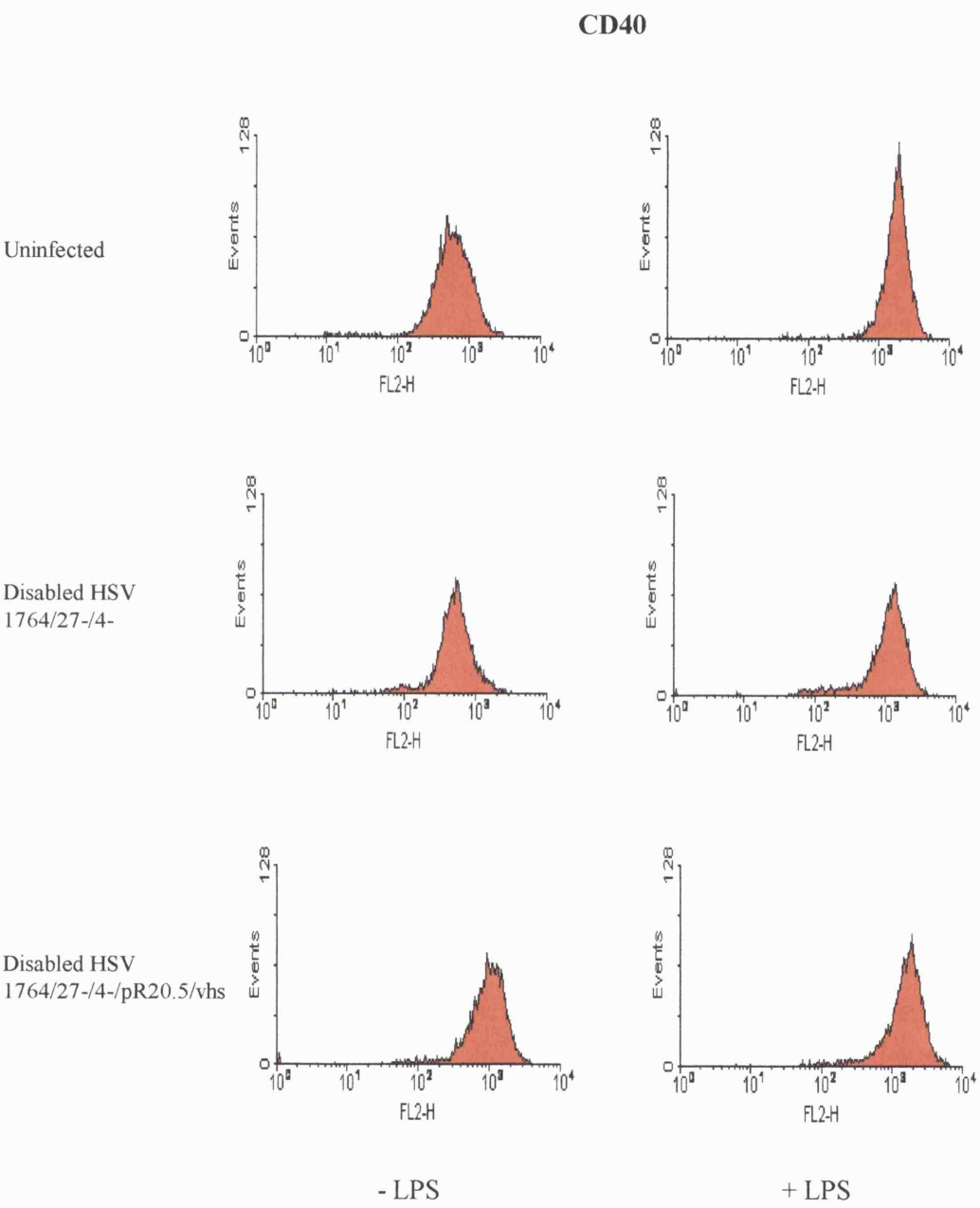


Figure VI.3: FACS analysis of DC population stained for CD40 following transduction with vectors 1764/27-/4- and 1764/27-/4-/pR20.5/vhs. Cells were either treated with lypo- polysaccharide (+LPS) or not (-LPS).

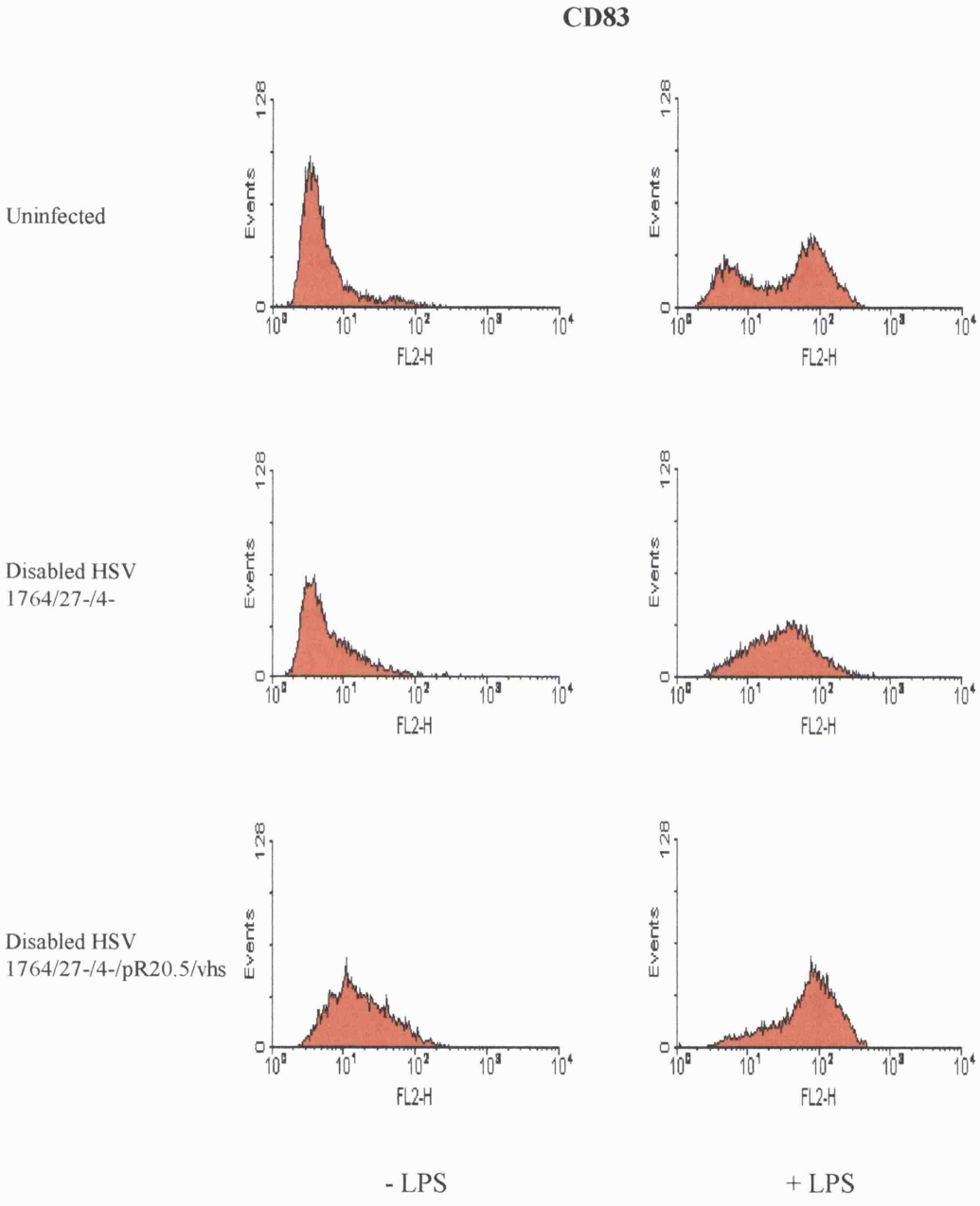


Figure VI.4: FACS analysis of DC population stained for CD83 following transduction with vectors 1764/27-/4- and 1764/27-/4-/pR20.5/vhs. Cells were either treated with lypo- polysaccharide (+LPS) or not (-LPS).

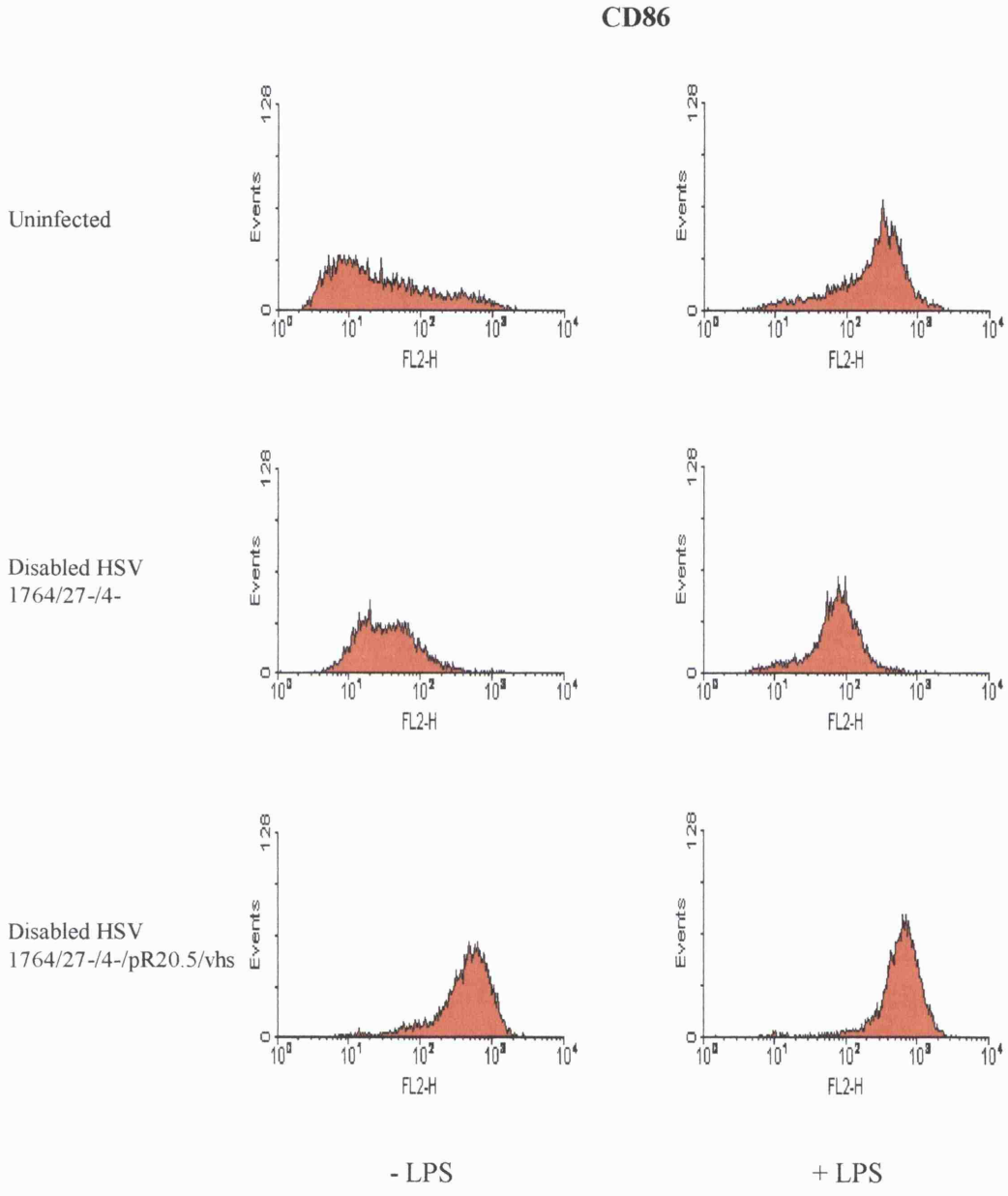


Figure VI.5: FACS analysis of DC population stained for CD86 following transduction with vectors 1764/27-/4- and 1764/27-/4-/pR20.5/vhs. Cells were either treated with lypo- polysaccharide (+LPS) or not (-LPS).

VI.2.5. Discussion

These results showed that DC transduced with a disabled HSV with vhs intact gave the same phenotype as a mock-infected cell population (expressing high levels of HLA-DR and CD40 and intermediate to low levels of CD83 and CD86) whereas DC transduced with an equivalent vector with vhs inactivated seem to generally up-regulate all these markers, in particular the activation marker CD86. These findings indicate that the disabled HSV vector 1764/27-/4- in which ICP34.5, ICP27 and ICP4 are deleted and vmw65 inactivated does not affect resting DC phenotype, whereas the further inactivation of UL41 from such a vector allows activation of the cells by the infection process. This suggests that vhs prevents DC activation, at least in the context of the disabled HSV vector tested. In order to further investigate this phenomenon, the phenotype of DC transduced with two minimally disabled vectors with or without vhs deleted were analysed.

VI.3. Effect of wild type HSV-1 and HSV with vhs inactivated on DC phenotype and activation markers

Wild type (wt) HSV-1 strain 17+ and the vector 17+/pR20.5/vhs (described in chapter III) were used in this section. vector for gene delivery to DC. 17+ HSV-1 vector strain is equivalent to 17+/pR20.5/vhs but does not contain an expression cassette and has the UL41 gene intact. The phenotype of DC transduced with both vectors was analysed by FACS for the expression of HLA-DR, CD40, CD83 and CD86, 24 hours after transduction with and without LPS treatment.

VI.3.1. HLA-DR

The FACS analysis results for HLA-DR are shown in figure VI.6. In the absence of LPS, mock-infected DC were positive for HLA-DR. DC infected with wt 17+ HSV-1 expressed similar levels of HLA-DR as did the cell population transduced with 17+/pR20.5/vhs. In the presence of LPS, a significant shift of cell population

was observed for mock-infected cells, but only a slight shift was observed with DC transduced with either 17+ or 17+/pR20.5/vhs. These results show that wt HSV transduced DC express HLA-DR at similar levels to non-infected DC and that these two viruses gave similar results. Thus, no significant difference was noted between DC transduced with 17+ (vhs intact) and 17+/pR20.5/vhs (vhs inactivated) in this case.

VI.3.2. CD40

The FACS analysis results for CD40 are shown in figure VI.7. In the absence of LPS, mock-infected DC are strongly positive for CD40 and DC infected with wt HSV also express high levels of CD40. Cells transduced with 17+/pR20.5/vhs express high levels of CD40 even though a split population is observed in this case. In the presence of LPS, a shift of cell population was observed for mock-infected cells and with DC infected with 17+ vector compared to the samples without LPS, but this shift was slightly less in cells transduced with 17+/pR20.5/vhs. Thus, these results indicate that DC transduced with the wild-type HSV express high levels of CD40 similarly to mock-infected DC. However, in DC transduced with the wild-type virus with the UL41 gene inactivated CD40 is somewhat reduced. The data suggests that in presence of LPS, wt HSV infected DC block CD40 expression compared to the mock-infected cells.

VI.3.3. CD83

The FACS analysis results for CD83 are shown in figure VI.8. In the absence of LPS, mock-infected DC express low levels of CD83. DC infected with 17+ and 17+/pR20.5/vhs both expressed intermediate levels of CD83. In the presence of LPS, a split in cell population was observed for mock-infected cells indicating that more than half of cells have shifted. DC infected with 17+ and 17+/pR20.5/vhs both expressed high levels of CD83 although the shift was more marked with cells transduced with 17+ compared to 17+/pR20.5/vhs, similar to the pattern observed

without LPS. These results showed that DC infected with wild-type HSV express relatively low levels of CD83, slightly higher than the non-infected DC. The data also indicate that in presence of LPS, transduced DC block CD83 expression. In addition, no significant difference was observed between 17+ with vhs intact and 17+/pR20.5/vhs with vhs inactivated.

VI.3.4. CD86

The FACS analysis results for CD86 are shown in figure VI.9. In the absence of LPS, mock-infected DC expressed low levels of CD86, DC infected with 17+ and 17+/pR20.5/vhs expressed an intermediate level of the marker. In the presence of LPS, a significant shift of cell population was observed for mock-infected cells whereas no significant increase of CD86 was observed with DC infected with either 17+ or 17+/pR20.5/vhs. The level of CD86 expression decreased with cells infected with 17+ and 17+/pR20.5/vhs compared to cells infected similarly in absence of LPS. These results showed that DC infected with wild-type HSV express intermediate levels of CD86, slightly higher than mock-infected DC. Also, these results show that in presence of LPS, transduced DC fail to up-regulate CD86. In addition, no difference was noted between DC transduced with the wild-type virus with the UL41 gene inactivated and the wild-type virus with vhs intact.

HLA DR

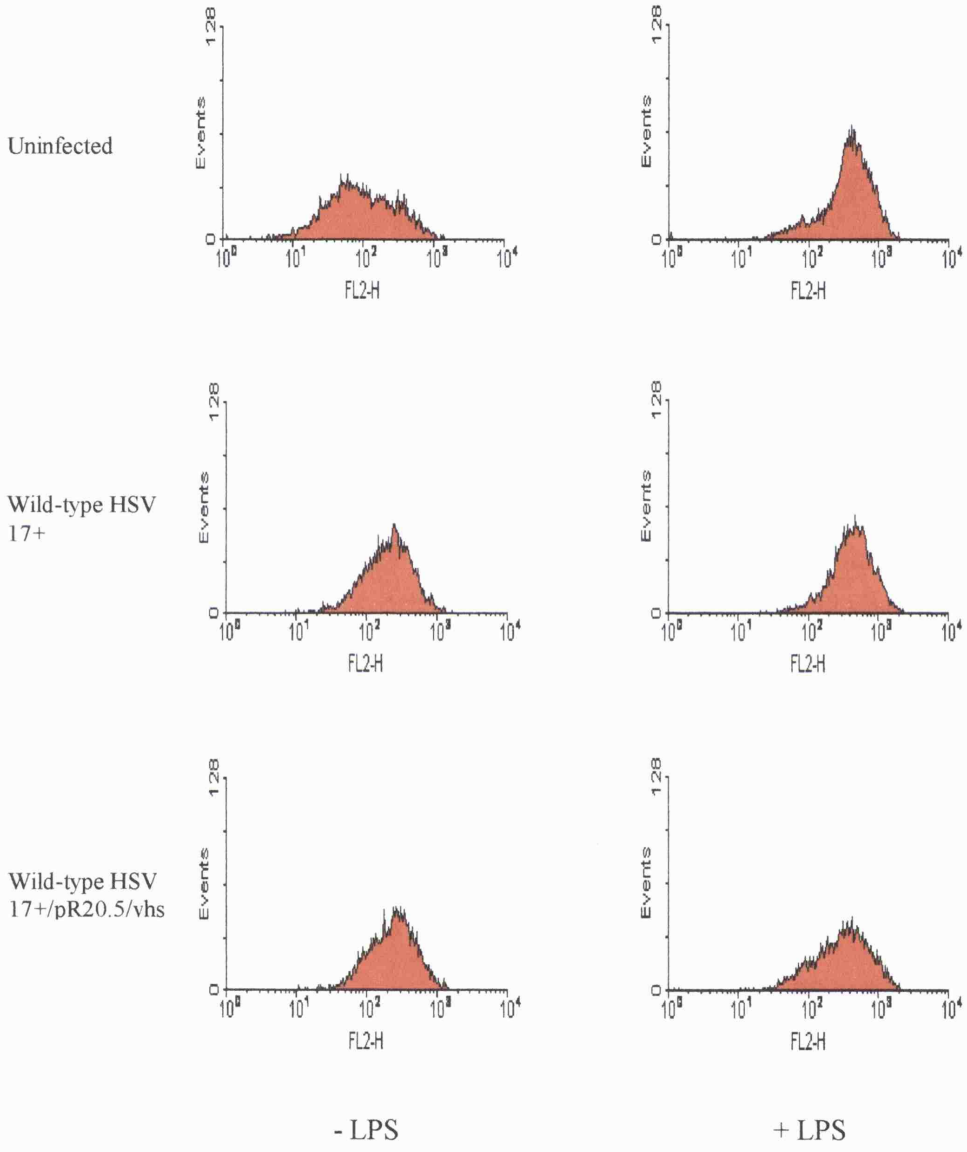


Figure VI.6: FACS analysis of DC population stained for HLA-DR following transduction with vectors 17+and 17+/pR20.5/vhs. Cells were either treated with lypopolysaccharide (+LPS) or not (-LPS).

CD40

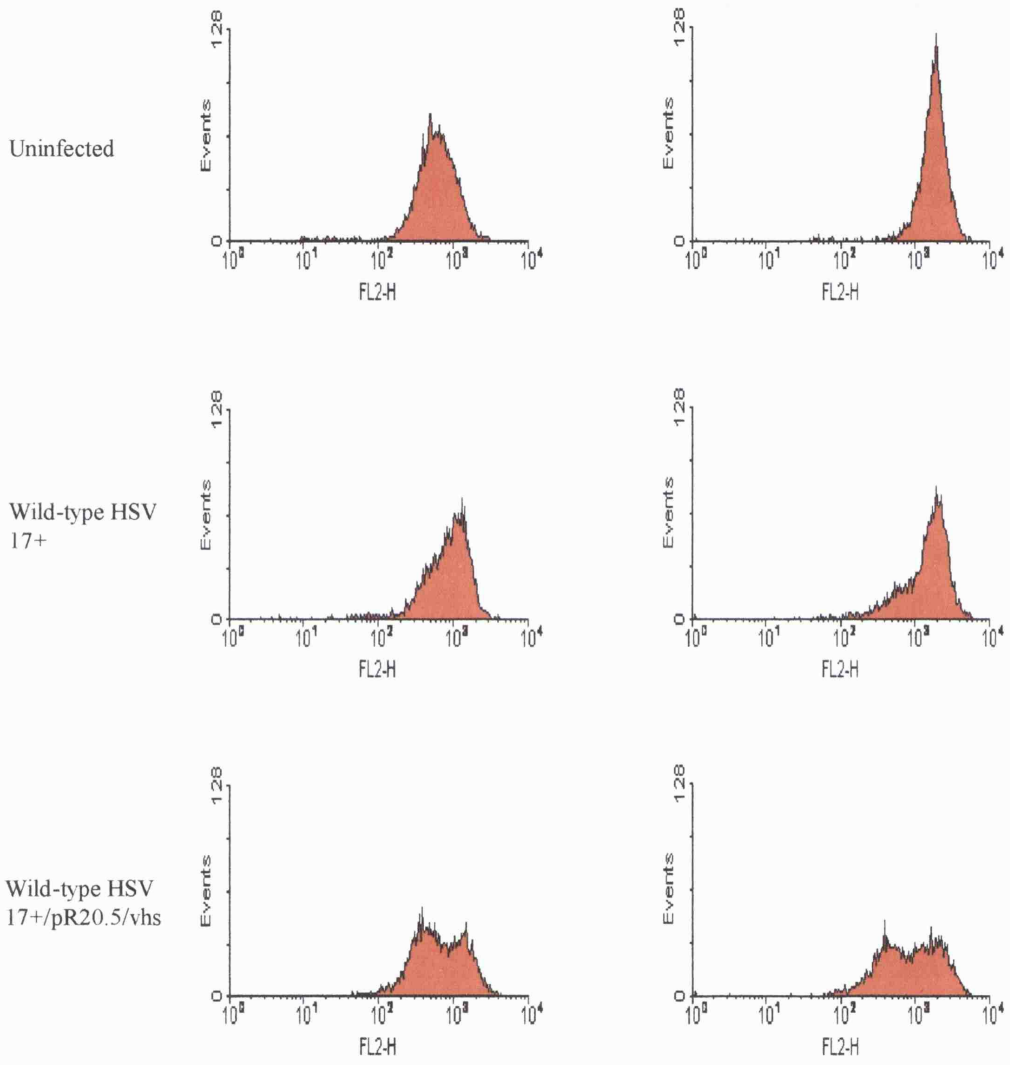


Figure VI.7: FACS analysis of DC population stained for CD40 following transduction with vectors 17+and 17+/pR20.5/vhs. Cells were either treated with lypopolysaccharide (+LPS) or not (-LPS).

