Selection of CMV-specific HLA-A2 restricted cytotoxic T lymphocytes using peptides from the CMV protein pp65

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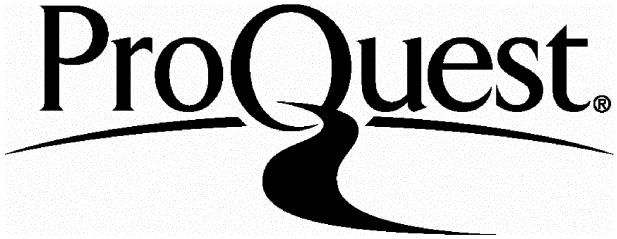

Dedicated to James

Abstract

The cytotoxic T lymphocyte (CTL) response to cytomegalovirus (CMV) is thought to play an important role in defence against this virus, and in those with compromised T cell immunity CMV is an important pathogen. Analysis of the antigen specificity of the CMV-specific CTL response has shown that it is directed almost entirely to a single tegument protein, the phosphoprotein pp65. The present study aimed to determine the nature of the CTL response to this CMV protein in the context of the HLA-A*0201 antigen.

Screening of the protein sequence of pp65 revealed 17 peptides which fulfilled the anchor binding motif requirements for HLA-A*0201. The binding affinity of these peptides to the HLA-A*0201 antigen was assessed using two methods. The peptides shown to bind were then assayed for the ability to induce an HLA-A*0201 restricted CTL response *in vitro*. Three epitopes were identified which stimulated *in vitro* CTL responses from the peripheral blood lymphocytes of CMV seropositive individuals. Such peptide-specific CTLs also recognised the naturally processed pp65 presented either in CMV infected cells, or in cells infected with an adenovirus construct expressing this protein. The three CTL epitopes identified were conserved in four clinical isolates and three laboratory strains of CMV.

The identification of these pp65 immunogenic epitopes may provide a useful basis for the development of future CMV immunotherapies, which could serve to generate a primary CTL response to CMV in seronegative individuals, or to amplify a memory CTL response in seropositive individuals. In the design of a potential peptide vaccine the ideal situation would be to target as high a number of the affected population as possible, and thus pp65 CTL epitopes for other HLA antigens need to be identified. The latter could be achieved using the strategy successfully employed in the present study for the HLA-A*0201 antigen.

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β_2m	β2-microglobulin
^{51}Cr	Chromium 51
7-deaza-dATP	7-deaza-2' deoxyadenosine-5' triphosphate
7-deaza-dGTP	7-deaza-2' deoxyguanosine-5' triphosphate
A	Adenosine
Ad5	Adenovirus type 5
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ATCC	American type culture collection
ATP	Adenosine triphosphate
B-LCL	B-lymphoblastoid cell line
BMT	Bone Marrow Transplantation
bp	Base pair
C	Cytosine
CD	Cluster of differntiation antigen
CIAP	Calf intestinal alkaline phosphatase
CLIP	Class II associated invariant chain peptide
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
dATP	Deoxyadenosine-5' triphosphate
dCTP	Deoxycytosine-5' triphosphate
ddH20	Double distilled H20
dGTP	Deoxyguanosine-5' triphosphate
dITP	2' deoxyinosine-5' triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
E. Coli	Eschericia Coli
EBNA-1	Epstein Barr virus nuclear antigen-1
EBV	Epstein Barr virus
ECACC	European Collection of Animal Cell Cultures
EDTA	Ethyl-tetra-acetic acid
EGFP	Red-shifted variant of wild type green fluorescencent protein
ELISA	Enzyme-linked immuno sorbent assay
EMEM	Earle's modified Eagle's medium
ER	Endoplasmic reticulum
FACS	Fluoresence activated cell sorter
FCS	Foetal calf serum
FITC	Fluoroscein isothyocynate
FL	Fluorescein
FSC	Forward scatter
G	Guanine
HBSS	Hepes buffered saline solution
HEL	Human embryonic lung
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
HSP70	Heat shock protein of 70kD
IE	Immediate early

IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP-30	30 kd intracellular peptide
IPTG	Isopropylthio-β-D-galactosidase
IU/L	International units per litre
kb	Kilobases
kd	Kilodaltons
LB	Luria Bertrani medium
MCF	Mean channel value of fluorescence
MCS	Multiple cloning site
MEM	Modified Eagle's medium
MF	Mean fluorescence
MHC	Major Histocompatability Complex
MOI	Multiplicity of infection
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
neor	Neomycin resistance cassette
NP-40	Nonident P-40
OD	Optical density
OPD	Ortho-phenylendiamine
ORF	Open reading frame
oriP	Origin of replication
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA	PBS containing 0.02% sodium azide
PCMV IE	Immediate early promoter of CMV
PCR	Polymerase chain reaction
PE	Phycoerithrin
PEG	Polyethyleneglycol
pfu	Plaque forming unit
PHA	Phytohaemaglutinin
pp65	Phospoprotein of 65kD
Rad	Recombinant adenovirus
RP-HPLC	Reverse phase high performance liquidchromatography
RPMI	Roswell Park Memorial Institute
RSV LTR	Rous sarcoma virus long terminal repeat
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SSC	Side scatter
SSOP	Sequence specific oligonucleotide probing
SV40	Simian virus 40
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TEMED	N, N, N', N' - Tetramethylethylenediamine
Th	T helper cell
X-gal	5-bromo-4-chloro-3indolyl-β-D-galctosidase

1. Chapter 1. Main Introduction

Cytomegalovirus (CMV), a member of the herpes virus group, is an agent which causes widespread infection in the human population. Although acquired CMV infection in immunocompetent individuals can produce a mononucleosis-like infection, in general CMV usually produces an asymptomatic infection, which is only detected serologically (Pass *et al.*, 1991). The virus is not usually eliminated from the body after primary infection, but can persist in the host throughout life. To maintain this life cycle, CMV uses mechanisms to evade the host immune response, one of which is the establishment of latency, a common feature of the herpesvirus family. The relatively low rate of reactivation of the virus in normal individuals seems to suggest a successful interaction between the host's immune system and the virus. However, a number of host and viral factors can affect this interaction. The most important is a shift in favour of the virus when the host's immune system is compromised or immature. Under these conditions, reactivation of latent virus, re-infection or primary infection can lead to the development of CMV disease. The populations at risk of CMV disease include neonates (Stagno *et al.*, 1991; Stagno *et al.*, 1982), patients who are immunossupressed due to pre-conditioning prior to solid organ or bone marrow transplantation, and patients infected with the human immunodeficiency virus (HIV). CMV is the most common congenital viral infection in humans, and is probably the most common infectious cause of mental retardation and non-hereditary sensoneural deafness in children in western countries (Boppana *et al.*, 1992). The dramatic and progressive cellular immunodeficiency induced by HIV infection creates optimal conditions for the reactivation of latent CMV. Clinical syndromes associated with CMV in HIV infected patients have included disease in almost every organ system, but most frequently it

is manifest by disease of the central nervous system, eyes, gastrointestinal system and lungs (Gallant *et al.*, 1992; Pertel *et al.*, 1992). CMV is also an important pathogen in allograft recipients, with symptomatic infection occurring in up to 40% of kidney, liver, heart and heart-lung transplant recipients (Ho, 1994). CMV infection has multiple effects on these patients, including pathology of infected organs (Paya *et al.*, 1989; Smyth *et al.*, 1991a), increased immunosuppression (Rook, 1988), and increased risk of allograft rejection, leading to a decreased patient survival (Glenn, 1981). CMV pneumonitis is a major cause of death in allogeneic bone marrow transplant recipients (Forman and Zaia, 1994). These conditions continue to be a problem despite the availability of effective antiviral agents such as ganciclovir and foscarnet (Bailey, 1993; Rubin, 1990). A greater understanding of the biology of CMV and its interactions with the host immune system is required to help combat the effects of infection with this virus in susceptible groups.

1.1 The biology of CMV

1.1.1 Virion characteristics

CMV has been classified as a member of the β subfamily of the herpesvirus based on its morphological appearance, its long replication cycle, cytopathic effects and host specificity. The virion consists of a core containing linear double-stranded DNA, an icosahedral capsid containing 162 capsomers surrounded by an amorphous layer of proteins called the tegument, and an envelope, which is derived from the host cell and contains virus-encoded glycoproteins. The genome of the laboratory strain AD169, has been sequenced, and contains 208 reading frames, encoding more than 200 different proteins (Chee *et al.*, 1990). Virally encoded proteins include those which control the expression of other viral genes, those which encode proteins required for viral replication, and those which are

structural components of the virion. However, only limited information is currently available regarding the functions of most of these proteins, their potential roles in infection, and their interaction with the host immune system.