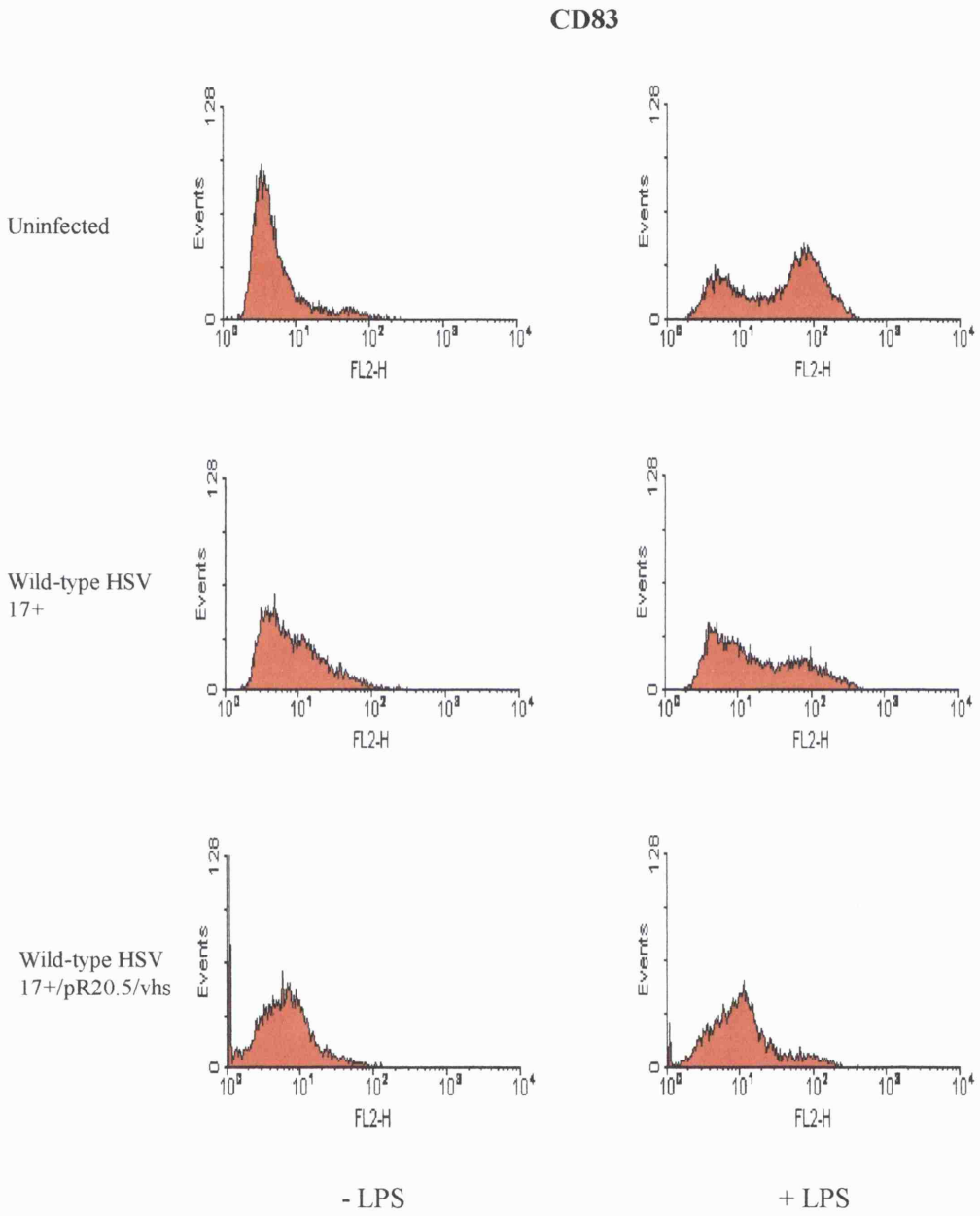


Figure VI.8: FACS analysis of DC population stained for CD83 following transduction with vectors 17+and 17+/pR20.5/vhs. Cells were either treated with lypopolysaccharide (+LPS) or not (-LPS).

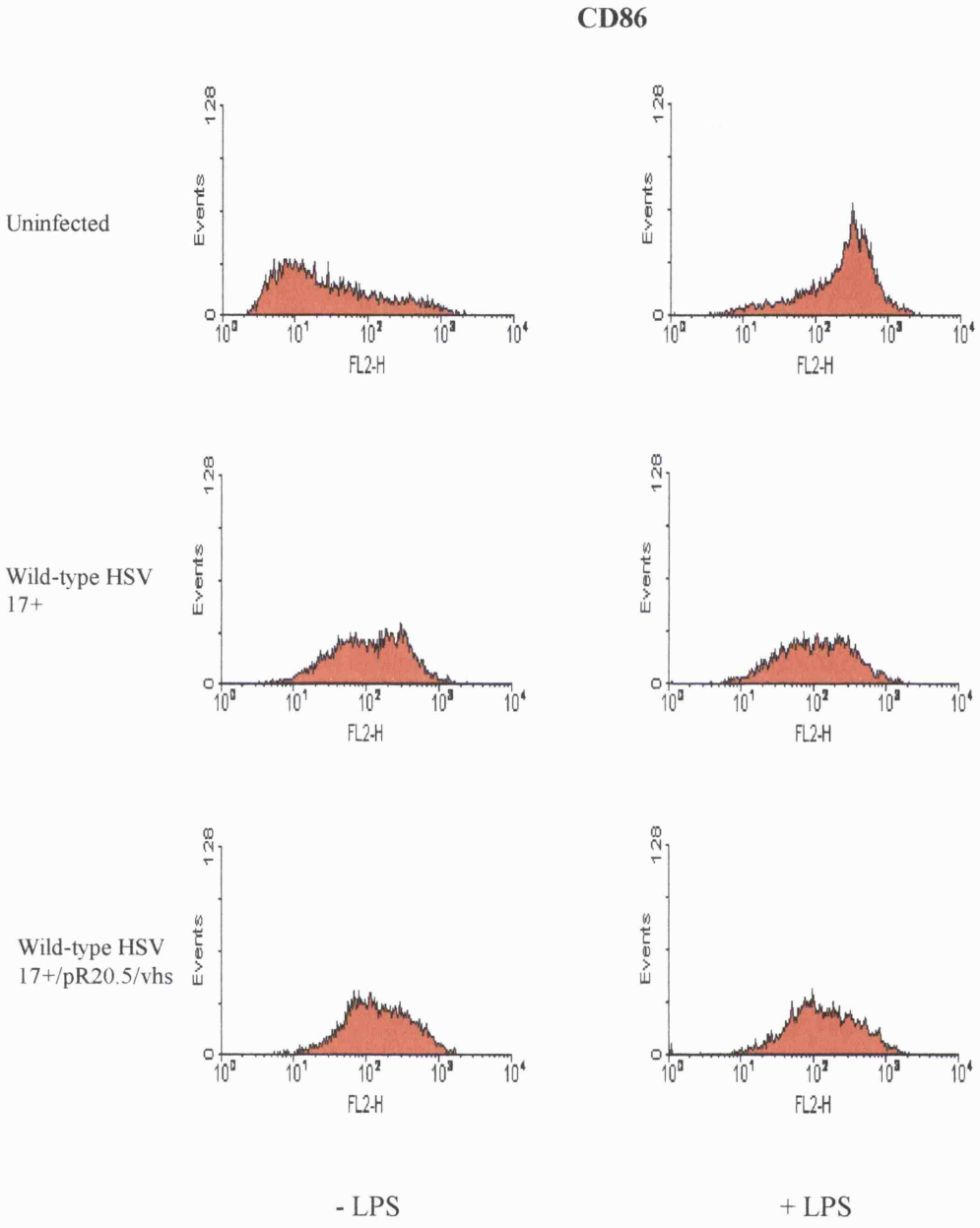


Figure VI.9: FACS analysis of DC population stained with CD86 following transduction with vectors 17+and 17+/pR20.5/vhs. Cells were either treated with lypopolysaccharide (+LPS) or not (-LPS).

VI.3.5. Discussion

A summary of FACS data presented in this chapter is shown in figures VI.10 and VI.11. These results showed that DC infected with wt HSV generally expressed slightly higher levels of surface markers than mock-infected cells. However, in the presence of LPS, DC infected with the wt HSV failed to up-regulate the surface markers tested compared to the mock-infected cells suggesting that wt HSV does not allow DC to fully activate - low levels of CD40, CD83 and CD86 are observed. However, the results obtained with these viruses showed no difference between wt viruses with and without vhs intact. This is in contrast to the effects of vhs deletion from a replication incompetent virus where there are significant effects on DC activation markers accompanying inactivation of vhs. Thus in wt HSV other genes as well as vhs may well contribute to the inactivation of DC by HSV which has been observed.

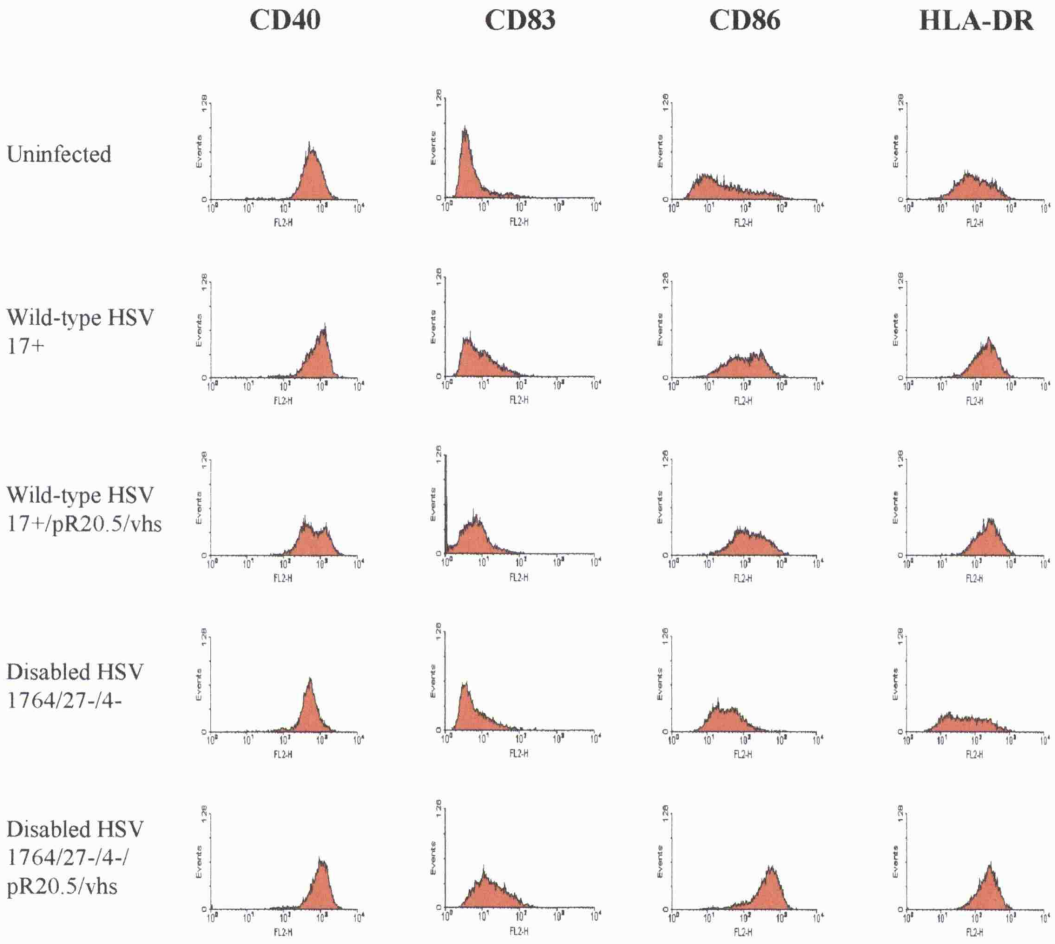


Figure VI.10: FACS analysis summary of DC population transduced with various HSV vectors, left untreated (no LPS) and stained for HLA-DR, CD40, CD83 and CD86.

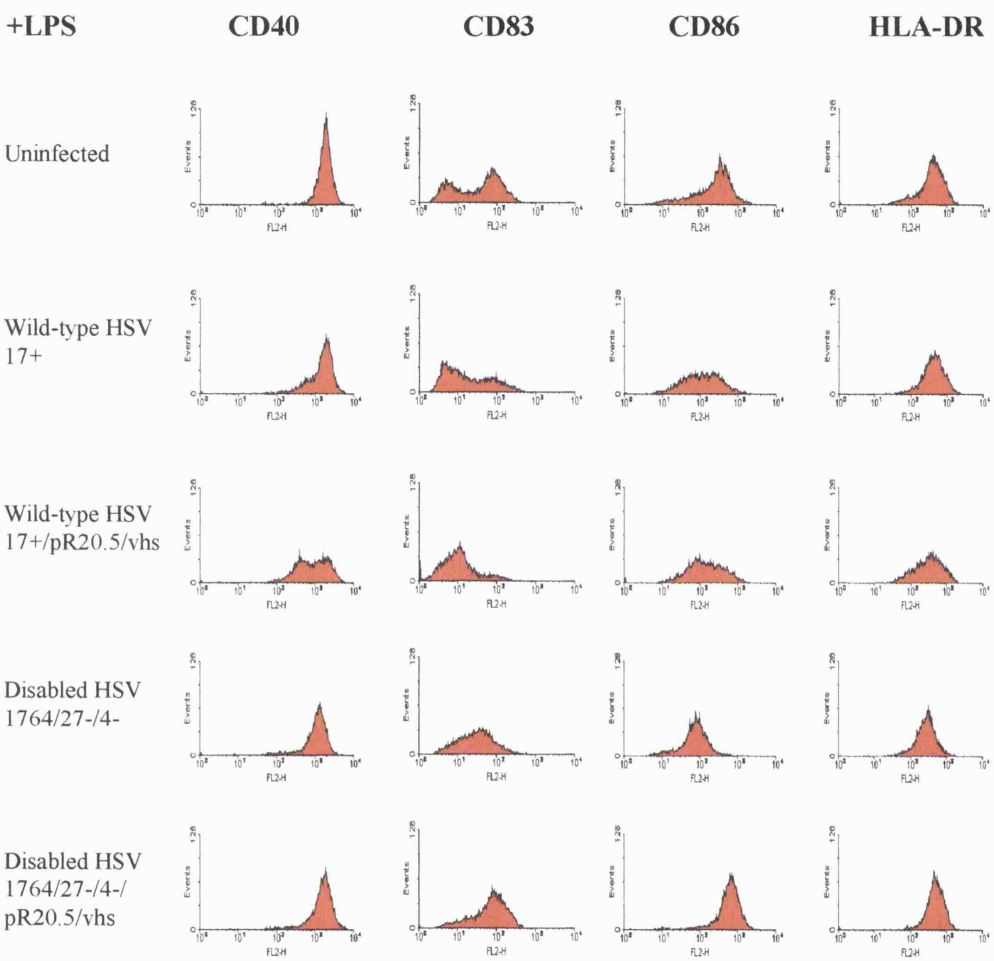


Figure VI.11: FACS analysis summary of DC population transduced with various HSV vectors, treated with LPS and stained for HLA-DR, CD40, CD83 and CD86.

CHAPTER VII

STIMULATION OF ANTIGEN SPECIFIC T CELL PROLIFERATION

BY HSV TRANSDUCED DENDRITIC CELLS

VII.1. Introduction

The objective of this chapter was to explore the ability of transduced DC to stimulate T cell responses in response to a foreign antigen delivered to the cells via an HSV vector. For this purpose, the gene coding for the hepatitis B surface antigen (HBsAg) was chosen as a model antigen. The results obtained in the first part of the results section indicated that two HSV-based vectors, one partially disabled and the other fully disabled gave good gene delivery efficiency to DC. The results in chapter VI in the second part of the results section showed that DC transduced with a fully disabled vector with vhs inactivated were able to activate surface markers important for T-cell stimulation. Based on these results, we chose to construct vectors encoding the HBsAg gene and study the ability of transduced DC to generate a specific T cell response. This would allow us to verify that when HSV vectors are used to deliver foreign antigens to DC they do not alter DC functioning from the point of view of T cell stimulation. Two HSV vectors expressing HBsAg were tested based on the following two viruses:

- 1764/pR15/UL43MSVGFP: partially disabled vector with ICP34.5 deleted, vmw65, UL43 and UL41 (vhs) inactivated.
- 1764/27-/4-/pR20.5/vhs: replication incompetent vector with ICP34.5, ICP27 and ICP4 deleted, and vmw65 and vhs inactivated. This virus strain also contains a deletion of the endogenous LAT P2 region to prevent recombinational instability which is caused by insertion of extra LAT P2 elements (contained in pR20.5) in vhs.

The first part of this chapter describes the cloning of HBsAg into the pR15 or pR20.5 cassettes, the insertion into the vectors and the confirmation of the genome structure by Southern blotting, and the expression of the protein in the vector producer cells. In the second part of this chapter, DC are isolated from donors who have been vaccinated against HBV and are transduced with the vectors constructed. DC function for antigen presentation to T cells was then assessed by measuring the CD4+ T cell

proliferation specific to HBsAg. The results from four vaccinated individuals are presented in this section. One unvaccinated individual was used as a negative control for these experiments.

VII.2. Construction of two HSV-based vectors encoding the hepatitis B surface antigen (HBsAg)

This section describes the construction of a partially disabled vector and a replication incompetent vector each encoding HBsAg. For this purpose, the HBsAg was cloned into shuttle plasmids pR15 or pR20.5 which were previously constructed in the laboratory for recombination into the HSV viral genome. In order to check that recombination occurred properly, the virus structure was confirmed by Southern blot. At the end of this section, the expression of HBsAg following infection with one of these viruses was determined in the virus producer cells.

VII.2.1. Cloning of the HBsAg gene into the pR15 or the pR20.5 cassettes

The cloning of HBsAg into the pR15 or pR20.5 cassettes was performed in two steps in order to facilitate subsequent manipulations. The HBsAg was first cloned into the plasmid pSP72 as described in figures VII.1 and VII.2. Briefly, the plasmid pHBV130 (provided by Kenneth Murray, University of Edinburgh) is the plasmid pBR322 containing a 3.2 kb HBV DNA fragment inserted at the Pst I site. The 939 bp HBsAg fragment (HBV nucleotides 1498 to 2437) was digested from pHBV130 with Xho I/Nsi I and cloned into pSP72 (Sal I/Sma I). The excision of a 971 bp fragment from pSP72 with Hind III/Eco RI showed that the HBsAg fragment was inserted into pSP72.

The HBsAg DNA fragment was then excised from pSP72 with Hind III/Eco RI and cloned into the pR15 cassette as indicated in figures VII.3 and VII.4. The pR15 cassette consisting of a LAP1 sequence and an MMLV-LTR promoter driving *LacZ* was inserted into a plasmid allowing recombination into the virion host shuttoff

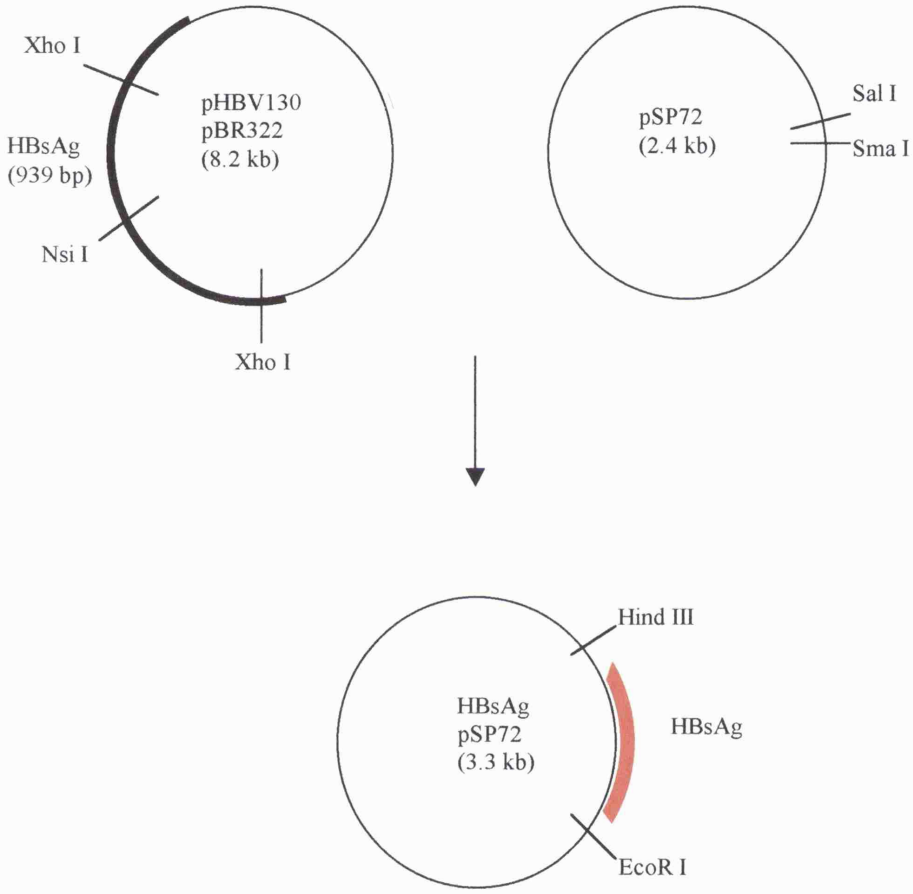


Figure VII.1: Schematic diagram for the cloning of the hepatitis B surface antigen (HBsAg) into pSP72 expression vector. pHBV130 was digested with XhoI/T4 polymerase/NsiI and cloned into pSP72 (SalI/T4 polymerase/SmaI).