1.1.2 The structural proteins of CMV

Prior to sequencing of the CMV genome, the nomenclature of CMV proteins was based on various characteristics of the protein, such as its molecular weight, post-translational modifications, structural and functional properties (i.e. localisation in the virion, temporal expression post infection, and the role in virus infectivity, replication or virion assembly), or homology with proteins in other human and animal herpesviruses. However this nomenclature has been confusing due to protein heterogeneity within CMV strains, and also has resulted in the same proteins being given several different names. It has now been decided to name CMV proteins according to the alphanumeric open reading frame designation of the protein (Chee et al., 1990), prefixed by pp for a phosphoprotein, gp for a glycoprotein, or p for a protein which is neither of the above, or whose post translational modification is as yet undefined (Landini and Spaete, 1993).

1.1.2.1 Capsid proteins

The mature CMV capsid consists of three abundant protein species, the 153 kd major capsid protein (pUL86), the 34 kd minor capsid protein (UL46) and the 10-12 kd small capsid protein, plus several less abundant proteins (Chee *et al.*, 1989; Gibson *et al.*, 1990).

1.1.2.2 Tegument proteins

The tegument is the area localised between the capsid and the final envelope of CMV virions, and is composed of a large number of proteins, particularly phosphoproteins (Gibson, 1983; Landini *et al.*, 1987; Stinski, 1976). Viral proteins which anchor the

envelope to the nucleocapsid are referred to as matrix proteins. The major tegument constituents are pp150 (also called the basic protein) which is the 1048 amino acid (aa) product of UL32, pp65 (the lower matrix protein) the 561 aa product of UL83 (Jahn *et al.*, 1987; Roby and Gibson, 1986; Ruger *et al.*, 1987), and pp71 (the upper matrix protein). Three other tegument proteins have been characterised, namely pUL48, ppUL99 and ppUL65. All the tegument proteins are phosphorylated with the exception of pUL48, the 212 kd high molecular weight protein (Spaete *et al.*, 1994). However, the pp65 protein has been found to be the major phosphate acceptor in infected cells and the primary target for phosphorylation *in vitro* by the virion associated protein kinase (Britt and Auger, 1986; Michelson *et al.*, 1984; Roby and Gibson, 1986). Furthermore, protein kinase activity may also be associated with pp65 itself (Somogyi *et al.*, 1990).

The tegument phosphoprotein pp150 is a major constituent of the virion, estimated to represent 20 % of its mass (Jahn et al., 1987; Roby and Gibson, 1986). The lower matrix protein pp65, another abundantly produced tegument protein, is rapidly translocated to the nucleus upon infection, and it is also present in the cytoplasm during later stages of infection (Britt and Vugler, 1987; Weiner *et al.*, 1985). Due to the high molecular mass of pp65 it can not reach the nucleus by passive diffusion (40 kd mass limit) (Dingwall and Laskey, 1991), and so it must exploit targeting signals. Such nuclear targeting sequences, called localisation signals, have been localised in two hydrophilic regions (HP1 and HP2) present in the C-terminal region of pp65 (Gallina *et al.*, 1996; Schmolke *et al.*, 1995a). Studies using a pp65 deletion mutant of CMV have demonstrated that pp65 is dispensable for the permissive replication of the virus and no morphological differences were found in comparison to the wild type virus, although the maturation process of the virion appeared to be delayed. However, at least one of the virion associated protein kinases appeared to be compromised by the deletion of pp65 (Schmolke *et al.*, 1995b), which supports the previous findings on the phosphorylative properties of pp65.

The function of pp65 in virus infection has remained elusive. Although its rapid nuclear transport immediately after infection suggests that pp65 is important in the initial events of viral replication, such as transcriptional activation of viral or cellular genes, no evidence of pp65 being a transcriptional transactivator has been found (Baldick *et al.*, 1997; Liu and Stinski, 1992). However, possibly the most relevant aspect of this protein is its immunogenicity and ability to target a cellular immune response against CMV (McLauglin-Taylor *et al.*, 1994).