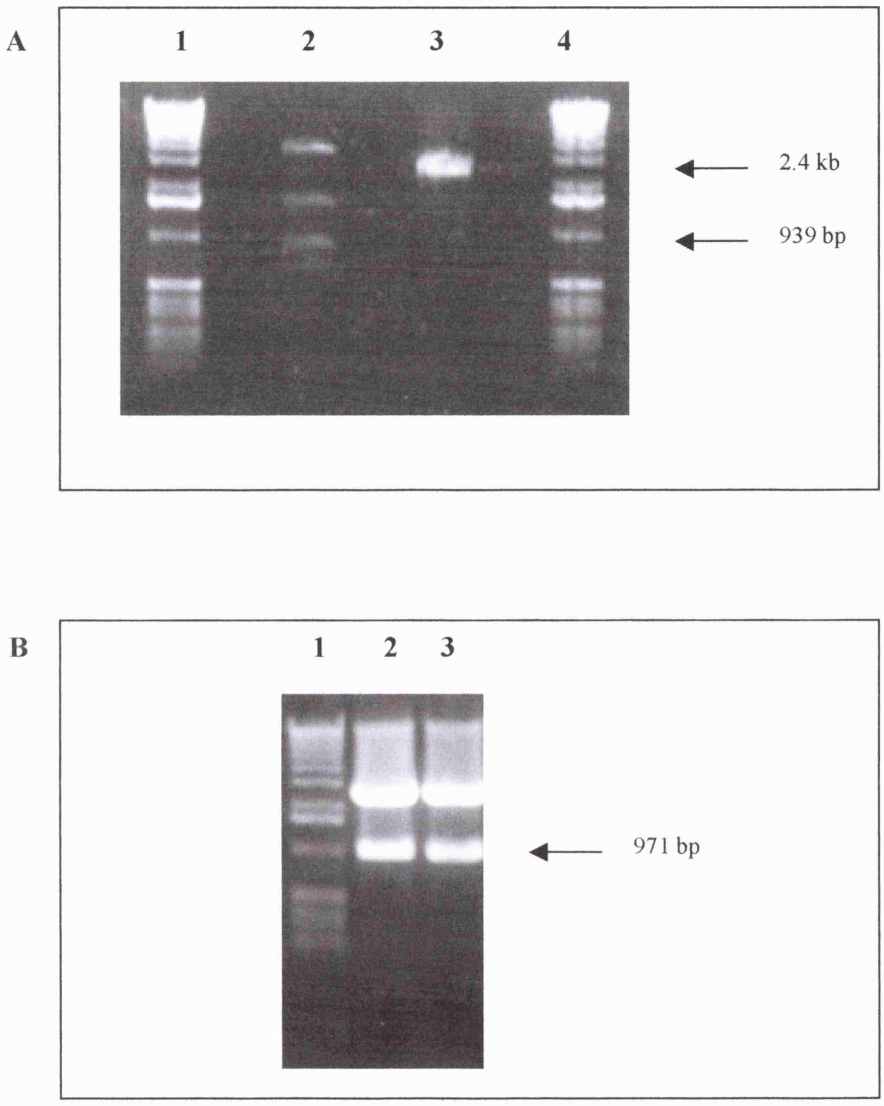


Figure VII.2: A. Restriction enzyme cleavage patterns for pHBV130 and pSP72. Tracks 1,4: 1 kb DNA ladder; track 2: pHBV130pBR322 digested with Xho I/ T4 polymerase/Nsi I; track 3: pSP72 digested with Sma I/Sal I.

B. Restriction enzyme cleavage patterns for HBsAgpSP72. Track 1: 1 kb DNA ladder; tracks 2,3: HBsAgpSP72 digested with Hind III/EcoR I.

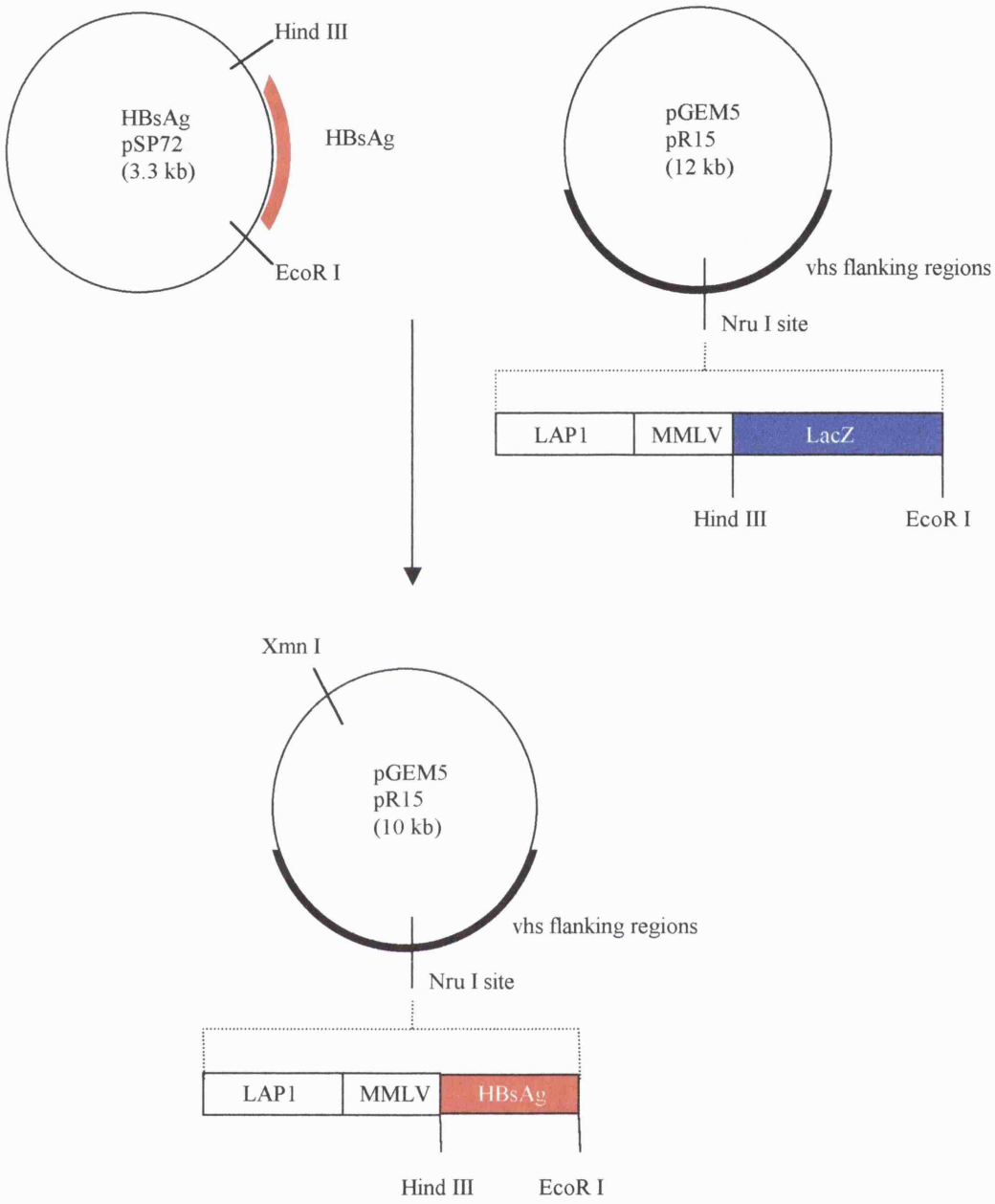


Figure VII.3: Schematic diagram for the cloning of the hepatitis B surface antigen (HBsAg) into the pR15 cassette. HBsAgpSP72 was digested with HindIII/EcoRI and cloned into pR15 instead of LacZ (HindIII/EcoRI). The unique XmnI site was used to linearise HBsAgpR15 for co-transfection with HSV viral DNA.

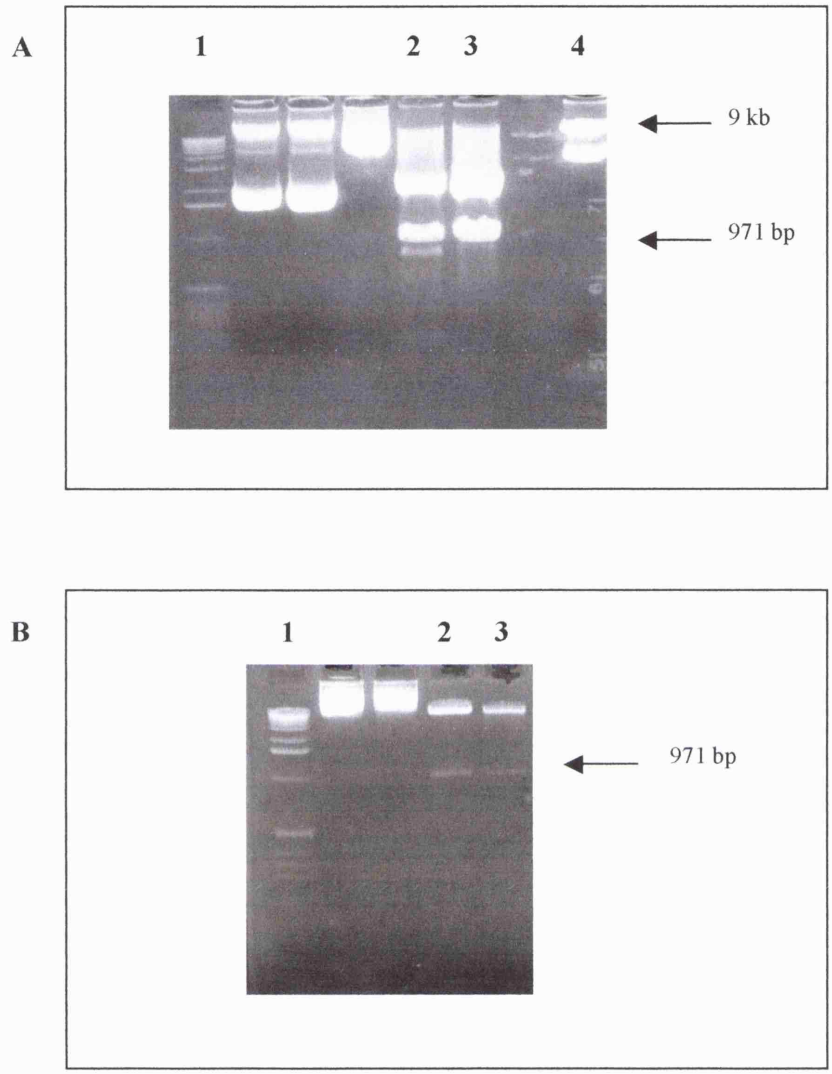


Figure VII.4: A. Restriction enzyme digestions for HBsAgpSP72 and pR15. Track 1: 1 kb DNA ladder; tracks 2,3: HBsAgpSP72 digested with Hind III/ T4 polymerase/EcoR I; track 4: pR15 digested with Hind III/T4 polymeras/EcoR I. **B.** Restriction enzyme digestions for HBsAgpR15. Track 1: 1 kb DNA ladder; tracks 2,3: HBsAgpR15 digested with Hind III/EcoR I.

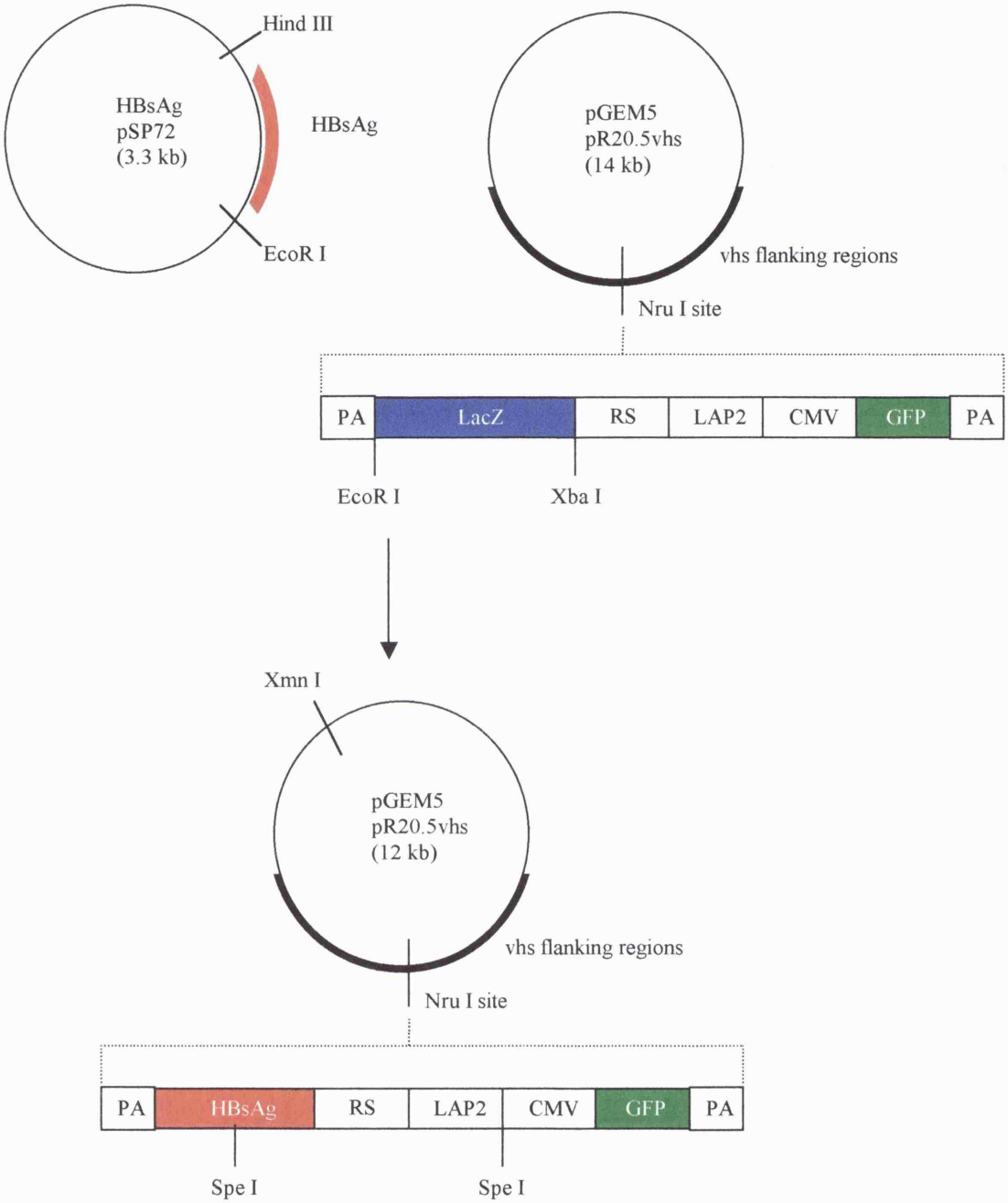


Figure VII.5: Schematic diagram for the cloning of the hepatitis B surface antigen (HBsAg) into the pR20.5vhs cassette. HBsAgpSP72 was digested with HindIII/EcoRI and cloned into pR20.5vhs instead of LacZ (HindIII/EcoRI). The unique XmnI site was used to linearise HBsAgpR20.5vhs for co-transfection with viral DNA.

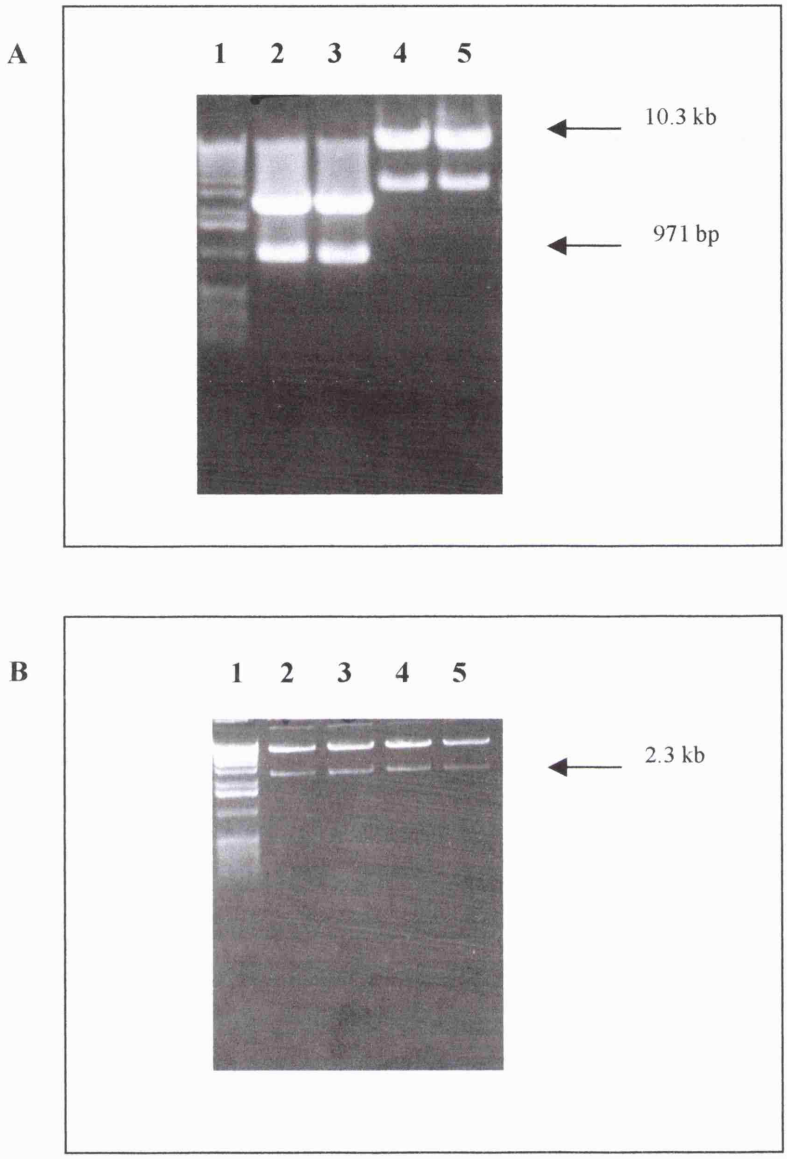


Figure VII.6: **A.** Restriction enzyme digestions for HBsAgpSP72 and pR20.5vhs. Track 1: 1 kb DNA ladder; tracks 2,3: HBsAgpSP72 digested with Hind III/ T4 polymerase/EcoR I; tracks 4,5: pR20.5vhs digested with Xba I/T4 polymerase/ EcoR I . **B.** Restriction enzyme digestions for HBsAgpR20.5vhs. Track 1: 1 kb DNA ladder; tracks 2,3,4,5: HBsAgpR20.5vhs digested with Spe I.

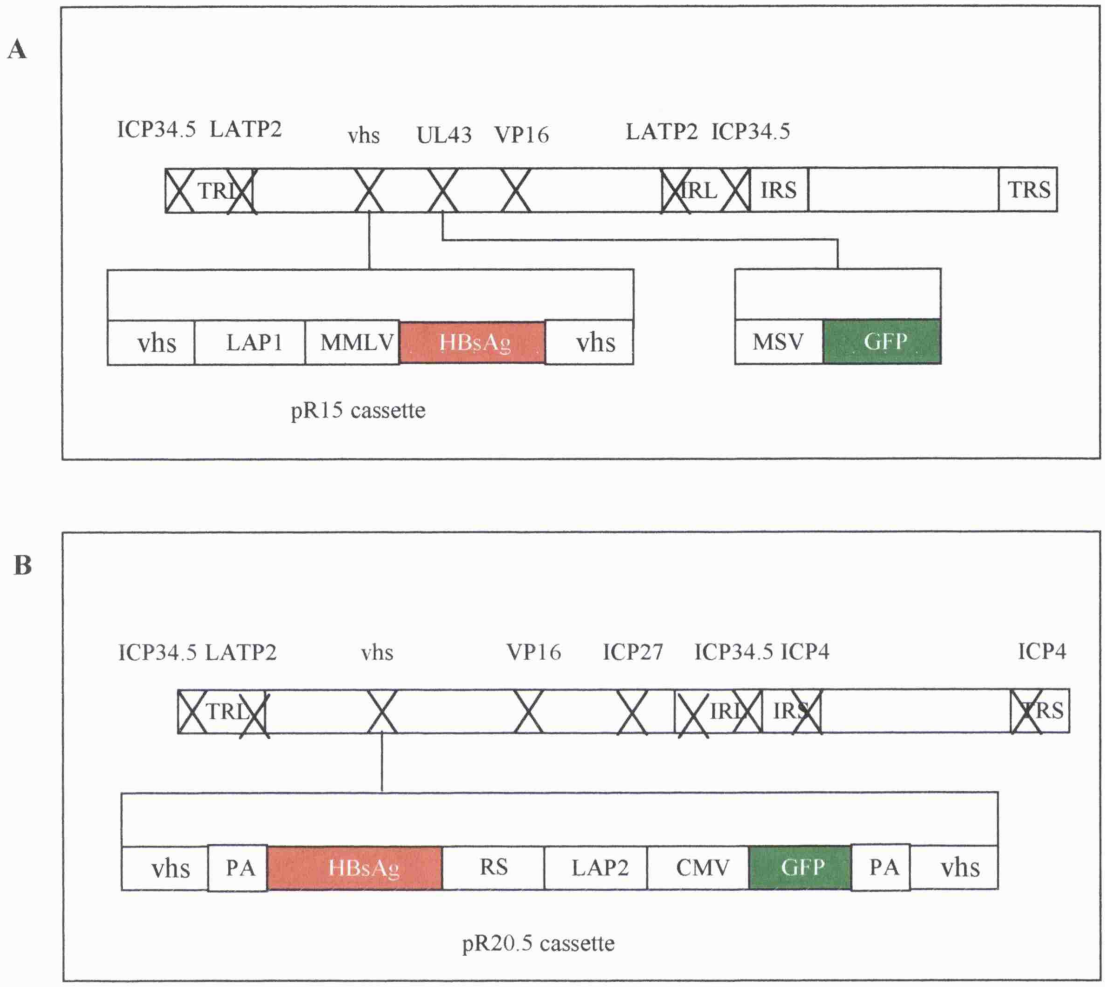


Figure VII.7: Schematic diagram of the HSV genome with the recombinant shuttle cassettes constructed. **A.** Diagram of the 1764/HBsAgpR15/UL43MSVGFP vector containing the recombinant shuttle cassette (HBsAgpR15) inserted in the UL41 gene (vhs). **B.** Diagram of the 1764/27-/4-/pR20.5/vhs vector containing the recombinant shuttle cassette (HBsAgpR20.5) inserted in the UL41 gene (vhs).

protein (vhs)-encoding gene (UL41) at the unique Nru I site (HSV nt 91854). An Eco RI site was also previously destroyed in order to give a unique Eco RI site in pR15. The HBsAg fragment was excised from pSP72 with Hind III/Eco RI and inserted into pR15 in place of *LacZ* (Hind III/Eco RI). The excision of a 971 bp fragment from pR15 with Hind III/Eco RI indicated that the pR15 plasmid contained the HBsAg DNA fragment.