The upper matrix protein, pp71, is transcribed from the same region of the genome as the lower matrix protein, but is less abundant in infected cells (Gibson, 1983; Irmiere and Gibson, 1985). The pp65 protein is encoded by the 5'-end of an abundant 4 kb mRNA, while the pp71 polypeptide corresponds to the single translational reading frame from a non-spliced 1.9 kb mRNA. The latter protein has been shown to enhance the transcription of promoters such as the major immediate early promoter, and it also enhances the infectivity of CMV (Liu and Stinski, 1992; Spaete et al., 1994). More recently it was also demonstrated that pp71 facilitates late gene expression, virus transmission to adjacent cells and plaque formation (Baldick et al., 1997).

1.1.2.3 Envelope proteins

The capsid and the tegument are enclosed by a lipid bilayer envelope, derived from either the inner nuclear or cytoplasmic membranes. At least 8 viral envelope glycoproteins have been identified, although there are likely to be more minor envelope glycoproteins present, as sequence analysis of the CMV strain AD169 genome has identified approximately 55 open reading frames potentially encoding glycoproteins (Chee et al., 1990). The three envelope glycoprotein complexes that have been characterised most fully have been designated gCI, gCII and gCIII (Cranage *et al.*, 1986; Farrar and Greenaway, 1986; Gretch *et al.*, 1988). The gCI is composed mainly of gB (gpUL55), the most abundant CMV envelope glycoprotein. gB is a multi-functional protein that is likely to play roles in

virion penetration into cells, virus transmission from cell to cell, and the fusion of infected cells (Myerson *et al.*, 1984). Furthermore gB has also been shown to be a prominent target for the neutralising antibody response (Cremer *et al.*, 1985; Pereira *et al.*, 1982; Pereira *et al.*, 1984). The gCII complex consists of at least two proteins, which are disulphide linked, designated gp47-52 (Kari *et al.*, 1990). These have been identified as the US10 and US6 gene products (Gretch et al., 1988). The gCIII complex includes the envelope protein gH (gpUL75) which is proposed to be in a stable complex with gL (UL115), as is the case for the herpes simplex virus homologues for these proteins (Spaete et al., 1994). gH appears to be involved in cell to cell spread of viruses and the fusion of the virion with the host cell plasma membrane. Furthermore, anti-gH antibodies can prevent cell-to-cell transmission of virus (Rasmussen *et al.*, 1991), a finding which is consistent with the role in membrane fusion suggested for the related herpes simplex virus gH protein. Based on the similarities to the herpes simplex virus, CMV gH is predicted to be essential for CMV replication.

1.1.3 Virus entry into host cells

CMV infects cells by sequential processes involving low affinity attachment, higher affinity attachment, fusion with the cell membrane and penetration of the capsid (Compton *et al.*, 1992; Compton *et al.*, 1993). Attachment proceeds rapidly and efficiently in both permissive and non-permissive cells, suggesting that the receptor(s)for CMV are widely distributed on many cell types (Albrecht *et al.*, 1976; Einhorn *et al.*, 1982; LaFemina and Hayward, 1983; LaFemina and Hayward, 1986; Rice *et al.*, 1984; Rosenthal *et al.*, 1981). The efficiency of penetration depends on the presence of particular receptors (Nowlin *et al.*, 1991). It has been suggested that cell surface proteins in the 30 and/or 92 kd size range may be involved as such CMV receptor(s) (Adlish *et al.*, 1990; Keay *et al.*, 1989; Taylor and Cooper, 1990). As in the case of other herpesviruses, heparin blocks virus attachment, and cell surface heparin sulphate may play a role in the initial interaction

between the virus and the host cell (Kimpton *et al.*, 1989; Neyts *et al.*, 1992). Multiple viral envelope glycoproteins are capable of binding to heparin (Compton et al., 1993), and therefore may play roles in attachment and penetration, but glycoproteins gB and gCII are the best candidates (Kari and Gehrz, 1992; Kari and Gehrz, 1993). Attachment to the cell surface is followed by virus penetration, and it has been demonstrated that CMV penetrates cells within 5 minutes of exposure to the virus (Smith and Harven . 1974). The virus entry is pH independent and occurs by direct fusion of the viral envelope with the plasma membrane (Compton et al., 1992), leading to the release of the nucleocapsid into the cytoplasm. After entry, viral nucleocapsids make their way to nuclear pores and deliver viral DNA to the nucleus leading to the initiation of a replicative cycle (Michelson *et al.*, 1977).