For the cloning into pR20.5, the HBsAg was excised from pSP72 with Hind III/Eco RI as described in figures VII.5 and VII.6. The pR20.5 cassette consisting of the gene for *GFP* and *LacZ* driven by the CMV and RSV promoters, respectively, in a back to back orientation and separated by LAT sequences was inserted into a plasmid allowing recombination into the virion host shuttoff protein (vhs)-encoding gene (UL41) at the unique Nru I site. The HBsAg fragment was excised from pSP72 with Hind III/Eco RI and inserted into pR20.5 in place of *LacZ* (Eco RI/Xba I). The excision of a 2.3 kb fragment from pRpR20.5 with spe I then indicated that the plasmid contained the HBsAg DNA fragment.

VII.2.2. Recombination of HBsAg containing plasmids into the 1764/pR15/ UL43MSVGFP or 1764/27-/4-/pR20.5/vhs viral backbones

The pR15 plasmid containing the HBsAg fragment was linearised using a unique Xmn I site in the plasmid backbone and cotransfected with infectious 1764/pR15/UL43MSVGFP viral DNA (partially disabled virus) for insertion into the vhs locus by homologous recombination. Recombinant plaques containing HBsAg were identified as white plaques (not expressing β-galactosidase, β-gal) from a background of blue plaques (expressing both GFP and β-gal).

Similarly the HBsAg encoding pR20.5 plasmid was linearised with a unique Xmn I site in the plasmid backbone and cotransfected with infectious 1764/27-/4-/pR20.5/vhs viral DNA. Recombinant plaques were then picked as green and not blue

plaques (expressing GFP but not β-gal) from a background of plaques that were green and blue (expressing both GFP and β-gal). The resulting virus constructs 1764/HBsAgpR15/ UL43MSVGFP and 1764/27-/4-/HBsAgpR20.5/vhs are shown respectively in figures VII.7A and VII.7B.

VII.2.3. Confirmation of the genome structure of 1764/HBsAgpR15/UL43MSV GFP

A southern blot was carried out in order to confirm the genome structure of virus 1764/HBsAgpR15/UL43MSVGFP. This was performed to show the presence of a 1 kb HBsAg DNA fragment within the 1764/HBsAgpR15/ UL43MSVGFP viral DNA following digestion with HindIII / EcoRI. The equivalent vector without HBsAg (1764/pR15/UL43MSVGFP) was used as a negative control and as shown in the blot no band was excised with Hind III/Eco RI. The plasmid pR15 was used as a positive control and a 1 kb fragment corresponding to HBsAg gene was excised with Hind III/Eco RI. Another positive control confirmed the presence of HBsAg in the pR20.5 plasmid after excision of a 2.3 kb fragment with Spe I. Thus, the genome structure of 1764/HBsAgpR15/UL43MSVGFP containing HBsAg was confirmed and this is shown in figure VII.8.

VII.2.3. The partially disabled virus 1764/HBsAgpR15/UL43MSVGFP expresses the HBsAg protein

A western blot was carried out to show the expression of HBsAg (25 kDa protein) after infection of BHKs with virus 1764/HbsAg15vhs/UL43MSVGFP. Figure VII.9 shows the expression of a 25 kDa protein corresponding to HBsAg from the virus. The negative and positive controls were protein extracts from BHKs respectively infected with the equivalent vector not containing HBsAg (1764/pR15/UL43MSVGPP) or after transient transfection with the HBsAg containing plasmid HBsAgpR15. No band was observed in the negative control

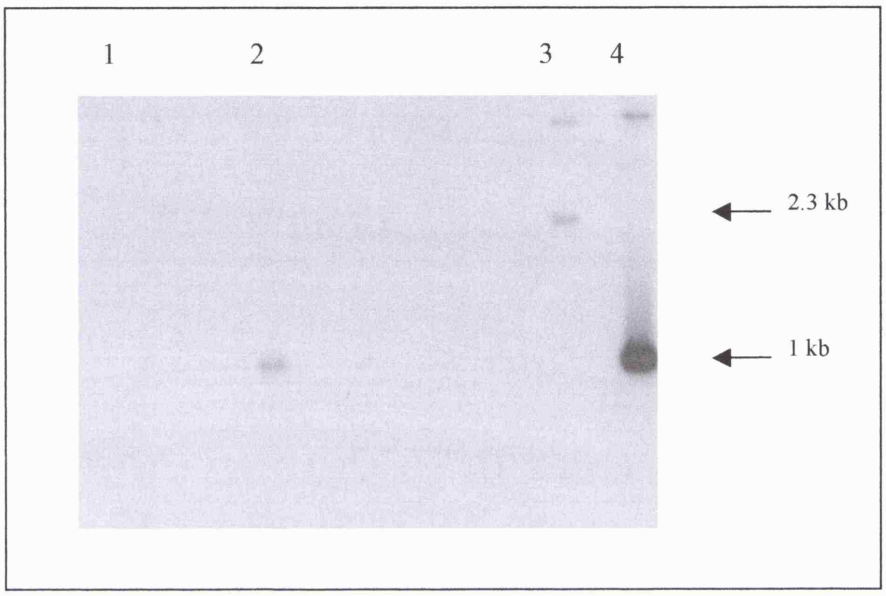


Figure VII.8: Southern blot showing the presence of HBsAg in 1764/pR15/UL43MSVGFP.
Track 1: negative control - 1764/pR15/UL43MSVGFP viral DNA digested with Hind III/EcoR I; track 2: 1764/HBsAgpR15/UL43MSVGFP viral DNA digested with Hind III/EcoR I; track 3: positive control - HBsAgpR20.5vhs plasmid DNA digested with spe I; track 4: positive control - HBsAgpR15 plasmid DNA digested with Hind III/EcoR I.

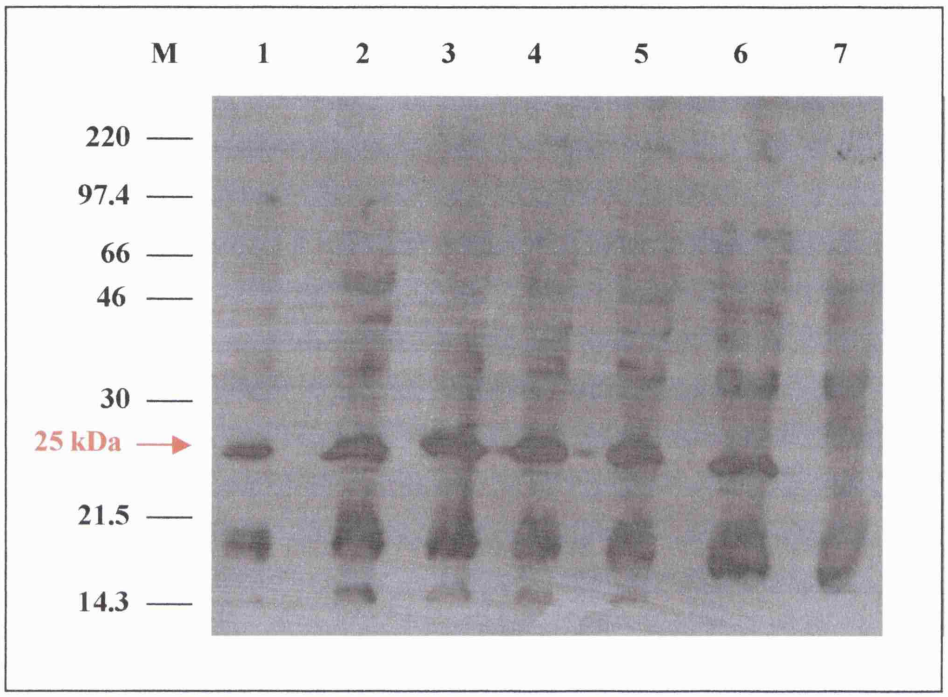


Figure VII.9: Western blot - 12% acrylamide gel showing the expression of HBsAg (25 kDa) in BHK cells. Track 1: positive control - protein extracts from BHK cells transfected with the recombinant plasmid DNA HBsAgpR15; tracks 2-6: protein extracts from BHK cells after infection with one individual plaque of virus. Track 7: negative control - protein extracts from BHK cells transfected with 17+/pR20.5/UL43. M = Molecular weight (kDa).

whereas a 25 kDa band was detected in the positive control. Thus, these results showed that the recombinant virus expressed high levels of HBsAg in BHKs.

In the first part of this chapter, the constructions of two recombinant viruses, 1764/HBsAgpR15/UL43MSVGFP and 1764/27-/4-/HBsAgpR20.5/vhs, were described and the genome structure confirmed. These vectors will now be used to transduce human DC isolated from vaccinated individuals in order to show a CD4+ T cell response specific to HBsAg. These results are presented in the following section.

VII.3. Stimulation of T cell responses using viruses 1764/HBsAgpR15/ UL43MSVGFP and 1764/27-/4-/HBsAgpR20.5/vhs

As about 90% of the world population has been exposed to HSV, the majority of people should respond to the virus even if it has been disabled. This could pose a problem for the use of HSV as a vector to deliver genes to DC as an ideal vector should deliver genes to the target cells without altering its function or survival. Thus, in this section the partially disabled and fully disabled vectors with the initial cassettes (not containing HBsAg) were used to transduce DC and show whether a further disabled vector could generate a weaker response in CD4+ proliferation assays. In parallel, the partially disabled and fully disabled vectors encoding the HBsAg were used as a model to stimulate a CD4+ T cell response specific to HBsAg in HBV vaccinated individuals. For this purpose, the viruses constructed in the previous section were used to deliver HBsAg into DC. Thus, DC transduced with HBsAg-encoding vectors were mixed with CD4+ T cells isolated from the peripheral blood of the same vaccinated individual and the affect on T cell proliferation assessed.

For each of the two vectors tested, the following conditions (in triplicate) were used:

- DC with T cells
- DC transduced with an HSV vector encoding reporter genes but no foreign

HBsAg and T cells

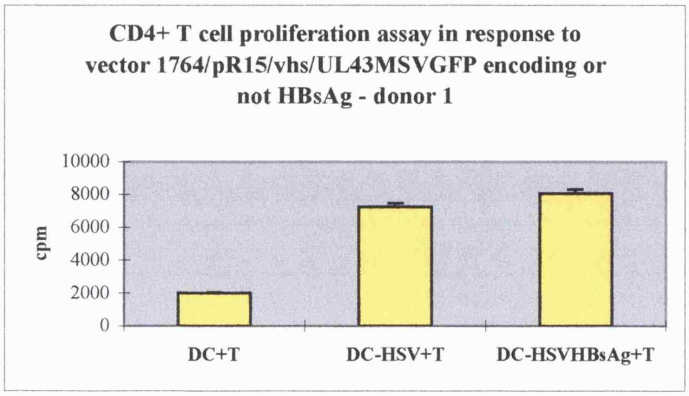
- DC transduced with an HSV vector encoding HBsAg with T cells

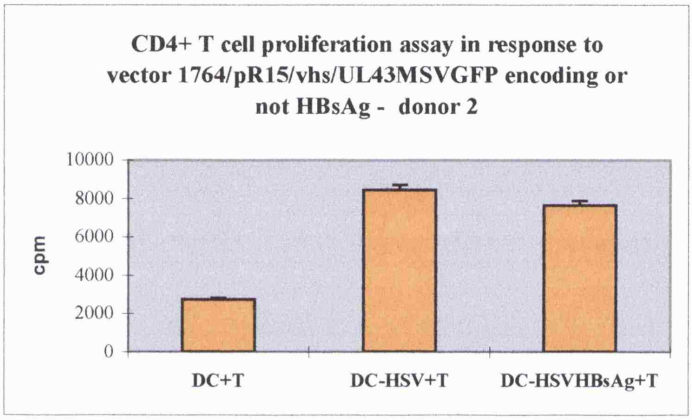
DC were used at two starting concentrations in order to determine the optimal number of DC to use per well ($1x10^4$ DC/well and $5x10^3$ DC/well) while the initial concentration of CD4+ T cells was fixed at $1x10^5$ cells/well. The optimal DC dilution of $1x10^4$ DC/well is shown here. Each set of proliferation assay was repeated for several donors and the results for five donors are described in this chapter.

As a positive control, the peripheral blood of donors was tested in order to confirm a CD4+ T cell response to the recall antigens purified protein derivative (PPD) and/or recombinant HBsAg protein. Thus, these were used to show that donors responded to recall antigens, such that the addition of antigen to DC and CD4+ T cells stimulated T lymphocyte proliferation *in vitro*. A strong proliferative response to PPD was seen for the five donors presented in this section whereas the response to HBsAg was not as high and variable. It was thus important that results from several individuals were assessed.

VII.3.1. CD4+ T cell stimulation in response to DC transduced with 1764/ HBsAgpR15/UL43MSVGFP

DC were isolated from peripheral blood of vaccinated donors and transduced with virus 1764/pR15/UL43MSVGFP containing or not containing HBsAg. Figure VII.10 presents the results for CD4+ proliferation assays using transduced DC for both donors. For donor 1, DC transduced with 1764/pR15/UL43MSVGFP induced proliferation of CD4+ T cells (mean counts per minute cpm=7255) and DC transduced with the equivalent vector encoding HBsAg stimulated slightly more CD4+ T cells (mean cpm=8075). Thus, DC transduced with both viruses 1764/pR15/UL43MSVGFP or 1764/HBsAgpR15/UL43MSVGFP generated a T cell response, respectively of 3,6 or 4 folds higher than the background (as DC alone





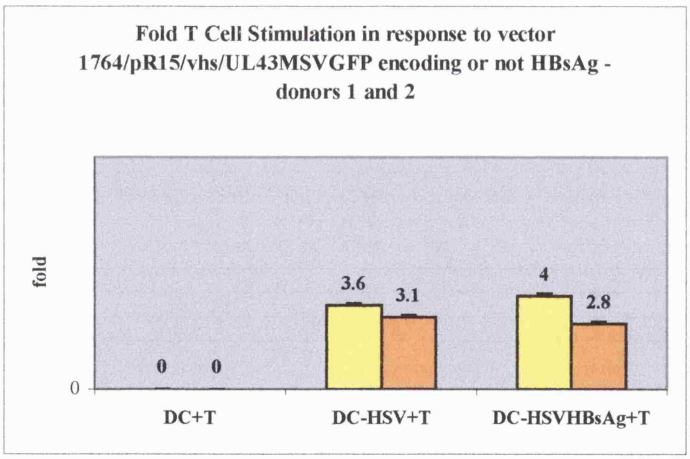


Figure VII.10: CD4+ T cell proliferation assay in response to HBsAg after transduction of DC with 1764/pR15/vhsUL43MSVGFP (HSV) or equivalent vector encoding HBsAg (HSVHBsAg).

induced CD4+ T cell proliferation of mean cpm=1994), but no significant difference was noted between these two viruses.

For donor 2, DC transduced with 1764/pR15/UL43MSVGFP induced proliferation of CD4+ T cells (mean counts per minute cpm=8454) whereas DC transduced with the equivalent vector encoding HBsAg induced slightly less CD4+ T cell proliferation (mean cpm=7668). Thus, DC transduced with both viruses 1764/pR15/UL43MSVGFP or 1764/HBsAgpR15/UL43MSVGFP generated a T cell response, respectively of 3,1 or 2.8 folds higher than the background (as DC alone induced CD4+ T cell proliferation of mean cpm=2768), but as seen for donor 1 no significant difference was noted between these two viruses.

These results indicated that DC transduced with the partially disabled HSV vector (1764/pR15/UL43MSVGFP) retained the ability to induce CD4+ T lymphocyte proliferation as shown in the results from two healthy individuals that responded well to HSV. However, no strong CD4+ T cell response specific to HBsAg was obtained using virus 1764/HBsAg15vhs/UL43MSVGFP which indicates that it was difficult to generate a recall response to the antigen via this vector. One reason for these results was the nature of the antigen itself and also the donor's response to that particular antigen. Indeed, the positive control for these experiments using recombinant HBsAg mixed with DC and T cells showed low cpm values for donor 2 (lower than the background) indicating that the individual did not respond well to HBsAg (data not shown). Thus, it was necessary to ensure that the next donors responded well to HBsAg in order to detect a specific response to HBsAg-encoding HSV vectors.

VII.3.2 CD4+ T cell proliferation in response to 1764/27-/4-/HBsAgpR20.5/vhs

DC were isolated from peripheral blood of two other vaccinated donors and transduced with virus 1764/27-/4-/pR20.5/vhs containing or not containing HBsAg. Figure VII.11 presents the results for CD4+ proliferation assays using transduced DC

for both donors. For donor 3, DC transduced with 1764/27-/4-/pR20.5/vhs induced proliferation of CD4+ T cells (mean counts per minute cpm=1032) and DC transduced with the equivalent vector encoding HBsAg stimulated twice more CD4+ T cells (mean cpm=1477). Thus, for donor 3, not only did DC transduced with both viruses 1764/27-/4-/pR20.5/vhs or 1764/27-/4-/pR20.5/vhs generate a T cell response, respectively, 3.9 or 8.4 folds higher than the background (as DC alone induced very low CD4+ T cell proliferation of mean cpm=262), but a stronger T cell proliferation was noted using the vector encoding HBsAg compared to the one without HBsAg.

Surprisingly for donor 4, DC transduced with 1764/27-/4-/HBsAgpR20.5/vhs induced weak proliferation of CD4+ T cells (mean counts per minute cpm=559) whereas DC transduced with the equivalent vector encoding HBsAg induced a very strong CD4+ T cell proliferation (mean cpm=4032). The fold stimulation obtained with DC transduced with 1764/27-/4-/pR20.5/vhs or 1764/27-4-/HBsAgpR20.5/vhs were respectively 0,1 or 1,1 folds the background (DC alone induced CD4+ T cell proliferation of mean cpm=3780). Thus similarly to donor 3, here a strong increase in proliferation was noted with the use of the fully disabled vector encoding HBsAg compared to the virus without HBsAg. Therefore, DC transduced with the fully disabled vector blocked T cell response whereas DC transduced with the equivalent vector encoding HBsAg stimulated it.

These results indicated that DC transduced with the replication incompetent HSV vector (1764/27-/4-/pR20.5/vhs) retained the ability to induce CD4+ T lymphocyte proliferation as shown in the results from donor 3 that responded well to HSV. However, this was not the case for donor 4 who was not a good responder to HSV. In both cases, a strong CD4+ T cell response specific to HBsAg was obtained using virus 1764/27-/4-/HBsAgpR20.5/vhs which showed a recall response to the antigen via this vector. These results also indicated that donors 3 and 4 were strong HBsAg responders as shown also by the use of recombinant HBsAg mixed with DC and T cells showed higher cpm values for both donors compared to for donors 1 and 2 indicating that they responded well to HBsAg (data not shown). In order to clearly

determine and confirm that the CD4+ T cell proliferation observed using DC transduced with the fully disabled vector encoding HBsAg was specific to the presence of HBsAg, another series of assays were carried out using cells from peripheral blood of an unvaccinated donor (5) in parallel with donor 3. As the unvaccinated individual has not previously been exposed to any HBV proteins, the CD4+ T lymphocytes have not been primed to HbsAg and thus it should not be possible to detect a recall response for donor 5. This allowed the use of donor 5 as a negative control of our experiments as a primary response to any foreign antigen was not expected to be strong.

Figure VII.12 shows the CD4+ T lymphocyte proliferation for the unvaccinated individual compared to donor 3. For donor 5, DC transduced with 1764/27-/4-/HBsAgpR20.5/vhs induced proliferation of CD4+ T cells (mean counts per minute cpm=2238) whereas DC transduced with the equivalent vector encoding HbsAg did not induce a CD4+ T cell proliferation (mean cpm=593). The fold stimulation obtained with DC transduced with 1764/27-/4-/pR20.5/vhs or 1764/27-4-/HBsAgpR20.5/vhs were respectively 2.2 or 0.6 folds the background (DC alone induced CD4+ T cell proliferation of mean cpm=1033). Thus, a response to HSV was noted with donor 5 whereas as expected a decrease in recall antigen response of about 4 fold was noted with the use of the fully disabled vector encoding HBsAg compared to the virus without HBsAg. Therefore, DC transduced with the fully disabled vector stimulated a T cell response to HSV whereas DC transduced with the equivalent vector encoding HBsAg blocked it. This confirmed that the unvaccinated individual did not respond to HBsAg in a CD4+ T cell proliferation assay but more importantly suggested that the increase in CD4+ T cell proliferation observed for donors 3 and 4 was indeed due to the presence of HBsAg within virus 1764/27-/4-/HBsAgpR20.5/vhs.

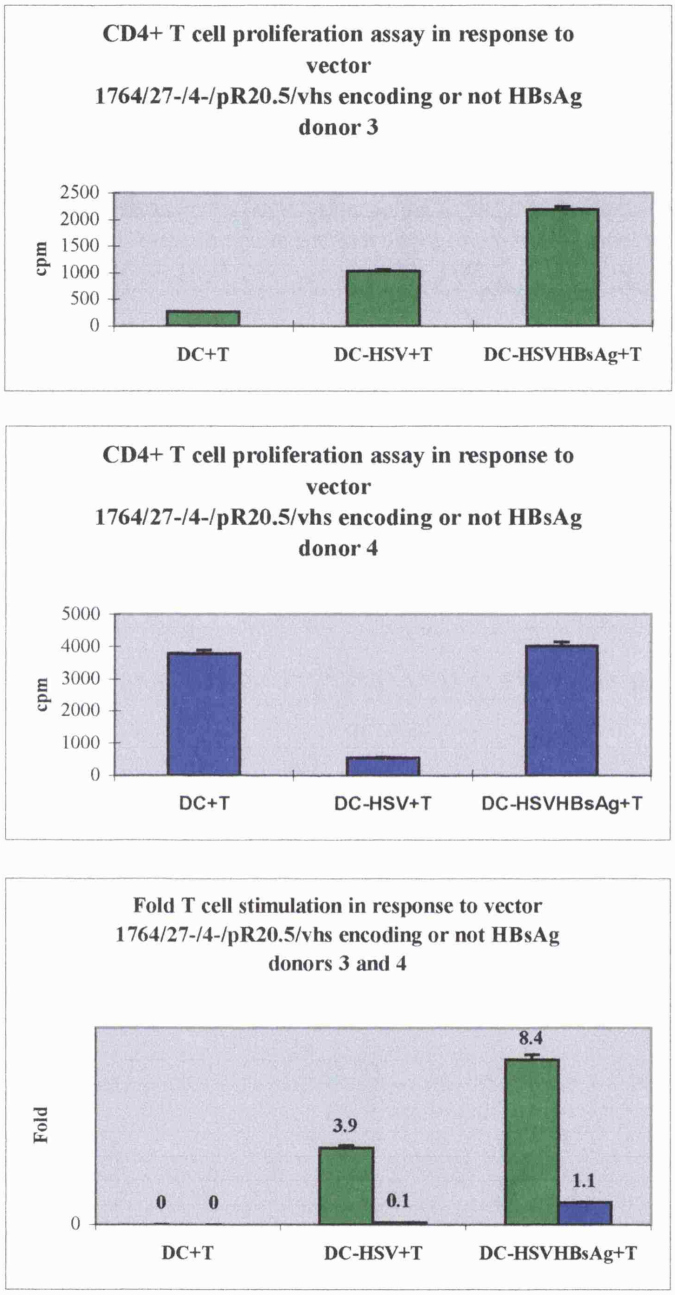
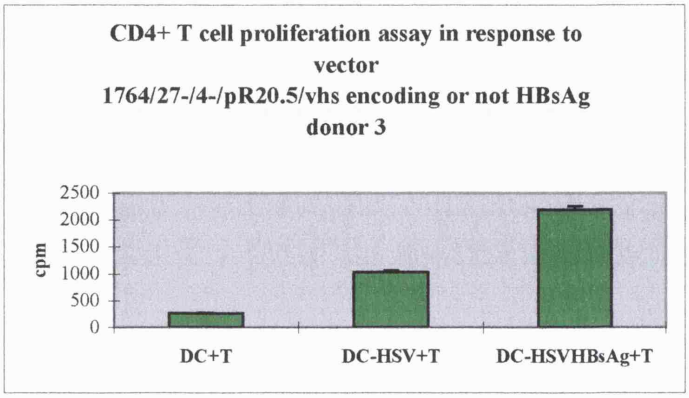
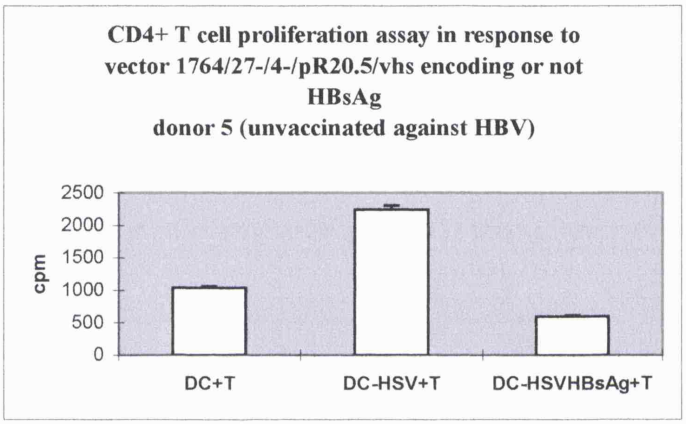


Figure VII.11: CD4+ T cell proliferation assay in response to HBsAg after transduction of DC with 1764/27-/4-/pR20.5/vhs (HSV) or equivalent vector encoding HBsAg (HSVHBsAg).





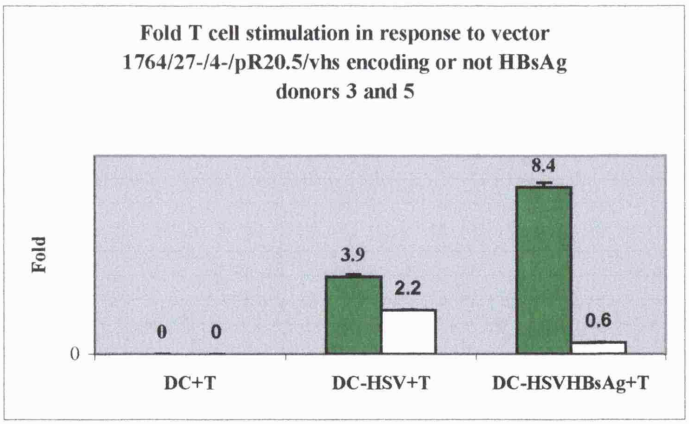


Figure VII.12: CD4+ T cell proliferation in response to HBsAg in which DC and T cells were prepared from hepatitis B vaccinated or non-vaccinated individuals. DC were transduced with vector 1764/27-/4-/pR20.5/vhs (HSV) or equivalent vector encoding HBsAg (HSVHBsAg).

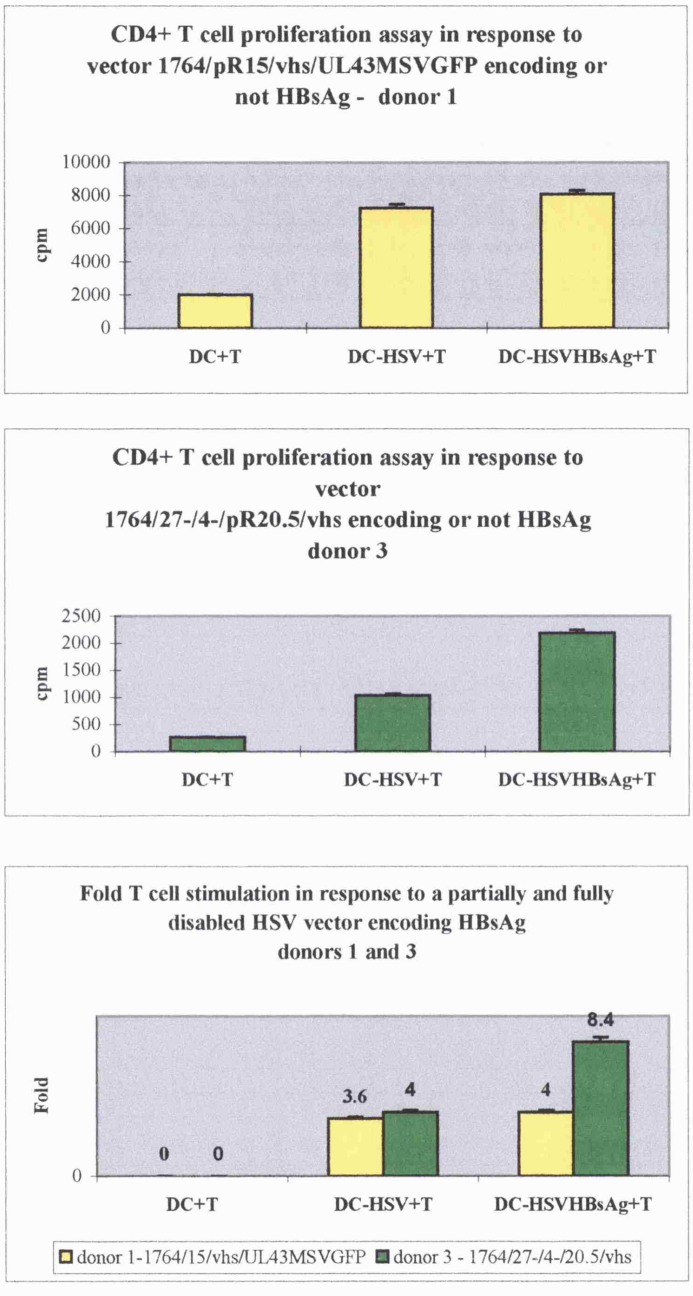


Figure VII.13: Comparison of CD4+ T cell proliferation assay obtained for donors 1 and 3 in response to HBsAg. DC were transduced with 1764/pR15/vhsUL43MSVGFP encoding or not HBsAg (donor 1) or with 1764/27-/4-/pR20.5/vhs encoding or not HBsAg (donor 3).

VII.4. Discussion

This chapter describes the construction and application to DC of two HSV-based vectors, one partially disabled and the other fully disabled each encoding the HBsAg as a model to stimulate a CD4+ T cell response after transduction of DC.

It was concluded from these results that the five donors responded differently to recall antigen, PPD, HBsAg or to HSV proteins due to their past exposure and tolerance to the pathogens. Thus, it was important to screen several donors in order to find some that would be suited for this study.

The results from donors 1 and 3 (figure VII.13) who both responded to recombinant HBsAg as well as HSV proteins clearly showed that DC transduced with the virus 1764/27-/4-/HBsAgpR20.5/vhs encoding HBsAg retained the ability to present the antigen to CD4+ T cells whereas such a response was not obtained when DC were transduced with virus 1764/HBsAgpR15/UL43MSVGFP encoding HBsAg. Therefore, it was concluded that DC transduced with the fully disabled vector stimulate a specific response while DC ability to stimulate CD4+ T cells was affected using the partially disabled vector.

This meant that the fully disabled HSV vector with ICP34.5, ICP27, ICP4 deleted and vmw65 and vhs inactivated may be a good candidate for use in immunotherapy as it allowed gene delivery of the foreign gene into DC and did not affect their ability to present the antigen to CD4+ T cells for T lymphocyte proliferation. Therefore, HBsAg was successfully used as a model to show that virus 1764/27-/4-/pR20.5/vhs might be used to safely and efficiently deliver other genes (e.g. genes encoding tumour antigens or genes encoding proteins critical in infectious disease) into DC without impairing their function.

CHAPTER VIII

CONSTRUCTION OF A REPLICATION INCOMPETENT HSV VECTOR EXPRESSING THE MUC-1 TUMOR ANTIGEN

VIII.1. Introduction

From the previous chapters, it was noticed that vector 1764/27-/4-/pR20.5/vhs with ICP34.5, ICP27 and ICP4 deleted and vmw65 and UL41 (vhs) inactivated, gave the overall best results as outlined below. This meant that this replication incompetent vector offered the best combination of deletions and inactivations and thus might be used to successfully deliver foreign genes to DC without altering DC function. Indeed, various properties of this replication incompetent virus may make it a good candidate for immunotherapy purposes. These are:

- good gene delivery to human DC
- lack of toxicity to DC
- lack of replication in DC as genes coding for ICP27 and ICP4 essential for viral replication have been deleted
- ability of transduced DC to process antigen
- ability to activate DC when vhs was inactivated from the virus by up-regulation of T-cell co-stimulatory molecules and other activation markers
- stimulation of a CD4+ T cell response specific to the HBsAg delivered to dendritic cells with the vector *in vitro*.

These results therefore offer potential tools for cancer immunotherapy. As a target for immunotherapy, the mucin-1 gene (MUC-1) was selected as its product (the polymorphic epithelial mucin, PEM) is overexpressed and aberrantly glycosylated in most breast tumors, resulting in an antigenically distinct molecule (Hareuveni *et al.* 1990). So far, recombinant vaccinia viruses encoding the MUC-1 gene have given encouraging results for immunotherapy in patients with breast cancer suggesting that MUC-1 may be used as an immunogen in the treatment of breast and other cancers (Scholl *et al.* 2000).

This chapter describes the construction of a recombinant HSV-based virus encoding MUC-1 to deliver the tumor-associated antigen to DC and the ability of DC

to express the antigen. The objective was to test this construct in DC isolated from peripheral blood of a patient with ovarian and/or breast cancer and to test the ability of transduced DC to stimulate a CD4+ T cell proliferation *in vitro*. However, ethical approval to undertake this work had not been obtained at the time of the project, thus attempts were made to study the effect of MUC-1-encoding vector in DC and T cells isolated from frozen peripheral blood of a patient who had died from breast cancer some years previously.

VIII.2. Construction of replication incompetent HSV vector containing the mucin-1 gene (MUC-1)

The MUC-1 gene was previously cloned into the plasmid pR15 (Immunology dept., UCL), and as the pR15 cassette and pR20.5 cassettes were each inserted in the vhs flanking regions, the MUC-1pR15 plasmid was directly inserted into 1764/27-/4-/pR20.5/vhs. The construction of virus 1764/27-/4-/MUC-1pR15 is briefly described here. The desired DNA fragment containing the MUC-1 gene was first excised from pBS-PEM (based upon pBluescript KS+ - provided by Ian McFarlane, ICRF, Guy's Hospital) with Cla I/BamH I and cloned into pSP72 (Sma I/BamH I). The MUC-1 gene was then excised from pSP72 with Hind III/EcoR I and inserted into the pR15 cassette instead of the *LacZ* reporter gene. The pR15 cassette consisting of a LAP1 sequence and MMLV-LTR promoter driving *LacZ* was previously inserted into a plasmid allowing recombination into the virion host shuttoff protein (vhs)-encoding gene (UL41) at the unique Nru I site (HSV nt 91854).

The MUC-1 encoding plasmid MUC-1pR15 was linearised using a unique Xmn I site present in the plasmid backbone and co-transfected with infectious 1764/27-/4-/pR20.5/vhs viral DNA for insertion into the vhs locus by homologous recombination. Recombinant plaques were identified as white plaques from a background of green and blue plaques (expressing GFP and β-gal present in the pR20.5 cassette from the viral DNA backbone). A genome structure diagram of the resulting virus 1764/27-/4-/MUC-1pR15 is shown in figure VIII.1.

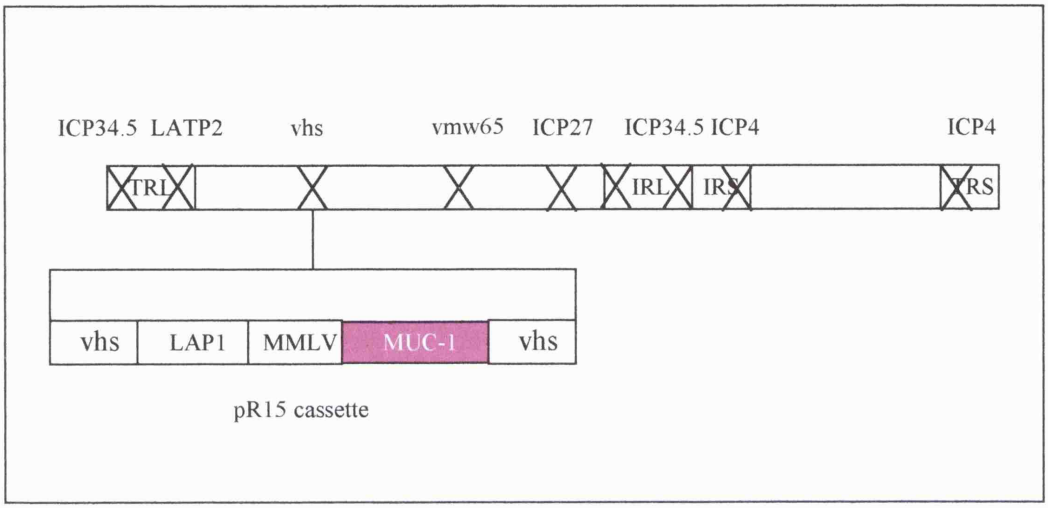


Figure VIII.1: Schematic diagram of the HSV genome with the recombinant shuttle cassette containing the MUC-1 gene. Diagram of 1764/27-/4-/MUC-1pR15 vector containing the recombinant shuttle cassette inserted in the UL41 gene (vhs).

VIII.3. Expression of MUC-1 in the vector producer cells and in DC

Here, the MUC-1 encoding vector was used to infect vector producer cells and DC in order to determine the expression of the protein from the recombinant virus. Thus a western blot was carried out to show the expression of MUC-1 (150-190kDa protein) after infection of the vector producer cells (MAM49 cells) with virus 1764/27-/4-/MUC-1 15/vhs. Figure VIII.2 shows the expression of 150-190 kDa size proteins corresponding to the product of the MUC-1 gene from the virus. The negative and positive controls were protein extracts from MAM49 cells respectively infected with the wild-type vector 17+/pR20.5/UL43 or the replication incompetent vector not containing MUC-1 (1764/27-/4-/pR20.5/vhs). No band was observed in the negative controls. Thus, these results showed that the recombinant virus expressed high levels of MUC-1 in producer cells. The smear obtained instead of a distinct band can be explained by the fact that MUC-1 has a large number of tandem repeats and thus several forms and sizes of the encoded PEM protein exist. Such a pattern has been demonstrated in the published literature (Scholl *et al.* 2000).

Following these results, a western blot was carried out to determine if DC transduced with vector 1764/27-/4-/MUC-1pR15 expressed the encoded protein (figure VIII.3). The western blot showed that a smear corresponding to 150-190 kDa size protein was detected with protein extracts from DC transduced with the recombinant vector. The negative controls were protein extracts from producer cells respectively infected with 17+/pR20.5/UL43 or the equivalent vector not containing MUC-1 (1764/27-/4-/pR20.5/vhs). No band was observed in the negative controls. The positive control was protein extracts from producer cells infected with the recombinant MUC-1-encoding vector used previously. A smear corresponding to the MUC-1 product of size 150-190 kDa was also obtained. Thus, DC transduced with virus 1764/27-/4-/MUC-1pR15 expressed the MUC-1 encoded protein.

In this chapter, the construction of the recombinant virus 1764/27-/4-/MUC-1pR15 was described and the expression of the tumor-associated protein was

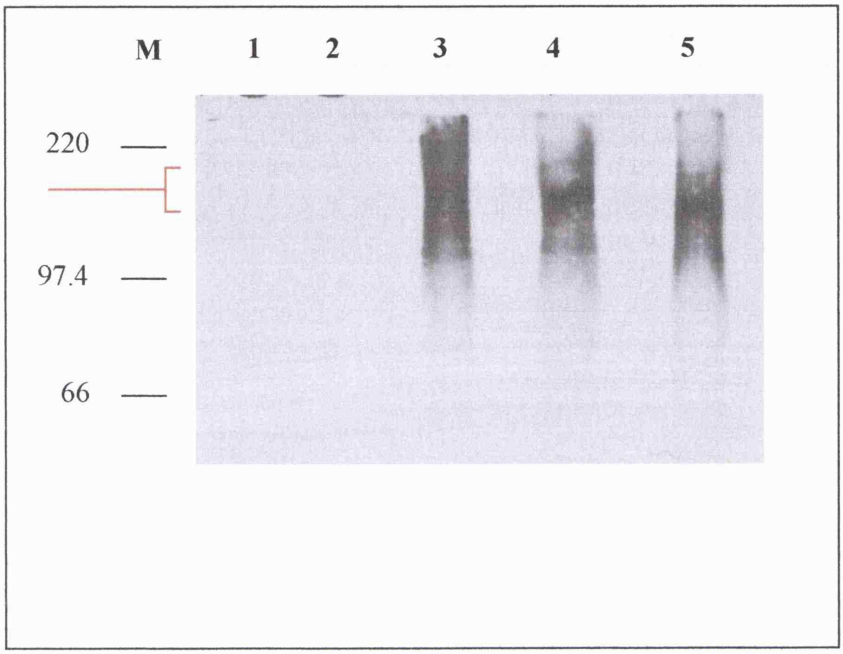


Figure VIII.2: Western blot - 10% acrylamide gel showing the expression of MUC-1 (150-190 kDa) in MAM49 cells. Tracks 1, 2: negative controls - protein extracts from MAM49 cells transduced respectively with 17+/pR20.5/UL43 and 1764/27-/4-/pR20.5/vhs vector; tracks 3-5: protein extracts from MAM cells after transfection with one individual plaque of virus 1764/27-/4-/MUC-1pR15. M = Molecular weight (kDa).

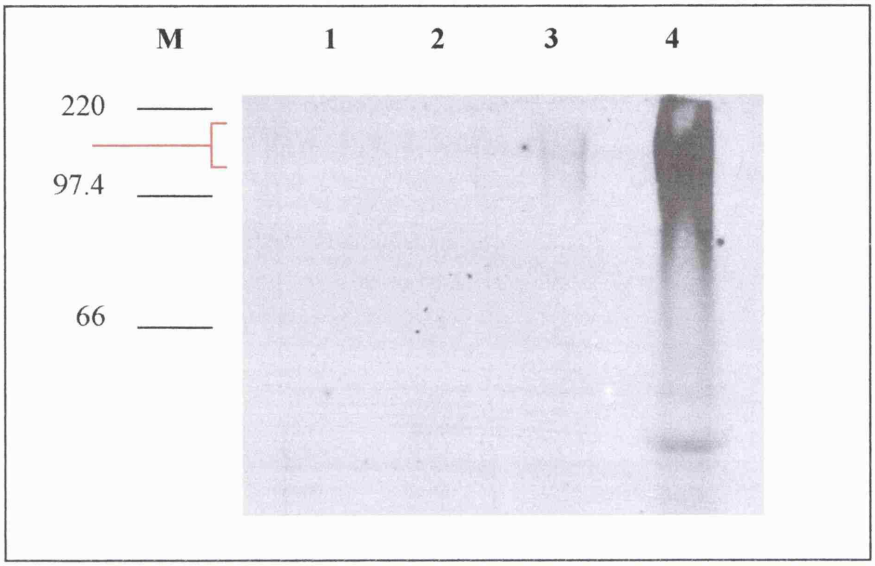


Figure VIII.3: Western blot - 10% acrylamide gel showing the expression of MUC-1 (150-190 kDa) in dendritic cells (DC). Tracks 1, 2: negative controls - protein extracts from DC transduced respectively with 17+/pR20.5/UL43 and 1764/27-/4-/ pR20.5/vhs vector; track 3: protein extracts from DC after transduction with the 1764/27-/4-/MUC-1pR15 vector; track 4: positive control - protein extracts from MAM49 cells infected with 1764/27-/4-/MUC-1pR15. M = Molecular weight (kDa).

confirmed in the vector producer cells and in DC. Having constructed such a virus, the objective was to use it in human DC isolated from cancer patients expressing the MUC-1 marker in order to show a CD4+ T cell response specific to MUC-1. However, as mentioned previously, it was not possible to obtain fresh blood from such patients, thus an alternative method was tested using frozen peripheral blood stored from patients. Attempts were made to optimise the isolation of DC and T cells from frozen peripheral blood of a breast cancer patient whose cancer expressed MUC-1 (this information was confirmed by Dr. Jonathan Ledermann). Frozen peripheral blood mononuclear cells (PBMC) of a breast cancer patient was kindly provided by Dr. Didra Driva, University College Hospital). Having isolated DC and T cells from frozen PBMC, CD4+ T cell proliferation assays were set up following the transduction of DC with vector 1764/27-/4-/MUC-1 pR15 at MOI=1. Unfortunately, no proliferation was detected as most of the T cells had not survived the incubation time in the plates for the assay. This suggested that the isolation procedure in which cells were freeze-thawed may have affected the cells and thus should be avoided for subsequent experiments using frozen PBMC.

CHAPTER IX

GENERAL DISCUSSION

Herpes simplex virus (HSV) is a large DNA enveloped virus which infects humans. Upon exposure to HSV, an initial acute infection stimulates a vigorous immune response. HSV initially infects the skin or mucosa where interaction with the host immune system would first be anticipated to occur. However, HSV has evolved to enter a latent state in infected individuals from which it can reactivate intermittently to cause disease (Latchman 1990). During the phase of chronic infection, the virus remains dormant within the host tissues and it co-exists with an ongoing host immune response (Fink *et al.* 1996). During this period, virus replication occurs, but the spread of virus is controlled by host immunity. Dendritic cells (DC) which are the key mediators of an effective immune response are present in tissues primarly exposed to pathogens e.g. HSV, and thus the study of the interaction of DC and HSV is important in order to develop HSV-1 based vectors for gene delivery to these cells and also for application in cancer immunotherapy.