1.1.4 The viral replication cycle

Productive replication follows the co-ordinate expression of ordered sets of viral genes. Gene expression may be divided into sequentially expressed stages, namely immediate-early (IE or α), early (E or β) and late (L or γ) stages, based on the time of synthesis after infection. Immediate early gene expression occurs within the first hour following infection. These immediate early genes are transcribed by the host polymerase in the absence of protein synthesis (Mach *et al.*, 1989; Malone *et al.*, 1990; Stenberg and Stinski, 1985). Immediate early gene products are synthesised throughout the replication cycle of the virus. They are non-structural viral proteins, which accumulate in the nucleus and control the switch to early gene expression. The most abundantly expressed immediate early region has two genes which generate the major IE1 and IE2 proteins. Such proteins play an important role in regulating both cellular (Hagemeier *et al.*, 1992), and viral gene expression (Malone et al., 1990).

Early gene expression can be divided into two classes, early transcription (β_1), which begins at 4-8 h post infection, and is not affected by inhibitors of DNA replication and delayed early transcription (β_2), which is first detectable from 8 to 24 h post infection. CMV E genes are transactivated by immediate early gene products, and their expression begins within 2 h of infection. Transcription of the E genes requires *de novo* protein synthesis but not viral DNA replication (Wathen and Stinski, 1982). Early viral gene products are intimately involved in viral DNA replication, for example the CMV-specific DNA polymerase (UL54) and DNA binding protein, p52 (UL44), which interact to form a stable complex (Mach et al., 1989).

Late gene expression is transactivated by both immediate early and early gene products, and occurs in parallel with virus DNA replication. Late CMV mRNA transcripts are first detected at 12-36 h post infection, but there is a temporal lag before proteins are detected. The majority of the late gene products encode structural proteins, such as the viral envelope, capsid and tegument proteins (Mach et al., 1989). The switch from early to late gene expression during CMV replication occurs between 24 and 36 h post-infection (Mocarski, 1988; Stinski *et al.*, 1983). Progeny virions accumulate by 48 h post infection and reach maximal levels at 72 to 96 h.

1.1.5 Cell tropism

CMV exhibits a highly restricted host range in cell culture. The restricted host cell range of CMV involves a blockage of viral gene expression which takes place after penetration (DeMarchi, 1983; Michelson *et al.*, 1979; Nelson *et al.*, 1987; Walker and Hudson, 1987; Wathen *et al.*, 1981). Cultured human skin fibroblasts and embryonic lung fibroblasts have been most commonly used to propagate laboratory strains of CMV, as well as clinical CMV isolates, to high titres. Besides skin and lung fibroblasts, permissive infection by CMV has also been demonstrated in bone marrow fibroblasts (Apperley *et al.*, 1989;

Torok-Storb *et al.*, 1993). Other mesenchymal cells such as vascular smooth muscle cells and endothelial cells are also susceptible to CMV infection in culture. After infection with the CMV laboratory strain AD169, human arterial smooth muscle cells express immediate early antigen, develop a cytopathogenic effect and release infectious virus (Hosenpud *et al.*, 1991; Tumilowicz, 1990). Cultured endothelial cells have been permissively infected by CMV laboratory strains and by recent clinical isolates of CMV. While in cultured brain capillary cells the laboratory CMV strain AD169 had been propagated to considerably high titres (Lathey *et al.*, 1990), a number of studies revealed only limited infection of human umbilical or human aorta endothelial cells by laboratory strains of CMV (Hosenpud et al., 1991; Waldman *et al.*, 1991; Wu *et al.*, 1994).

Since it was recognised that CMV transmission by blood transfusion can be prevented by the depletion of the white blood cells, human leukocytes have been a source for studies on CMV infection. In the 1980's various researchers had reported immediate early antigen expression and early antigen expression in lymphocytes (Einhorn and Ost, 1984; Rice et al., 1984). While monocytes appear to be abortively infected by CMV *in vitro*, they apparently become permissive after differentiation into macrophages (Ibanez *et al.*, 1991; Lathey and Spector, 1991). Cells of the monocyte /macrophage lineage therefore might well be involved in CMV latency and in reactivation and dissemination.

There are some reports of permissive infection *in vitro* of retinal pigment epithelial cells, renal tubular epithelium, and renal glomerular epithelium (Heieren *et al.*, 1988). In addition there are reports on abortive infection of an epithelial cell line which becomes permissive after treatment with sodium butyrate (Tanaka *et al.*, 1991).