A number of strategies have been used to deliver genes to DC aimed at tumor vaccination with some considerable success. Each specific method has been hampered by the lack of an efficient means of loading antigen to DC (described in the general introduction). Amongst viral gene delivery methods, retroviruses except lentivirus based vectors have been shown to infect DC poorly and adenoviruses have required very high multiplicities of infection for efficient infection of DC (Dietz and Vuk-Pavlovic 1998). HSV has been demonstrated to be capable of delivering genes to DC at high efficiency which suggests that HSV might be developed as a vector for gene delivery to these cells (Coffin *et al.* 1998). Studies have generally used minimally disabled HSV based vectors which have been shown to affect DC function (Kruse *et al.* 2000), (Salio *et al.* 1999). We have developed and optimised gene delivery to DC using a range of HSV-1 based vectors that were disabled in non-essential and essential genes in order to reduce virus mediated effects on DC. Such vectors were minimally disabled, partially disabled or replication incompetent viruses and our initial studies of HSV-1 and DC interactions showed that DC can generally be infected by the virus very efficiently. This implies that these cells express high

levels of surface receptors that allows viral entry and that the virus may naturally infect these cells as part of its lifecycle.

Vectors were tested in DC at a range of MOI and over a period of four days in order to assess gene delivery to DC. Levels of detectable gene expression as measured by levels of GFP and β-gal were increased with higher doses of virus and the dose effect in relation to time showed that gene delivery was the highest at 24 hours and then decreased with time in the following three days. Minimally disabled vectors gave efficient gene delivery to DC and relatively low toxicity to cells. This suggests that wild-type HSV naturally infects DC as part of its lifecycle as it would seem unlikely that such high gene delivery efficiencies would be observed otherwise. However, partially disabled vectors gave two types of result. The first set of viruses gave similar levels of gene delivery as the minimally disabled vectors, while the second result was with viruses which gave extremely high gene delivery with in one case differential expression of GFP and β-gal. This virus (1764/pR15/vhs/ UL43MSVGFP) has ICP34.5 deleted, and vmw65, UL43 and UL41 (vhs) inactivated, and also gave very good cell survival. Thus, this virus, even though not entirely replication-incompetent, would be a potential candidate for gene delivery to DC. It was hypothesised that DC transduced with this vector process delivered proteins more effectively than other partially disabled vectors. The results for the replication incompetent vectors showed that apparent gene delivery was the lowest in DC while all cells remained alive. Thus, these vectors were reduced in toxicity due to the deletion of genes coding for IE proteins known to be toxic to cells which may have further increased the antigen processing capability of DC. Virus 1764/27-/4-/pR20.5/vhs (ICP34.5, ICP27, ICP4 deleted, and vmw65 and UL41 inactivated) gave the highest gene delivery results with 99% live cells at 24 hours suggesting that this vector may be the best candidate replication incompetent vector for gene delivery to DC.

DC transduced with HSV vectors retained the ability to process the antigens as β-gal levels were increased following treatment with MG132 proteasome inhibitor.

This suggests that the differential GFP/β-gal levels detectable with some of the viruses may indeed be due to antigen processing by the transduced cells which is only apparent when the vectors are appropriately disabled. Thus, DC transduced with HSV-1 proteolytically process delivered antigens, this being a likely explanation for the differential level of GFP and β-gal obtained with some of the vectors. This implies that the actual gene delivery was generally higher than the levels observed.

DC transduced with wild-type HSV vectors but not the partially disabled vectors allowed slight viral growth. Dendritic cells are thus essentially non-permissive for the growth of HSV, even though DC are infected very efficiently. As it would seem unlikely that HSV would have evolved the ability to enter DC without some purpose (e.g. prevention of induction of cellular immune responses to HSV by DC), it is possible that DC are in some way affected by HSV infection in a way not involving lytic replication. Furthermore, DC transduced with HSV expressed only low levels of IE gene proteins. Surprisingly, the partially disabled vector 1764/pR15/vhs/UL43MSVGFP which contains an inactivating mutation in the gene encoding vmw65 gave similar results to the 17+/20.5/UL43. This might suggest that even with the 17+/pR20.5/UL43 vector vmw65 transactivated IE gene expression is not occuring, which requires cellular transcription factors which may well not be present in DC, the expression levels observed being the background 'un-transactivated' expression levels from the ICP0, ICP22 and ICP47 promoters. This explanation may be the case as the *in1814* mutation to vmw65 present in virus 1764/pR15/vhs/UL43MSVGFP has been shown to effectively abolish the transactivating activity of vmw65 and there was no difference in IE gene expression levels with and without the mutation. As ICP47 inhibits antigen presentation, it was useful to show that an HSV vector is not expressing this protein in DC.

The initiation of an immune response is dependent on the activation of DC. This process is triggered by surface receptors specific for inflammatory cytokines or for T-cell activation. Viruses have evolved mechanisms to avoid detection by the host immune system. The evasive functions of four HSV-1 proteins have been

demonstrated so far although none of these have been shown to affect DC function (described in the general introduction). The vhs protein has been suggested to be a fifth protein mediating evasion of host defence mechanisms although this was demonstrated in non-specific defence mechanisms (Suzutani *et al.* 2000). We and others have found that HSV-1 infects DC very efficiently and inhibited to some extent maturation of DC which may reveal another strategy used by HSV-1 to evade the immune response. Indeed, DC infected with minimally disabled HSV-1 are inhibited in their T-cell stimulatory capacity, generate some infectious particles to allow slight viral growth and some IE protein gene products were detected (Kruse *et al.* 2000). We have found that DC transduced with wild-type HSV vectors fail to up-regulate surface activation markers or at least present a mixed phenotype population. Furthermore, DC transduced with a disabled HSV with vhs intact gave the same phenotype as a mock-infected cell population whereas DC transduced with an equivalent vector with vhs inactivated seem to generally up-regulate all these markers, in particular the activation marker CD86. This suggests that vhs prevents DC activation, at least in the context of the disabled HSV vector tested and that vhs plays a key role in virus escape from host immunity. Thus, the presence of vhs which works to destabilise host cellular and viral RNA rapidly, resulting in the shutoff of protein synthesis, interferes with DC activation and functioning.

However, the results obtained with wild-type vectors with or without vhs intact showed no difference between these viruses. This is in contrast to the effects of vhs deletion from a replication incompetent virus where there are significant effects on DC activation markers accompanying inactivation of vhs. Thus, in wild-type HSV other genes as well as vhs may well contribute to the inactivation of DC by HSV observed. Thus, it can be speculated that proteins encoded by immediate-early proteins or present at the start of IE gene transcription might be responsible for the interference with DC function. Furthermore, replication defective mutants of HSV have been proposed as vaccine candidates and as vehicles for gene therapy because of their inability to produce infectious progeny. The immunogenicity of these HSV replication mutants, both qualitative and quantitative levels, will directly determine

the effectiveness of either of these applications. Studies have shown that a single immunization with any individual mutant containing one or more mutations in HSV alpha genes (ICP4, ICP27, ICP22 and ICP0) is able to generate a strong CTL response and immune protection against HSV challenge suggesting that such mutants can be used as effective immunogens in vivo (Brehm *et al.* 1999). Consequently, it has become evident that novel strategies have to be developed in order to use HSV-1 based vectors as a tool for cancer immunotherapy.

The functionality of transduced DC was further studied by measuring T cell proliferation in response to a specific antigen. For this purpose, the hepatitis B surface antigen (HBsAg) was used as a model to test the ability of transduced DC to elicit a specific T cell response to the antigen delivered via the vector. We have shown that a replication incompetent vector with deletions in essential and non-essential genes and with vhs inactivated can stimulate CD4+ T cell response specific to HBsAg encoded in the vector. Two vectors, one partially disabled and the other replication incompetent each encoding the HBsAg, were constructed and used to stimulate a CD4+ T cell response after transduction of DC. Two donors responded to recombinant HBsAg and HSV proteins clearly showed that DC transduced with virus 1764/27-/4-/HBsAgpR20.5/vhs encoding HBsAg retained the ability to present the antigen to CD4+ T cells whereas such a strong response was not obtained when DC were transduced with virus 1764/HBsAgpR15/vhs/UL43MSVGFP encoding HBsAg. Thus, DC transduced with the replication incompetent vector stimulate a specific response while DC ability to stimulate CD4+ T cells was affected using the partially disabled vector. This meant that the replication incompetent HSV vector (1764/27-/4-/pR20.5/vhs) with ICP34.5, ICP27 and ICP4 deleted, and vmw65 and UL41 (vhs) inactivated may be a good candidate for use in immunotherapy as it allowed safe and efficient gene delivery to DC without impairing their T-cell stimulatory capacity.

The discovery that HSV infects DC at high efficiency suggests that HSV might be developed for use as a vector for these cells. However, we and others have found that DC transduced with wild-type HSV become inactivated and are inhibited

in their T-cell stimulatory capacity. The identification of vhs as a key mediator of this inactivation process has provided more insight into possible ways through which the virus escapes from immune surveillance. This has allowed the development of replication incompetent HSV vectors with deletions in essential and non-essential genes and with vhs inactivated that gave efficient gene delivery to DC, allowed antigen processing, activation of DC and did not affect their ability to stimulate CD4+ T cells. Based on these results that are summarised in figure IX.1, a replication incompetent virus encoding the MUC-1 tumor-antigen which is over-expressed in breast/ovarian cancer patient's cells was constructed. The expression of MUC-1 from virus 1764/27-/4-/MUC-1pR15/vhs was confirmed in the vector producer cells and in DC. Attempts were made to use this virus in DC isolated from frozen blood packs stored from cancer patients expressing the MUC-1 marker in order to show a specific response to the tumor antigen. For future work, proliferation assays and CTL responses specific to the delivered antigen could be performed from peripheral blood mononuclear cells of cancer patients expressing the specific marker. The specific tumor lysis by CTL that have been exposed to HSV transduced DC would then further determine if such HSV vectors are suited for application in cancer immunotherapy. Further work which is currently being undertaken in our laboratory aim at constructing other replication incompetent vectors with UL41 (vhs) deleted and containing various antigens for the immunotherapy of cancer or infectious diseases.

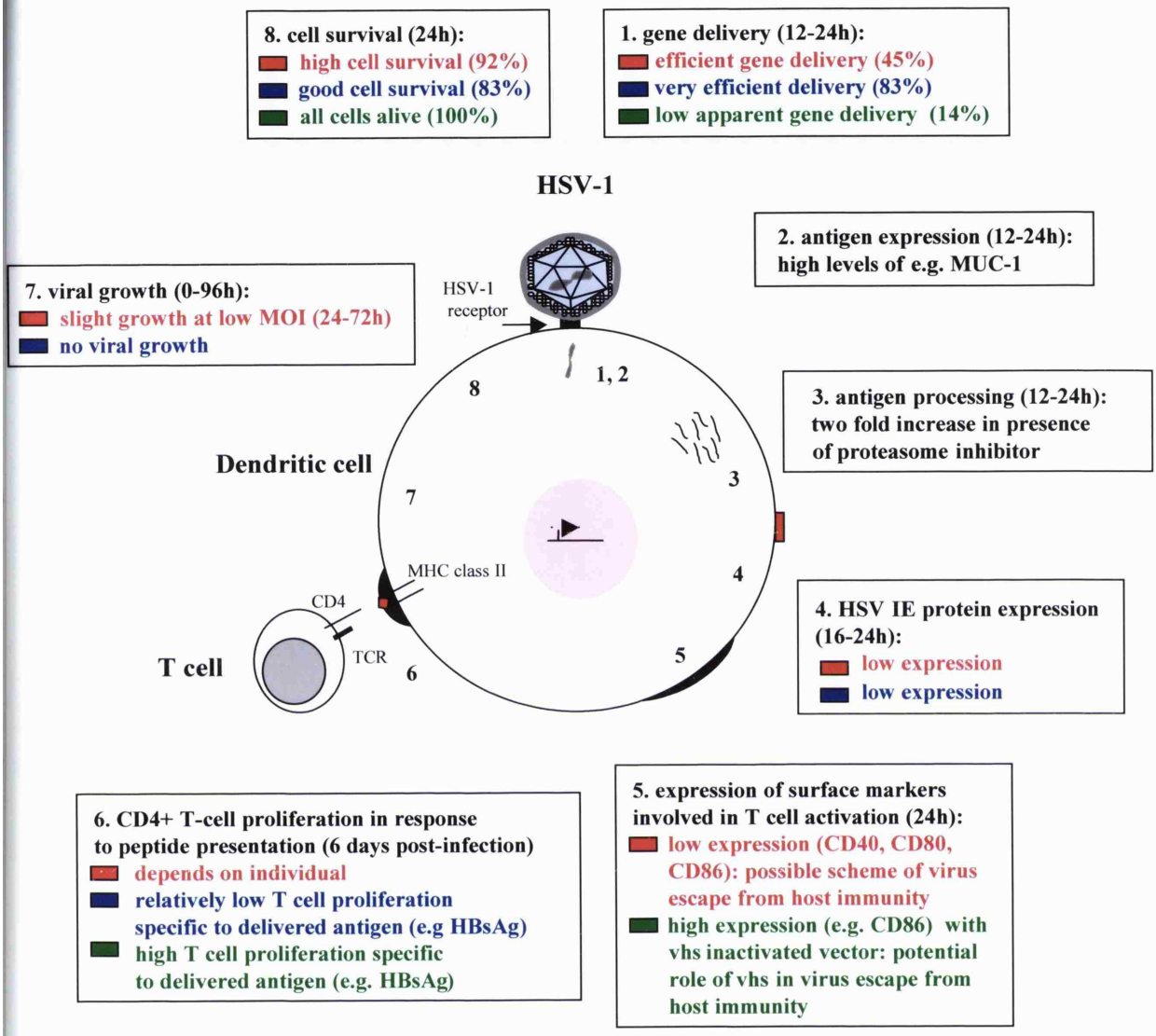


Figure IX.1: Schematic diagram summarising the results presented in this thesis. The diagram indicates a possible scheme (steps 1 to 8) for the transduction of dendritic cells with HSV-1 based vectors at multiplicity of infection (MOI) of one (unless otherwise stated) and their effect on DC functions. IE: immediate early.

- minimally disabled vector
- partially disabled vector
- replication incompetent vector

References

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793.

Ace, C. I., McKee, T. A., Ryan, J. M., Cameron, J. M., and Preston, C. M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J.Virol.* **63**, 2260-2269.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J.Exp.Med.* **188** , 1359-1368.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-89.

Alcami, A. and Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. *Mol.Med.Today* **6**, 365-372.

Alters, S. E., Gadea, J. R., Sorich, M., O'Donoghue, G., Talib, S., and Philip, R. (1998). Dendritic cells pulsed with CEA peptide induce CEA-specific CTL with restricted TCR repertoire. *J.Immunother.* **21**, 17-26.

Arthur, J. F., Butterfield, L. H., Roth, M. D., Bui, L. A., Kiertscher, S. M., Lau, R., Dubinett, S., Glaspy, J., McBride, W. H., and Economou, J. S. (1997). A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther.* **4**, 17-25.

Aubert, M. and Blaho, J. A. (1999b). The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. *J.Virol.* **73**, 2803-2813.

Aubert, M. and Blaho, J. A. (1999a). The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. *J.Virol.* **73**, 2803-2813.

Aubert, M., O'Toole, J., and Blaho, J. A. (1999). Induction and prevention of apoptosis in human HEp-2 cells by herpes simplex virus type 1. *J.Virol.* **73**, 10359-10370.

Aung, S., Tang, Y. W., and Graham, B. S. (1999). Interleukin-4 diminishes CD8(+) respiratory syncytial virus-specific cytotoxic T-lymphocyte activity in vivo. *J.Virol.* **73**, 8944-8949.

Austyn, J. M., Kupiec-Weglinski, J. W., Hankins, D. F., and Morris, P. J. (1988). Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. *J.Exp.Med.* **167**, 646-651.

Baggiolini, M. (1998). Chemokines and leukocyte traffic. *Nature* **392**, 565-568.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J. P., Trepo, C., and Inchauspe, G. (2001). Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* **120**, 512-524.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (1994). An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *J.Gen.Virol.* **75 (Pt 6)**, 1245-1258.

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annu.Rev.Immunol.* **12**, 881-922.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000b). Immunobiology of dendritic cells. *Annu.Rev.Immunol.* **18**, 767-811.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000a). Immunobiology of dendritic cells. *Annu.Rev.Immunol.* **18**, 767-811.

Banchereau, J. and Steinman, R. M. (1998b). Dendritic cells and the control of immunity. *Nature* **392**, 245-252.

Banchereau, J. and Steinman, R. M. (1998a). Dendritic cells and the control of immunity. *Nature* **392**, 245-252.

Banfield, B. W., Leduc, Y., Esford, L., Schubert, K., and Tufaro, F. (1995). Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. *J.Virol.* **69**, 3290-3298.

Barcy, S. and Corey, L. (2001). Herpes simplex inhibits the capacity of lymphoblastoid B cell lines to stimulate CD4+ T cells. *J.Immunol.* **166**, 6242-6249.

Batterson, W., Furlong, D., and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. *J.Virol.* **45**, 397-407.

Batterson, W. and Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *J.Virol.* **46**, 371-377.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513-1523.

Blaho, J. A., Zong, C. S., and Mortimer, K. A. (1997). Tyrosine phosphorylation of the herpes simplex virus type 1 regulatory protein ICP22 and a cellular protein which shares antigenic determinants with ICP22. *J.Virol.* **71**, 9828-9832.

Boczkowski, D., Nair, S. K., Snyder, D., and Gilboa, E. (1996). Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J.Exp.Med.* **184**, 465-472.

Bogyo, M., Gaczynska, M., and Ploegh, H. L. (1997). Proteasome inhibitors and antigen presentation. *Biopolymers* **43**, 269-280.

Boon, T., Cerottini, J. C., Van den, E. B., van der, B. P., and Van Pel, A. (1994). Tumor antigens recognized by T lymphocytes. *Annu.Rev.Immunol.* **12**, 337-365.

Boothman, D. A., Geller, A. I., and Pardee, A. B. (1989). Expression of the E. coli Lac Z gene from a defective HSV-1 vector in various human normal, cancer-prone and tumor cells. *FEBS Lett.* **258**, 159-162.

Boris-Lawrie, K. and Temin, H. M. (1994). The retroviral vector. Replication cycle and safety considerations for retrovirus-mediated gene therapy. *Ann.N.Y.Acad.Sci.* **716**, 59-70.

Borrow, P., Evans, C. F., and Oldstone, M. B. (1995). Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. *J.Virol.* **69**, 1059-1070.

Borrow, P. and Oldstone, M. B. (1995). Measles virus-mononuclear cell interactions. *Curr.Top.Microbiol.Immunol.* **191**, 85-100.

Boursnell, M. E., Entwisle, C., Ali, S. A. Sivasubramaniam, S. D., Reeder, S., MacLean, C. S., Blakeley, D. M., Miller, J., Hill, S., Shields, J. G., Inglis, S. C., and Rees, R. C. (1998). Disabled infectious single cycle (DISC) herpes simplex virus as a vector for immunotherapy of cancer. *Adv. Exp. Med. Biol.* **451**, 379-384.

Brehm, M., Samaniego, L. A., Bonneau, R. H., DeLuca, N. A., and Tevethia, S. S. (1999). Immunogenicity of herpes simplex virus type 1 mutants containing deletions in one or more alpha-genes: ICP4, ICP27, ICP22, and ICP0. *Virology* **256**, 258-269.

Bruun, T., Kristoffersen, A. K., Rollag, H., Beck, S., and Degre, M. (1998). Herpes simplex virus type 1 inhibits in vitro differentiation and selected functions of human blood-derived monocytes. *APMIS* **106**, 1194-1203.

Bubenik, J. (2001). Genetically engineered dendritic cell-based cancer vaccines (review). *Int.J.Oncol.* **18**, 475-478.

Cameron, P. U., Freudenthal, P. S., Barker, J. M., Gezelter, S., Inaba, K., and Steinman, R. M. (1992). Dendritic cells exposed to human immunodeficiency virus

type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science* **257**, 383-387.

Campbell, J. J., Bowman, E. P., Murphy, K., Youngman, K. R., Siani, M. A., Thompson, D. A., Wu, L., Zlotnik, A., and Butcher, E. C. (1998). 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J.Cell Biol.* **141**, 1053-1059.

Carroll, W. L., Lowder, J. N., Streifer, R., Warnke, R., Levy, S., and Levy, R. (1986). Idiotype variant cell populations in patients with B cell lymphoma. *J.Exp.Med.* **164**, 1566-1580.

Carrozza, M. J. and DeLuca, N. A. (1996). Interaction of the viral activator protein ICP4 with TFIID through TAF250. *Mol.Cell Biol.* **16**, 3085-3093.

Carter, K. L., Ward, P. L., and Roizman, B. (1996). Characterization of the products of the U(L)43 gene of herpes simplex virus 1: potential implications for regulation of gene expression by antisense transcription. *J.Virol.* **70**, 7663-7668.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Durand, I., Cella, M., Lanzavecchia, A., and Banchereau, J. (1997). CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood* **90**, 1458-1470.

Caux, C., Vanbervliet, B., Massacrier, C., Azuma, M., Okumura, K., Lanier, L. L., and Banchereau, J. (1994). B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J.Exp.Med.* **180**, 1841-1847.

Cella, M., Facchetti, F., Lanzavecchia, A., and Colonna, M. (2000). Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. **1**, 305-310.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. (1999a). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat.Med.* **5**, 919-923.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. (1999b). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat.Med.* **5**, 919-923.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J.Exp.Med.* **184**, 747-752.

Celluzzi, C. M., Mayordomo, J. I., Storkus, W. J., Lotze, M. T., and Falo, L. D., Jr. (1996). Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J.Exp.Med.* **183**, 283-287.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Chan, A. W., Luetjens, C. M., Dominko, T., Ramalho-Santos, J., Simerly, C. R., Hewitson, L., and Schatten, G. (2000). Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. *Mol.Hum.Reprod.* **6**, 26-33.

Chen, Y., Emtage, P., Zhu, Q., Foley, R., Muller, W., Hitt, M., Gauldie, J., and Wan, Y. (2001). Induction of ErbB-2/neu-specific protective and therapeutic antitumor immunity using genetically modified dendritic cells: enhanced efficacy by cotransduction of gene encoding IL-12. *Gene Ther.* **8**, 316-323.

Chinnasamy, N., Chinnasamy, D., Toso, J. F., Lapointe, R., Candotti, F., Morgan, R. A., and Hwu, P. (2000). Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. *Hum.Gene Ther.* **11**, 1901-1909.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal.Biochem.* **162**, 156-159.

Christ, M., Lusky, M., Stoeckel, F., Dreyer, D., Dieterle, A., Michou, A. I., Pavirani, A., and Mehtali, M. (1997). Gene therapy with recombinant adenovirus vectors: evaluation of the host immune response. *Immunol.Lett.* **57**, 19-25.

Coffin, R. S., MacLean, A. R., Latchman, D. S., and Brown, S. M. (1996). Gene delivery to the central and peripheral nervous systems of mice using HSV1 ICP34.5 deletion mutant vectors. *Gene Ther.* **3**, 886-891.