1.1.6 CMV strain differences

The majority of clinical isolates of CMV exhibit substantial heterogeneity by restriction endonuclease analysis (Chandler and McDougall, 1986; Huang *et al.*, 1980). Studies in

CMV vaccine development have suggested that this genetic variability confers differences in the pathogenesis of CMV infection. Infection by the cell culture-adapted CMV strain Towne, caused only local reactions at the injection site both in seropositive and seronegative volunteers. In contrast, the low-passage isolate Toledo caused mild self-limited systemic disease in 3 of 5 seropositive recipients. The CMV strain Towne, although able to replicate in the host, seemed to be restricted to the site of application, whereas the isolate Toledo seemed to disseminate hematogenously (Sinzger and Jahn, 1996). Studies on the endothelial cell pathogenicity of clinical isolates of CMV versus laboratory strains indicated that differences in cell tropism might determine the virulence of CMV strains. As discussed above, growth differences between various CMV isolates in endothelial cell culture had been described by several investigators (Ho *et al.*, 1984; Waldman et al., 1991). These studies demonstrated that the endothelial cell cytopathogenicity of a recent clinical isolate was lost during 20 passages in fibroblasts, while the same isolate kept its endotheliotropism when propagated in endothelial cells. A recent publication described dramatic sequence variations between CMV isolates and laboratory strains of the virus (Cha *et al.*, 1996). A region of more than 13 kbp was found in recent isolates as well as the low passages strain Toledo, which was absent in the high-passage laboratory strains AD169 and Towne. This region contains 22 previously unknown open reading frames, mainly putative glycoproteins. Possibly the study of glycoprotein expression in various CMV strains might provide a clue to variation in cell tropism. In this context it is noteworthy that genomic variations in the gB gene of clinical isolates have been correlated with the outcome of infection in the respective patients (Fries *et al.*, 1994).

1.2 The host immune response to viral infection

The immune system has evolved many mechanisms to protect the host against a wide range of bacteria, parasites, fungi, and viruses, to which it may be exposed. The immune response to foreign antigens can be divided in two broad categories known as innate immunity and acquired immunity. Innate immunity refers to defence systems already in place in the organism, such as complement, interferon, phagocytic cells (neutrophils and macrophages), and natural killer cells. An innate immune response can be activated rapidly (within a few hours to a few days) following exposure to a pathogen, and its response is characteristically broadly specific Acquired immunity is mediated mainly by B and T lymphocytes, and provides the host with humoral and cellular mechanisms capable of recognising and eliminating a large range of unique microbial pathogens in an antigen-specific fashion. Following exposure to the pathogen, specific memory is imprinted on the acquired immune system, leading to rapid responses to a secondary infection with that pathogen. The innate and acquired immune responses are interconnected through cytokine networks.

1.2.1 Natural Killer cell responses

Natural killer (NK) cells are bone marrow derived lymphocytes with a large granular morphology which play an important role in the lysis of virus infected cells and also some tumour cells (Trincheri 1989). NK cells are developmentally related to T cells but differ by the absence of CD3 and the T cell receptor. They express the adhesion molecules CD2 and LFA-1, and are characterised by the expression of CD16 (an Fcγ receptor), and/or CD56. Whereas activation of conventional T cells requires target expression of major histocompatibility complex (MHC) antigens (Described in section 1.2.7), NK cells are able to efficiently lyse target cells without a need for such MHC antigens. The mechanisms and molecular interactions between NK cells and their targets are still poorly understood.

However, in an individual lacking NK cells, recurrent viral infections have been shown to be a major problem. NK cells may kill target cells, and inhibit viral replication in a number of ways, working in concert with other arms of the innate and acquired immune system. The NK response occurs rapidly, and takes place early in viral infection, and importantly can occur before replication of the virus has taken place. NK cells contain cytotoxic granules, including perforin, and can also secrete cytokines, and mediate antibody dependant cellular cytotoxicity, due to their expression of Fcγ receptors (Moretta *et al.*, 1994).