Coffin, R. S., Thomas, S. K., Thomas, N. S., Lilley, C. E., Pizzey, A. R., Griffiths, C. H., Gibb, B. J., Wagstaff, M. J., Inges, S. J., Binks, M. H., Chain, B. M., Thrasher, A. J., Rutault, K., and Latchman, D. S. (1998). Pure populations of transduced primary human cells can be produced using GFP expressing herpes virus vectors and flow cytometry. *Gene Ther.* **5**, 718-722.

Cook, M. L., and Stevens, J. G. (1973). Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence of intra-axonal transport of infection. *Infect. Immun.* **7**, 272-288.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33-38.

Croyle, M. A., Cheng, X., and Wilson, J. M. (2001). Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther.* **8**, 1281-1290.

Cyster, J. G. (1999). Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. *J.Exp.Med.* **189**, 447-450.

Deatly, A. M., Spivack, J. G., Lavie, and Fraser, N. W. (1987). RNA from an immediate-early region of the type I herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. *Proc. Natl. Acad. Sci. USA.* **84**, 3204-3208.

de Fraissinette, A., Schmitt, D., and Thivolet, J. (1989). Langerhans cells of human mucosa. *J.Dermatol.* **16**, 255-262.

DeLuca, N. A., McCarthy, A. M., and Schaffer, P. A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J.Virol.* **56**, 558-570.

DeLuca, N. A. and Schaffer, P. A. (1985). Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. *Mol.Cell Biol.* **5**, 1997-208.

Devi-Rao, G. B., Goodart, S. A., Hecht, L. M., Rochford, R., Rice, M. K., and Wagner, E. K. (1991). Relationship between polyadenylated and nonpolyadenylated herpes simplex virus type 1 latency-associated transcripts. *J.Virol.* **65**, 2179-2190.

Diao, J., Smythe, J. A., Smyth, C., Rowe, P. B., and Alexander, I. E. (1999). Human PBMC-derived dendritic cells transduced with an adenovirus vectorinduce cytotoxic T-lymphocyte responses against a vector-encoded antigen in vitro. *Gene Ther.* **6**, 845-853.

DiDonato, J. A., Spitzner, J. R., and Muller, M. T. (1991). A predictive model for DNA recognition by the herpes simplex virus protein ICP4. *J.Mol.Biol.* **219**, 451-470.

Dietz, A. B. and Vuk-Pavlovic, S. (1998). High efficiency adenovirus-mediated gene transfer to human dendritic cells. *Blood* **91**, 392-398.

Doherty, P. C., Knowles, B. B., and Wettstein, P. J. (1984). Immunological surveillance of tumors in the context of major histocompatibility complex restriction of T cell function. *Adv.Cancer Res.* **42**, 1-65.

Donahue, R. E., Kessler, S. W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R., and . (1992). Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J.Exp.Med.* **176**, 1125-1135.

Drake, D. R., III, Shawver, M. L., Hadley, A., Butz, E., Maliszewski, C., and Lukacher, A. E. (2001). Induction of polyomavirus-specific CD8(+) T lymphocytes by distinct dendritic cell subpopulations. *J.Virol.* **75**, 544-547.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc.Natl.Acad.Sci.U.S.A* **90**, 3539-3543.

Drillien, R., Spehner, D., Bohbot, A., and Hanau, D. (2000). Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. *Virology* **268**, 471-481.

Dyall, J., Latouche, J. B., Schnell, S., and Sadelain, M. (2001). Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. *Blood* **97**, 114-121.

Ehrmann, M. A., Scheyhing, C. H., and Vogel, R. F. (2001). In vitro stability and expression of green fluorescent protein under high pressure conditions. *Lett.Appl.Microbiol.* **32**, 230-234.

Elgadi, M. M., Hayes, C. E., and Smiley, J. R. (1999). The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. *J.Virol.* **73**, 7153-7164.

Engelmayer, J., Larsson, M., Subklewe, M., Chahroudi, A., Cox, W. I., Steinman, R. M., and Bhardwaj, N. (1999). Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J.Immunol.* **163**, 6762-6768.

Everett, R. D. (1984). Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO J.* **3**, 3135-3141.

Everett, R. D. (1987). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products (review). *Anticancer Res.* **7**, 589-604.

Fagnoni, F. F., Takamizawa, M., Godfrey, W. R., Rivas, A., Azuma, M., Okumura, K., and Engleman, E. G. (1995). Role of B70/B7-2 in CD4+ T-cell immune responses induced by dendritic cells. *Immunology* **85**, 467-474.

Farassati, F., Yang, A. D., and Lee, P. W. (2001). Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. *Nat.Cell Biol.* **3**, 745-750.

Fearnley, D. B., McLellan, A. D., Mannering, S. I., Hock, B. D., and Hart, D. N. (1997). Isolation of human blood dendritic cells using the CMRF-44 monoclonal

antibody: implications for studies on antigen-presenting cell function and immunotherapy. *Blood* **89**, 3708-3716.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Feldman, L. (1996). In vivo expression of reporter genes from HSV-1 recombinant vectors in protocols for gene transfer in neuroscience: towards gene therapy for neurological disorders. Ed. P. R. Lowenstein and L. W. Enquist. Publishers Chichester J. Wiley Funds Ltd. 203-211.

Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., ter Schegget, J., Melief, C. J., and Kast, W. M. (1993). Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur.J.Immunol.* **23**, 2242-2249.

Fenwick, M. L. and Clark, J. (1982). Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J.Gen.Virol.* **61 (Pt l)**, 121-125.

Fink, D. J., DeLuca, N. A., Goins, W. F., and Glorioso, J. C. (1996). Gene transfer to neurons using herpes simplex virus-based vectors. *Annu.Rev.Neurosci.* **19**, 265-287.

Fink, D. J. and Glorioso, J. C. (1997). Engineering herpes simplex virus vectors for gene transfer to neurons. *Nat.Med.* **3**, 357-359.

Finkelman, F. D. (1995). Relationships among antigen presentation, cytokines, immune deviation, and autoimmune disease [comment]. *J.Exp.Med.* **182**, 279-282.

Flamand, V., Sornasse, T., Thielemans, K., Demanet, C., Bakkus, M., Bazin, H., Tielemans, F., Leo, O., Urbain, J., and Moser, M. (1994). Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. *Eur.J.Immunol.* **24**, 605-610.

Fong, L. and Engleman, E. G. (2000b). Dendritic cells in cancer immunotherapy. *Annu.Rev.Immunol.* **18**, 245-273.

Fong, L. and Engleman, E. G. (2000a). Dendritic cells in cancer immunotherapy. *Annu.Rev.Immunol.* **18**, 245-273.

Fraefel, C., Song, S., Lim, F., Lang, P., Yu, L., Wang, Y., Wild, P., and Geller, A. I. (1996). Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *J.Virol.* **70**, 7190-7197.

Franks, L. M., Bollen, A., Seeger, R. C., Stram, D. O., and Matthay, K. K. (1997). Neuroblastoma in adults and adolescents: an indolent course with poor survival. *Cancer* **79**, 2028-2035.

Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Seidel, C. A., and Cines, D. B. (1984). Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature* **309**, 633-635.

Fugier-Vivier, I., Servet-Delprat, C., Rivailler, P., Rissoan, M. C., Liu, Y. J., and Rabourdin-Combe, C. (1997). Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. *J.Exp.Med.* **186**, 813-823.

Fushimi, T., Kojima, A., Moore, M. A., and Crystal, R. G. (2000). Macrophage inflammatory protein 3alpha transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J.Clin.Invest* **105**, 1383-1393.

Gaffney, D. F., McLauchlan, J., Whitton, J. L., and Clements, J. B. (1985). A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. *Nucleic Acids Res.* **13**, 7847-7863.

Galvan, V. and Roizman, B. (1998). Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. *Proc.Natl.Acad.Sci.U.S.A* **95**, 3931-3936.

Gardner, J. P., Frolov, I., Perri, S., Ji, Y., MacKichan, M. L., zur, M. J., Chen, M., Belli, B. A., Driver, D. A., Sherrill, S., Greer, C. E., Otten, G. R., Barnett, S. W., Liu, M. A., Dubensky, T. W., and Polo, J. M. (2000). Infection of human dendritic cells by a sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. *J.Virol.* **74**, 11849-11857.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597.

Geiss, B. J., Smith, T. J., Leib, D. A., and Morrison, L. A. (2000). Disruption of virion host shutoff activity improves the immunogenicity and protective capacity of a replication-incompetent herpes simplex virus type 1 vaccine strain. *J.Virol.* **74**, 11137-11144.

Geller, A. I. and Breakefield, X. O. (1988). A defective HSV-1 vector expresses Escherichia coli beta-galactosidase in cultured peripheral neurons. *Science* **241**, 1667-1669.

Geller, A. I. and Freese, A. (1990). Infection of cultured central nervous system neurons with a defective herpes simplex virus 1 vector results in stable expression of Escherichia coli beta-galactosidase. *Proc.Natl.Acad.Sci.U.S.A* **87**, 1149-1153.

Gerster, T. and Roeder, R. G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc.Natl.Acad.Sci.U.S.A* **85**, 6347-6351.

Gilboa, E. (1999). How tumors escape immune destruction and what we can do about it [see comments]. *Cancer Immunol.Immunother.* **48**, 382-385.

Goins, W. F., Sternberg, L. R., Croen, K. D., Krause, P. R., Hendricks, R. L., Fink, D. J., Straus, S. E., Levine, M., and Glorioso, J. C. (1994). A novel latency-active promoter is contained within the herpes simplex virus type 1 UL flanking repeats. *J.Virol.* **68** , 2239-2252.

Goldsmith, K., Chen, W., Johnson, D. C., and Hendricks, R. L. (1998). Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the CD8+ T cell response. *J.Exp.Med.* **187**, 341-348.

Goletz, T. J., Klimpel, K. R., Leppla, S. H., Keith, J. M., and Berzofsky, J. A. (1997). Delivery of antigens to the MHC class I pathway using bacterial toxins. *Hum.Immunol.* **54**, 129-136.

Graham, F. L. and Prevec, L. (1992). Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology* **20**, 363-390.

Granelli-Piperno, A., Zhong, L., Haslett, P., Jacobson, J., and Steinman, R. M. (2000b). Dendritic cells, infected with vesicular stomatitis virus-pseudotyped HIV-1, present viral antigens to CD4+ and CD8+ T cells from HIV-1-infected individuals. *J.Immunol.* **165**, 6620-6626.

Granelli-Piperno, A., Zhong, L., Haslett, P., Jacobson, J., and Steinman, R. M. (2000a). Dendritic cells, infected with vesicular stomatitis virus-pseudotyped HIV-1, present viral antigens to CD4+ and CD8+ T cells from HIV-1-infected individuals. *J.Immunol.* **165**, 6620-6626.

Greenberg, P. D., Cheever, M.A., and Fefer, A. (1981). H-2 restriction of adoptive immunotherapy of advanced tumors. *J. Immunol.* **126**, 2100-2103.

Grosjean, I., Caux, C., Bella, C., Berger, I., Wild, F., Banchereau, J., and Kaiserlian, D. (1997). Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. *J.Exp.Med.* **186**, 801-812.

Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y. J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J.Exp.Med.* **185**, 1101-1111.

Gruber, A., Kan-Mitchell, J., Kuhen, K. L., Mukai, T., and Wong-Staal, F. (2000). Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. *Blood* **96**, 1327-1333.

Gruenheid, S., Gatzke, L., Meadows, H., and Tufaro, F. (1993). Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. *J.Virol.* **67**, 93-100.

Habib, N. A., Ding, S. F., el Masry, R., Mitry, R. R., Honda, K., Michail, N. E., Dalla, S. G., Izzi, G., Greco, L., Bassyouni, M., el Toukhy, M., and Abdel-Gaffar, Y. (1996). Preliminary report: the short-term effects of direct p53 DNA injection in primary hepatocellular carcinomas. *Cancer Detect.Prev.* **20**, 103-107.

Hahn, G., Jores, R., and Mocarski, E. S. (1998). Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc.Natl.Acad.Sci.U.S.A* **95**, 3937-3942.

Hardy, W. R. and Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. *J.Virol.* **68**, 7790-7799.

Hareuveni, M., Tsarfaty, I., Zaretsky, J., Kotkes, P., Horev, J., Zrihan, S., Weiss, M., Green, S., Lathe, R., and Keydar, I. (1990). A transcribed gene, containing a variable number of tandem repeats, codes for a human epithelial tumor antigen. cDNA cloning, expression of the transfected gene and over-expression in breast cancer tissue. *Eur.J.Biochem.* **189**, 475-486.

Hart, D. N. (1997). Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* **90**, 3245-3287.

Hart, D. N. and Prickett, T. C. (1993). Intercellular adhesion molecule-2 (ICAM-2) expression on human dendritic cells. *Cell Immunol.* **148**, 447-454.

Henderson, R. A., Nimgaonkar, M. T., Watkins, S. C., Robbins, P. D., Ball, E. D., and Finn, O. J. (1996). Human dendritic cells genetically engineered to express high levels of the human epithelial tumor antigen mucin (MUC-1). *Cancer Res.* **56**, 3763-3770.

Hengel, H., Lindner, M., Wagner, H., and Heeg, K. (1987). Frequency of herpes simplex virus-specific murine cytotoxic T lymphocyte precursors in mit. *J.Immunol.* **139**, 4196-4202.

Hermens, W. T. and Verhaagen, J. (1998). Viral vectors, tools for gene transfer in the nervous system. *Prog.Neurobiol.* **55**, 399-432.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and Johnson, D. (1995). Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**, 411-415.

Holt, P. G., Haining, S., Nelson, D. J., and Sedgwick, J. D. (1994). Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J.Immunol.* **153**, 256-261.

Holzer, G. W., Remp, G., Antoine, G., Pfleiderer, M., Enzersberger, O. M., Emsenhuber, W., Hammerle, T., Gruber, F., Urban, C., Falkner, F. G., and Dorner, F. (1999). Highly efficient induction of protective immunity by a vaccinia virus vector defective in late gene expression. *J.Virol.* **73**, 4536-4542.

Honess, R. W. and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J.Virol.* **14**, 8-19.

Howard, M. K., Coffin, R. S., MacLean, A. R., Brown, S. M., Bailey, D., Anderson, P. N., Burnstock, G., and Latchman, D. S. (1997). Gene delivery to rat enteric neurons using herpes simplex virus-based vectors. *J.Mol.Neurosci.* **9**, 65-74.

Howard, M. K., Kershaw, T., Gibb, B., Storey, N., MacLean, A. R., Zeng, B. Y., Tel, B. C., Jenner, P., Brown, S. M., Woolf, C. J., Anderson, P. N., Coffin, R. S., and Latchman, D. S. (1998). High efficiency gene transfer to the central nervous system of rodents and primates using herpes virus vectors lacking functional ICP27 and ICP34.5. *Gene Ther.* **5**, 1137-1147.

Hsu, F. J., Benike, C., Fagnoni, F., Liles, T. M., Czerwinski, D., Taidi, B., Engleman, E. G., and Levy, R. (1996). Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat.Med.* **2**, 52-58.

Huang, W. Y., Aramburu, J., Douglas, P. S., and Izumo, S. (2000). Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat.Med.* **6**, 482-483.

Ibrahim, J., Griffin, P., Coombe, D. R., Rider, C. C., and James, W. (1999). Cell-surface heparan sulfate facilitates human immunodeficiency virus Type 1 entry into some cell lines but not primary lymphocytes. *Virus Res.* **60**, 159-169.

Imler, J. L. (1995). Adenovirus vectors as recombinant viral vaccines. *Vaccine* **13**, 1143-1151.

Inaba, K., Metlay, J. P., Crowley, M. T., and Steinman, R. M. (1990). Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ [published erratum appears in J Exp Med 1990 Oct 1;172(4):1275]. *J.Exp.Med.* **172**, 631-640.

Izutsu, K., Yoshioka, S., and Terao, T. (1993). Decreased protein-stabilizing effects of cryoprotectants due to crystallization. *Pharm.Res.* **10**, 1232-1237.

Jacob, R. J., Morse, L. S., and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J.Virol.* **29**, 448-457.

Jenne, L., Hauser, C., Arrighi, J. F., Saurat, J. H., and Hugin, A. W. (2000b). Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. *Gene Ther.* **7**, 1575-1583.

Jenne, L., Hauser, C., Arrighi, J. F., Saurat, J. H., and Hugin, A. W. (2000a). Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. *Gene Ther.* **7**, 1575-1583.

Jenne, L., Schuler, G., and Steinkasserer, A. (2001). Viral vectors for dendritic cell-based immunotherapy. *Trends Immunol.* **22**, 102-107.

Jerome, K. R., Chen, Z., Lang, R., Torres, M. R., Hofmeister, J., Smith, S., Fox, R., Froelich, C. J., and Corey, L. (2001). HSV and Glycoprotein J Inhibit Caspase Activation and Apoptosis Induced by Granzyme B or Fas. *J.Immunol.* **167**, 3928-3935.

Jerome, K. R., Fox, R., Chen, Z., Sears, A. E., Lee, H., and Corey, L. (1999). Herpes simplex virus inhibits apoptosis through the action of two genes, Us5 and Us3. *J.Virol.* **73**, 8950-8957.

Jones, F. E., Smibert, C. A., and Smiley, J. R. (1995). Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. *J.Virol.* **69**, 4863-4871.

Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat.Genet.* **17**, 314-317.

Kaiserlian, D. and Dubois, B. (2001). Dendritic cells and viral immunity: friends or foes? *Semin.Immunol.* **13**, 303-310.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., and Hori, M. (1999). Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J.Immunol.* **162**, 5584-5591.

Karp, C. L., Wysocka, M., Wahl, L. M., Ahearn, J. M., Cuomo, P. J., Sherry, B., Trinchieri, G., and Griffin, D. E. (1996). Mechanism of suppression of cell-mediated immunity by measles virus. *Science* **273**, 228-231.

Kast, W. M., Boog, C. J., Roep, B. O., Voordouw, A. C., and Melief, C. J. (1988). Failure or success in the restoration of virus-specific cytotoxic T lymphocyte response defects by dendritic cells. *J.Immunol.* **140**, 3186-3193.

Kelsall, B. L. and Strober, W. (1996). Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J.Exp.Med.* **183**, 237-247.

Kikuchi, T., Akasaki, Y., Irie, M., Homma, S., Abe, T., and Ohno, T. (2001). Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. *Cancer Immunol.Immunother.* **50**, 337-344.

Kim, D. T., Mitchell, D. J., Brockstedt, D. G., Fong, L., Nolan, G. P., Fathman, C. G., Engleman, E. G., and Rothbard, J. B. (1997). Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. *J.Immunol.* **159**, 1666-1668.

King, C., Mueller, H. R., Malo, C. M., Murali-Krishna, K., Ahmed, R., King, E., and Sarvetnick, N. (2001). Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T-cell responses. *Nat.Med.* **7**, 206-214.

Kirk, C. J., Hartigan-O'Connor, D., Nickoloff, B. J., Chamberlain, J. S., Giedlin, M., Aukerman, L., and Mule, J. J. (2001). T cell-dependent antitumor immunity mediated by secondary lymphoid tissue chemokine: augmentation of dendritic cell-based immunotherapy. *Cancer Res.* **61**, 2062-2070.

Klemm, R. D., Goodrich, J. A., Zhou, S., and Tjian, R. (1995). Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. *Proc.Natl.Acad.Sci.U.S.A* **92**, 5788-5792.

Koppers-Lalic, D., Rijsewijk, F. A., Verschuren, S. B., Gaans-Van den Brink, J. A., Neisig, A., Ressing, M. E., Neefjes, J., and Wiertz, E. J. (2001). The UL41-encoded virion host shutoff (vhs) protein and vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1. *J.Gen.Virol.* **82**, 2071-2081.

Krestel, H. E., Mayford, M., Seeburg, P. H., and Sprengel, R. (2001). A GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. *Nucleic Acids Res.* **29**, E39.

Kruse, M., Rosorius, O., Kratzer, F., Stelz, G., Kuhnt, C., Schuler, G., Hauber, J., and Steinkasserer, A. (2000). Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J.Virol.* **74**, 7127-7136.

Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C. A., Becker, V., Gross, A. J., Hemmerlein, B., Kanz, L., Muller, G. A., and Ringert, R. H. (2000). Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nat.Med.* **6**, 332-336.

Kwak, L. W., Campbell, M. J., Czerwinski, D. K., Hart, S., Miller, R. A., and Levy, R. (1992). Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N.Engl.J.Med.* **327**, 1209-1215.

Kwong, A. D. and Frenkel, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc.Natl.Acad.Sci.U.S.A* **84**, 1926-1930.

La Boissiere, S., Hughes, T., and O'Hare, P. (1999). HCF-dependent nuclear import of VP16. *EMBO J.* **18**, 480-489.

Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685.

Larregina, A. T., Watkins, S. C., Erdos, G., Spencer, L. A., Storkus, W. J., Beer, S. D., and Falo, L. D., Jr. (2001). Direct transfection and activation of human cutaneous dendritic cells. *Gene Ther.* **8**, 608-617.

Larsson, M., Messmer, D., Somersan, S., Fonteneau, J. F., Donahoe, S. M., Lee, M., Dunbar, P. R., Cerundolo, V., Julkunen, I., Nixon, D. F., and Bhardwaj, N. (2000). Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8+ T cells. *J.Immunol.* **165**, 1182-1190.

Latchman, D. S. (1990). Current status review: molecular biology of herpes simplex virus latency. *J.Exp.Pathol.(Oxford)* **71**, 133-141.

Leib, D. A., Bogard, C. L., Kosz-Vnenchak, M., Hicks, K. A., Coen, D. M., Knipe, D. M., and Schaffer, P. A. (1989). A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. *J.Virol.* **63**, 2893-2900.

Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. (1996). CD28/B7 system of T cell costimulation. *Annu.Rev.Immunol.* **14**, 233-258.

Leopardi, R., Van Sant, C., and Roizman, B. (1997). The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. *Proc.Natl.Acad.Sci.U.S.A* **94**, 7891-7896.

Lilley, C. E., Groutsi, F., Han, Z., Palmer, J. A., Anderson, P. N., Latchman, D. S., and Coffin, R. S. (2001). Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system in vivo. *J.Virol.* **75**, 4343-4356.

Littman, D. R. (1996). The CD4 molecule. Roles in T lymphocytes and in HIV disease. Introduction. *Curr.Top.Microbiol.Immunol.* **205**, v-x.

Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H., and Ke, N. J. (1999). Is green fluorescent protein toxic to the living cells? *Biochem.Biophys.Res.Commun.* **260**, 712-717.

Long, M. C., Leong, V., Schaffer, P. A., Spencer, C. A., and Rice, S. A. (1999). ICP22 and the UL13 protein kinase are both required for herpes simplex virus-

induced modification of the large subunit of RNA polymerase II. *J.Virol.* **73**, 5593-5604.

Lotze, M. T., Hellerstedt, B., Stolinski, L., Tueting, T., Wilson, C., Kinzler, D., Vu, H., Rubin, J. T., Storkus, W., Tahara, H., Elder, E., and Whiteside, T. (1997). The role of interleukin-2, interleukin-12, and dendritic cells in cancer therapy. *Cancer J.Sci.Am.* **3 Suppl 1**, S109-S114.

Maio, M., Altomonte, M., Tatake, R., Zett, R. A. and Ferrone, S. (1991). Susceptibility to natural killer cell mediated lysis of human FO-1 melanoma cells after induction of HLA class I antigen expression by transfection with β2m gene. *J. Clin. Invest.* **88**, 282-289.

Maloney, D. G., Kaminski, M. S., Burowski, D., Haimovich, J., and Levy, R. (1985). Monoclonal anti-idiotype antibodies against the murine B cell lymphoma 38C13: characterization and use as probes for the biology of the tumor in vivo and in vitro. *Hybridoma* **4**, 191-209.

Manickan, E., Kanangat, S., Rouse, R. J., Yu, Z., and Rouse, B. T. (1997). Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. *J.Leukoc.Biol.* **61**, 125-132.

Marchesi, S. L., Steers, E., and Shifrin, S. (1969). *Biochim. Biophys. Acta* **181**, 20-34.

Marie, J. C., Kehren, J., Trescol-Biemont, M. C., Evlashev, A., Valentin, H., Walzer, T., Tedone, R., Loveland, B., Nicolas, J. F., Rabourdin-Combe, C., and Horvat, B. (2001). Mechanism of measles virus-induced suppression of inflammatory immune responses. *Immunity.* **14**, 69-79.

Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ildstad, S. T., Kast, W. M., and Deleo, A. B. (1995a). Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat.Med.* **1**, 1297-1302.

Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ildstad, S. T., Kast, W. M., and Deleo, A. B. (1995b). Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat.Med.* **1**, 1297-1302.

McCarthy, A. M., McMahan, L., and Schaffer, P. A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *J.Virol.* **63**, 18-27.

McFarlane, M., Daksis, J. I., and Preston, C. M. (1992). Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65. *J.Gen.Virol.* **73 (Pt 2)**, 285-292.

McLachlin, J. R., Cornetta, K., Eglitis, M. A., and Anderson, W. F. (1990). Retroviral-mediated gene transfer. *Prog.Nucleic Acid Res.Mol.Biol.* **38**, 91-135.

Merson, M. H. (1993). Slowing the spread of HIV: agenda for the 1990s. *Science* **260**, 1266-1268.

Molnar-Kimber, K. L., Sterman, D. H., Chang, M., Kang, E. H., ElBash, M., Lanuti, M., Elshami, A., Gelfand, K., Wilson, J. M., Kaiser, L. R., and Albelda, S. M. (1998). Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. *Hum.Gene Ther.* **9**, 2121-2133.

Monahan, P. E., Samulski, R. J., Tazelaar, J., Xiao, X., Nichols, T. C., Bellinger, D. A., Read, M. S., and Walsh, C. E. (1998). Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. *Gene Ther.* **5** , 40-49.

Morse, M. A., Lyerly, H. K., Gilboa, E., Thomas, E., and Nair, S. K. (1998). Optimization of the sequence of antigen loading and CD40-ligand-induced maturation of dendritic cells. *Cancer Res.* **58**, 2965-2968.

Morse, M. A., Zhou, L. J., Tedder, T. F., Lyerly, H. K., and Smith, C. (1997). Generation of dendritic cells in vitro from peripheral blood mononuclear cells with granulocyte-macrophage-colony-stimulating factor, interleukin-4, and tumor necrosis factor-alpha for use in cancer immunotherapy. *Ann.Surg.* **226**, 6-16.

Mortarini, R., Anichini, A., Di Nicola, M., Siena, S., Bregni, M., Belli, F., Molla, A., Gianni, A. M., and Parmiani, G. (1997). Autologous dendritic cells derived from CD34+ progenitors and from monocytes are not functionally equivalent antigen-presenting cells in the induction of melan-A/Mart-1(27-35)-specific CTLs from peripheral blood lymphocytes of melanoma patients with low frequency of CTL precursors. *Cancer Res.* **57**, 5534-5541.

Moss, B. (1996). Poxviridae: the viruses and their replication. Fields Virology. Ed. Fields, B. N., Knipe, D. M., Howley, P. M. Philadelphia: Lippincott-Raven. 2637-2672.

Murphy, S. (1999). Viral delivery systems for gene therapy in understanding gene therapy. Ed. N. R. Lemoine. BIOS scientific puplishers Ltd. 21-42.

Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr.Top.Microbiol.Immunol.* **158**, 97-129.

Nair, S. K., Boczkowski, D., Morse, M., Cumming, R. I., Lyerly, H. K., and Gilboa, E. (1998). Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. *Nat.Biotechnol.* **16**, 364-369.

Nair, S. K., Hull, S., Coleman, D., Gilboa, E., Lyerly, H. K., and Morse, M. A. (1999). Induction of carcinoembryonic antigen (CEA)-specific cytotoxic T-lymphocyte responses in vitro using autologous dendritic cells loaded with CEA peptide or CEA RNA in patients with metastatic malignancies expressing CEA. *Int.J.Cancer* **82**, 121-124.

Negre, D., Mangeot, P. E., Duisit, G., Blanchard, S., Vidalain, P. O., Leissner, P., Winter, A. J., Rabourdin-Combe, C., Mehtali, M., Moullier, P., Darlix, J. L., and Cosset, F. L. (2000). Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. *Gene Ther.* **7**, 1613-1623.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998b). Vaccination of melanoma patients with pep. *Nat.Med.* **4**, 328-332.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998a). Vaccination of melanoma patients with pep. *Nat.Med.* **4**, 328-332.

Nestle, F. O., Banchereau, J., and Hart, D. (2001). Dendritic cells: On the move from bench to bedside. *Nat.Med.* **7**, 761-765.

Nonacs, R., Humborg, C., Tam, J. P., and Steinman, R. M. (1992). Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J.Exp.Med.* **176**, 519-529.

Ogle, W. O. and Roizman, B. (1999). Functional anatomy of herpes simplex virus 1 overlapping genes encoding infected-cell protein 22 and US1.5 protein. *J.Virol.* **73**, 4305-4315.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Olweus, J., BitMansour, A., Warnke, R., Thompson, P. A., Carballido, J., Picker, L. J., and Lund-Johansen, F. (1997). Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc.Natl.Acad.Sci.U.S.A* **94**, 12551-12556.

Paglia, P., Chiodoni, C., Rodolfo, M., and Colombo, M. P. (1996). Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J.Exp.Med.* **183**, 317-322.

Palella, J. D.,Silverman, L. J., Homa, F.L., Levine, M, and Kelley, W. N. (1989). Transfer of human HPRT gene sequences into neuronal cells by a herpes simplex virus derived vector. *Adv. Exp. Med. Biol.* **253A**, 549-554.

Palmer, J. A., Branston, R. H., Lilley, C. E., Robinson, M. J., Groutsi, F., Smith, J., Latchman, D. S., and Coffin, R. S. (2000). Development and optimization of herpes

simplex virus vectors for multiple long-term gene delivery to the peripheral nervous system. *J.Virol.* **74**, 5604-5618.

Palucka, K. and Banchereau, J. (1999). Dendritic cells: a link between innate and adaptive immunity. *J.Clin.Immunol.* **19**, 12-25.

Pamer, E. and Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. *Annu.Rev.Immunol.* **16**, 323-358.

Para, M.F., Baucke, R. and Spear, P. G. (1982). Glycoprotein gE of herpes simplex virus type 1: effects of anti-gE on virion infectivity and on virus-induced Fc-binding receptors. *J. Virol.* **41**, 129-136.

Pardoll, D. (1992). Immunotherapy with cytokine gene-transduced tumor cells: the next wave in gene therapy for cancer. *Curr.Opin.Oncol.* **4**, 1124-1129.

Pardoll, D. M. (1993). New strategies for enhancing the immunogenicity of tumors. *Curr.Opin.Immunol.* **5**, 719-725.

Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R., and Piston, D. W. (1997). Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys.J.* **73**, 2782-2790.

Peebles, R. S., Jr. and Graham, B. S. (2001b). Viruses, dendritic cells and the lung. *Respir.Res.* **2**, 245-249.

Peebles, R. S., Jr. and Graham, B. S. (2001a). Viruses, dendritic cells and the lung. *Respir.Res.* **2**, 245-249.

Pikal-Cleland, K. A., Rodriguez-Hornedo, N., Amidon, G. L., and Carpenter, J. F. (2000). Protein denaturation during freezing and thawing in phosphate buffer systems: monomeric and tetrameric beta-galactosidase. *Arch.Biochem.Biophys.* **384**, 398-406.

Pisani, P., Parkin, D. M., Bray, F., and Ferlay, J. (1999). Erratum: Estimates of the worldwide mortality from 25 cancers in 1990. Int. J. Cancer, 83, 18-29 (1999). *Int.J.Cancer* **83**, 870-873.

Purves, F. C., Longnecker, R. M., Leader, D. P., and Roizman, B. (1987). Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. *J.Virol.* **61**, 2896-2901.

Purves, F. C., Spector, D., and Roizman, B. (1991). The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene. *J.Virol.* **65**, 5757-5764.

Rammensee, H. G., Falk, K., and Rotzschke, O. (1993). MHC molecules as peptide receptors. *Curr.Opin.Immunol.* **5**, 35-44.

Rampling, R., Cruickshank, G., Papanatassiou, V., Nicoll, J., Hadley, D., Brennan, D., Petty, R., MacLean, A., Harland, J., McKie, E., Mabbs, R., and Brown, M. (1998). Toxicity evaluation of replication competent herpes simplex virus (ICP34.5 null mutant 1764) in patients with recurrent malignant glioma. *Gene Ther.* **10**, 859-866.

Randazzo, B. P., Tal-Singer, R., Zabolotny, J. M., Kesari, S., and Fraser, N. W. (1997). Herpes simplex virus 1716, an ICP 34.5 null mutant, is unable to replicate in CV-1 cells due to a translational block that can be overcome by coinfection with SV40. *J.Gen.Virol.* **78 (Pt 12)**, 3333-3339.

Reeves, M. E., Royal, R. E., Lam, J. S., Rosenberg, S. A., and Hwu, P. (1996). Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. *Cancer Res.* **56**, 5672-5677.

Reis, M. M., Higuchi, M., Benvenuti, L. A., Aiello, V. D., Gutierrez, P. S., Bellotti, G., and Pileggi, F. (1997). An in situ quantitative immunohistochemical study of cytokines and IL-2R+ in chronic human chagasic myocarditis: correlation with the presence of myocardial Trypanosoma cruzi antigens. *Clin.Immunol.Immunopathol.* **83**, 165-172.

Restifo, N. P., Esquivel, F., Asher, A. L., Stotter, H., Barth, R. J., Bennink, J. R., Mule, J. J., Yewdell, J. W., and Rosenberg, S. A. (1991). Defective presentation of endogenous antigens by a murine sarcoma. Implications for the failure of an anti-tumor immune response. *J.Immunol.* **147**, 1453-1459.

Rettig, M. B., Ma, H. J., Vescio, R. A., Pold, M., Schiller, G., Belson, D., Savage, A., Nishikubo, C., Wu, C., Fraser, J., Said, J. W., and Berenson, J. R. (1997). Kaposi's sarcoma-associated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients. *Science* **276**, 1851-1854.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393**, 474-478.

Roizman, B. and Jenkins, F. J. (1985). Genetic engineering of novel genomes of large DNA viruses. *Science* **229**, 1208-1214.

Roizman, B. and Sear, A. E. (1996). Herpes simplex viruses and their replication. Fields Virology. Ed. Fields, B. N., Knipe, D. M., Howley, P. M. Philadelphia: Lippincott-Raven. 2231-2295.

Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. (1994). Proliferating dendritic cell progenitors in human blood. *J.Exp.Med.* **180**, 83-93.

Rosenberg, S. A., Anderson, W. F., Blaese, M., Hwu, P., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Ettinghausen, S. E., and .

(1993). The development of gene therapy for the treatment of cancer. *Ann.Surg.* **218**, 455-463.

Rudensky, A. Y. (1995). Endogenous peptides associated with MHC class II and selection of CD4 T cells. *Semin.Immunol.* **7**, 399-409.

Sacchetti, A., Cappetti, V., Marra, P., Dell'Arciprete, R., El Sewedy, T., Crescenzi, C., and Alberti, S. (2001). Green fluorescent protein variants fold differentially in prokaryotic and eukaryotic cells. *J.Cell Biochem.* **Suppl 36**, 117-128.

Sacks, W. R., Greene, C. C., Aschman, D. P., and Schaffer, P. A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J.Virol.* **55**, 796-805.

Sadanaga, N., Nagashima, H., Mashino, K., Tahara, K., Yamaguchi, H., Ohta, M., Fujie, T., Tanaka, F., Inoue, H., Takesako, K., Akiyoshi, T., and Mori, M. (2001). Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. *Clin.Cancer Res.* **7**, 2277-2284.

Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563-564.

Saeki, H., Moore, A. M., Brown, M. J., and Hwang, S. T. (1999). Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J.Immunol.* **162**, 2472-2475.

Salio, M., Cella, M., Suter, M., and Lanzavecchia, A. (1999). Inhibition of dendritic cell maturation by herpes simplex virus. *Eur.J.Immunol.* **29**, 3245-3253.

Sallusto, F. and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J.Exp.Med.* **179**, 1109-1118.

Sallusto, F. and Lanzavecchia, A. (2000). Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol.Rev.* **177**, 134-140.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). Molecular Cloning. A laboratory Manual. 2nd ed., New York, 2 vol, Cold Spring Harbor Laboratory.

Schilde, C., Wostemeyer, J., and Burmester, A. (2001). Green fluorescent protein as a reporter for gene expression in the mucoralean fungus Absidia glauca. *Arch.Microbiol.* **175**, 1-7.

Schoell, W. M., Mirhashemi, R., Liu, B., Janicek, M. F., Podack, E. R., Penalver, M. A., and Averette, H. E. (1999). Generation of tumor-specific cytotoxic T lymphocytes

by stimulation with HPV type 16 E7 peptide-pulsed dendritic cells: an approach to immunotherapy of cervical cancer. *Gynecol.Oncol.* **74**, 448-455.

Scholl, S. M., Balloul, J. M., Le Goc, G., Bizouarne, N., Schatz, C., Kieny, M. P., Mensdorff-Pouilly, S., Vincent-Salomon, A., Deneux, L., Tartour, E., Fridman, W., Pouillart, P., and Acres, B. (2000). Recombinant vaccinia virus encoding human MUC1 and IL2 as immunotherapy in patients with breast cancer. *J.Immunother.* **23**, 570-580.

Sears, A. E., Halliburton, I. W., Meignier, B., Silver, S., and Roizman, B. (1985). Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. *J.Virol.* **55**, 338-346.

Servet-Delprat, C., Vidalain, P. O., Bausinger, H., Manie, S., Le Deist, F., Azocar, O., Hanau, D., Fischer, A., and Rabourdin-Combe, C. (2000). Measles virus induces abnormal differentiation of CD40 ligand-activated human dendritic cells. *J.Immunol.* **164**, 1753-1760.

Smith, T. J., Ackland-Berglund, C. E., and Leib, D. A. (2000). Herpes simplex virus virion host shutoff (vhs) activity alters periocular disease in mice. *J.Virol.* **74**, 3598-3604.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.

Sozzani, S., D'Alessandro, F., Capsoni, F., Luini, W., Barcellini, W., Guidi, G., and Spreafico, F. (1988). In vitro modulation of human monocytes functions by RU 41740 (Biostim). *Int.J.Immunopharmacol.* **10**, 93-102.

Spaete, R. R. and Frenkel, N. (1982). The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**, 295-304.

Steinman, R. M, Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **5**, 1142-1162.

Steinman, R. M, Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 2. Functional proerties in vitro. *J. Exp. Med.* **2**, 380-397.

Steinman, R. M., Lustig, D. S., Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *J. Exp. Med.* **36**, 1431-1445.

Steinman, R. M., Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leucocyte reaction in mice. *Proc. Natl. Acad. Sci. USA.* **10,** 5132-5136.

Stockhammer, G., Brotchi, J., Leblanc, R., Bernstein, M., Schackert, G., Weber, F., Ostertag, C., Mulder, N. H., Mellstedt, H., Seiler, R., Yonekawa, Y., Twerdy, K., Kostron, H., De Witte, O., Lambermont, M., Velu, T., Laneuville, P., Villemure, J. G., Rutka, J. T., Warnke, P., Laseur, M., Mooij, J. J., Boethius, J., Mariani, L., Gianella-Borradori, A., and . (1997). Gene therapy for glioblastoma [correction of gliobestome] multiform: in vivo tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. *J.Mol.Med.* **75**, 300-304.

Stripecke, R., Carmen, V. M., Skelton, D., Satake, N., Halene, S., and Kohn, D. (1999). Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther.* **6**, 1305-1312.

Strobel, I., Berchtold, S., Gotze, A., Schulze, U., Schuler, G., and Steinkasserer, A. (2000a). Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. *Gene Ther.* **7**, 2028-2035.

Strobel, I., Krumbholz, M., Menke, A., Hoffmann, E., Dunbar, P. R., Bender, A., Hobom, G., Steinkasserer, A., Schuler, G., and Grassmann, R. (2000b). Efficient expression of the tumor-associated antigen MAGE-3 in human dendritic cells, using an avian influenza virus vector. *Hum.Gene Ther.* **11**, 2207-2218.

Strom, T. and Frenkel, N. (1987). Effects of herpes simplex virus on mRNA stability. *J.Virol.* **61**, 2198-2207.

Sutter, G. and Moss, B. (1992). Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc.Natl.Acad.Sci.U.S.A* **89**, 10847-10851.

Suzutani, T., Nagamine, M., Shibaki, T., Ogasawara, M., Yoshida, I., Daikoku, T., Nishiyama, Y., and Azuma, M. (2000). The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. *J.Gen.Virol.* **81 Pt 7**, 1763-1771.

Svensson, M., Stockinger, B., and Wick, M. J. (1997). Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. *J.Immunol.* **158**, 4229-4236.

Svensson, M. and Wick, M. J. (1999). Classical MHC class I peptide presentation of a bacterial fusion protein by bone marrow-derived dendritic cells. *Eur.J.Immunol.* **29**, 180-188.

Szabolcs, P., Gallardo, H. F., Ciocon, D. H., Sadelain, M., and Young, J. W. (1997). Retrovirally transduced human dendritic cells express a normal phenotype and potent T-cell stimulatory capacity. *Blood* **90**, 2160-2167.

Tanaka, K., Gorelik, E., Watanabe, M., Hozumi, N., and Jay, G. (1988). Rejection of B16 melanoma induced by expression of a transfected major histocompatibility complex class I gene. *Mol. Cell. Biol.* **8**, 1857-1861.

Tanaka, Y., Kagamiishi, A., Kiuchi, A., and Horiuchi, T. (1975). *J. Biochem.* **77**, 241-247.

Temin, H. M. (1989). Retrovirus vectors: promise and reality. *Science* **246**, 983.

Thomas, S. K., Lilley, C. E., Latchman, D. S., and Coffin, R. S. (1999). Equine herpesvirus 1 gene 12 can substitute for vmw65 in the growth of herpes simplex virus (HSV) type 1, allowing the generation of optimized cell lines for the propagation of HSV vectors with multiple immediate-early gene defects. *J.Virol.* **73**, 7399-7409.

Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den, D. P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., and Schuler, G. (1999). Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J.Exp.Med.* **190**, 1669-1678.

Tjoa, B. A., Erickson, S. J., Bowes, V. A., Ragde, H., Kenny, G. M., Cobb, O. E., Ireton, R. C., Troychak, M. J., Boynton, A. L., and Murphy, G. P. (1997b). Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* **32**, 272-278.

Tjoa, B. A., Erickson, S. J., Bowes, V. A., Ragde, H., Kenny, G. M., Cobb, O. E., Ireton, R. C., Troychak, M. J., Boynton, A. L., and Murphy, G. P. (1997a). Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* **32**, 272-278.

Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J., and Ploegh, H. L. (2000). Viral subversion of the immune system. *Annu.Rev.Immunol.* **18**, 861-926.

Townsend, S. E. and Allison, J. P. (1993). Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* **259**, 368-370.

Tsien, R. Y. (1998). The green fluorescent protein. *Annu.Rev.Biochem.* **67**, 509-544.

van Kooten, C. and Banchereau, J. (1996). CD40-CD40 ligand: a multifunctional receptor-ligand pair. *Adv.Immunol.* **61**, 1-77.

Vile, R. G. (1991). The retroviral life cycle and the molecular construction of retroviral vectors in methods in molecular biology, vol 8: Practical molecular virology: viral vectors for gene expression. Ed. M. Collins. The humana press, inc. 1-15.

Vile, R. G. (1996). Gene therapy for cancer, the course ahead. *Cancer Metastasis Rev.* **15**, 403-410.

Vile, R. G., Schulz, T. F., Danos, O. F., Collins, M. K., and Weiss, R. A. (1991). A murine cell line producing HTLV-I pseudotype virions carrying a selectable marker gene. *Virology* **180**, 420-424.

Walker, J. and Leib, D. A. (1998). Protection from primary infection and establishment of latency by vaccination with a herpes simplex virus type 1 recombinant deficient in the virion host shutoff (vhs) function. *Vaccine* **16**, 1-5.

Watts, C. (1997). Immunology. Inside the gearbox of the dendritic cell [news; comment]. *Nature* **388**, 724-725.

Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce, d. L., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J.Virol.* **71**, 6083-6093.

Wu, N., Watkins, S. C., Schaffer, P. A., and DeLuca, N. A. (1996). Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J.Virol.* **70**, 6358-6369.

Wu, S. J., Grouard-Vogel, G., Sun, W., Mascola, J. R., Brachtel, E., Putvatana, R., Louder, M. K., Filgueira, L., Marovich, M. A., Wong, H. K., Blauvelt, A., Murphy, G. S., Robb, M. L., Innes, B. L., Birx, D. L., Hayes, C. G., and Frankel, S. S. (2000). Human skin Langerhans cells are targets of dengue virus infection. *Nat.Med.* **6**, 816-820.

Xiang, Z. Q., Yang, Y., Wilson, J. M., and Ertl, H. C. (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219**, 220-227.

Yang, L., Voytek, C. C., and Margolis, T. P. (2000). Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1. *J.Virol.* **74**, 209-217.

York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L., and Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* **77**, 525-535.

Zhang, W. W. (1999). Development and application of adenoviral vectors for gene therapy of cancer. *Cancer Gene Ther.* **6**, 113-138.

Zhong, L., Granelli-Piperno, A., Choi, Y., and Steinman, R. M. (1999). Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. *Eur.J.Immunol.* **29**, 964-972.

Zhou, L. J. and Tedder, T. F. (1995). Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J.Immunol.* **154**, 3821-3835.

Zinkernagel, R. M. and Doherty, P. C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702.

Zitvogel, L., Mayordomo, J. I., Tjandrawan, T., Deleo, A. B., Clarke, M. R., Lotze, M. T., and Storkus, W. J. (1996). Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J.Exp.Med.* **183**, 87-97.

Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat.Biotechnol.* **15**, 871-875.