1.2.2 Interferons

Interferons are a family of proteins produced during viral infection. They can be classified as type 1 or type 2. Type 1 interferons, known as α and β interferons, are produced by virally infected cells. Type 2 interferon, also known as interferon-γ, is produced by activated T cells and NK cells in response to antigenic stimulation. Interferon α and β are released from infected cells within 24 hours of infection, and bind to receptors on surrounding uninfected cells. An antiviral state is induced in these cells, via the generation of a protein kinase which inhibits protein synthesis, and a 2'.5'-oligadenylate synthetase, which activates endonucleases and leads to degradation of viral mRNA. Interferons can also induce an upregulation of cell surface expression of MHC molecules in several cell types (Pestka *et al.*, 1987). This induction of MHC molecules by interferon may enhance the cellular immune response to virus by increasing the probability of recognition of viral antigens by T cells.

1.2.3 Macrophages

Tissue macrophages develop from circulating blood monocytes, and are concentrated in the lung, liver, lymph nodes and spleen. They also function by the phagocytosis and digestion of foreign material and contain lytic enzymes which are able to digest this material. Phagocytosis can, in certain cases, be mediated by complement. Macrophages, produce a variety of inflammatory mediators with antiviral effects. They also possess Fc receptors and can mediate antibody-dependent cellular cytotoxicity. Macrophages are activated by interferon-γ, resulting in increased phagocytosis, microbiocidal activity, cytotoxicity and the release of cytokines such as Interleukin-1. However, cytokine activated macrophages are non-specific in their target cell recognition, and can potentially cause tissue damage in areas of inflammation (Kurtz and Fujinami, 1993). They play a central role in cell mediated immunity to viruses via their capacity to act as professional antigen presenting cells in the tissues, and their ability to control the activation of specific T cell clones.

1.2.4 Humoral immunity

B cells are responsible for the antigen-specific humoral response which involves the production of antigen-specific receptors called antibodies or immunoglobulins. Infection by many types of viruses induces the production of virus-specific protective antibodies. Antibody has three important roles during viral infection: virus neutralisation, antibody-dependent cellular cytotoxicity, and complement mediated lysis of virus and virally infected cells.

Antibodies neutralise virus by interfering at different stages of the viral infection. Most commonly, antibodies block the attachment of the virus to specific cellular receptors by binding to antigenic determinants on the virus capsid or envelope. Many viral infections are known to produce neutralising antibodies. Neutralising antibodies produced during

rabies virus, influenza virus and rhinovirus infections play an important role in the host's recovery from disease, as well as in the protection of the host from re-infection.

1.2.5 T cell mediated immunity

Like B lymphocytes, T lymphocytes employ highly effective diversity-generating machinery to ensure that their receptors, the T cell receptor (TCR), have the capacity to recognise a vast number of antigens. Cellular immune responses to viruses and their interactions with humoral immune responses are complex. T lymphocytes can generate highly specific immune responses during viral infection and play an important role in the clearance of viruses. Furthermore, they are able to provide long lasting immunity to re-infection, by the generation of memory cells which respond rapidly to a second exposure to viral antigens.

The T lymphocyte lineage is produced in the bone marrow and then undergoes a process of education in the thymus. The majority of mature T cells can be divided on the basis of cell surface markers into two broad subsets called CD8 and CD4, based on their cell surface phenotype.

$CD4^+$ T lymphocytes are often mediators of help for both humoral immune responses and cytotoxic responses, by the release of cytokines. Development of the $CD4^+$ T helper (Th) cells releasing the appropriate cytokines is critical for the eradication of infectious organisms (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Sher and Coffman, 1992). Th1 cells produce interferon-γ and Interleukin-12, and play a critical role in directing cell mediated immune responses which are important for the clearance of viruses and other intracellular pathogens. Th2 cells which produce Interleukin-4, 5, 10 and 13 have been associated with allergy, and are important for humoral responses (Mosmann et al., 1986; Mosmann and Coffman, 1989; Parrochi *et al.*, 1991; Romagniani, 1991; Sher

and Coffman, 1992; Yssel *et al.*, 1992). $CD4^+$ Th cells can provide the help to $CD8^+$ T cells required for the generation of cytotoxic responses against virally infected cells. Furthermore, $CD4^+$ T cells are also capable of a cytolytic response, although this function is not considered to play a major role in the defence against most viral infections.

$CD8^+$ T cells are generally mediators of cytotoxic T lymphocyte (CTL) responses. CTLs produce cytokines such as interferon-γ, which induce inflammation, further stimulate antigen presentation, and may also interrupt a viral replication cycle or help to reduce the spread of virus to adjacent cells, particularly when the virus itself may prove to be a weak inducer of interferon-γ or β (York and Rock, 1996). However, the cytotoxic function of $CD8^+$ T cells is considered to be their major antiviral activity *in vivo*. Furthermore, CTLs are considered to be the main antiviral effector cells, and their mechanism of action and their roles will be detailed in the following sections.

1.2.6 The T cell receptor

The TCR consists of an immunoglobulin -like structure which is encoded by genes with a similar genetic organisation to that of the B cell receptor, with variable (V), diversity (D) and joining (J) regions (Davis, 1990). Assembly of the TCR takes place by rearrangement of the appropriate DNA segments, making a functional gene (Davis, 1990). The TCR is composed of two different chains α and β or γ and δ, which are united into heterodimers, of either αβ or γδ chains. The chains are synthesised in the cytoplasm independently of each other. They associate with their partner (α with β and γ with δ chains) to migrate to the cell surface. They must associate with another complex molecule, CD3, which is intimately involved in signal transduction during activation of the cell. At the cell surface, the TCR molecules are co-expressed with CD4 or CD8 molecules which recognise invariant parts of the MHC antigens (Salter *et al.*, 1990). T cells only recognise foreign antigen via the TCR, when it is associated with host or "self" encoded proteins known as

major histocompatibility complex (MHC) antigens, which are present on the surface of the antigen presenting cell.

1.2.7 The major histocompatibility complex

Human leukocyte antigens (HLA) are glycoproteins encoded by genes within the MHC, which complex with peptide within the cell, before being transported to the cell surface. On the surface the HLA-peptide complex is able to interact with T cells. During T cell development those cells which are capable of reacting with peptides derived from "self proteins complexed with self-HLA are normally deleted. During infection however, new pathogen derived peptides are complexed with self-HLA, presenting a novel structure on the cell surface. As the T cells have not been tolerised to such structures, and if the immunological environment is suitable, a T cell response will follow. This phenomenon is known as "MHC-restriction" and forms a central component of antigen recognition (Zinkernagel and Doherty, 1974; Zinkernagel and Doherty, 1975).

The major characteristics of the HLA system which makes it suitable for sampling the antigenic environment are 1) its extreme level of polymorphism, 2) that it is a multi-locus system, with 2 antigens commonly expressed at each locus, and 3) there are two classes of HLA molecule with different modes of assembly, adapted to perform distinct roles within the acquired immune system.

1.2.8 Genetics and polymorphism of the MHC

The MHC is contained on the short arm of chromosome 6. This complex is divided into three major groups. The HLA class I region contains genes encoding the so called "classical" class I antigens, HLA-A, -B and -C and the "non-classical" classical antigens, HLA-E, -F and -G. The class II region encodes the HLA class II molecules HLA-DP, -

DQ and -DR, which are structurally and functionally related to the class I locus products. This region also encodes the transporters associated with processing (TAP) molecules and some proteasome proteins. The class III region lying between the class I and class II clusters, is densely packed with genes coding for a variety of functions, including some components of the complement system (C2, C4, Bf), heat shock proteins (Hsp70), and tumour necrosis factor (TNF).

The MHC class I and class II loci are the most polymorphic genetic system known in the human. This variation in MHC class I and class II genes, and therefore proteins, constitutes the basis of tissue rejection in transplantation. Due to their close proximity on chromosome 6, alleles at different class I and class II loci tend to occur in a non-random fashion within a population and are inherited en bloc, as a haplotype. The number of alleles is such that most unrelated individuals express significantly different phenotypes. The total number of HLA alleles is unknown, as their identification and structural characterisation is still in progress. However, to date, the nucleotide sequences of 399 HLA class I alleles (109 HLA-A, 222 HLA-B and 68 HLA-C, 5 HLA-E, 14 HLA-G) and 401 HLA class II alleles have been identified (Marsh, 1998 personal communication). This clearly indicates that traditional serological typing greatly underestimates the number of antigens which exist, and many serologically indistinguishable variants, or subtypes, are known (Bodmer *et al.*, 1997). The frequency of each allele in a population varies according to the ethnic group. The HLA-A2 antigen is common in many populations world-wide; for example the frequency of HLA-A2 in the British population is approximately 45%, of which over 90% are of the HLA-A*0201 subtype, whereas in the Chinese population the representative HLA-A2 variant is HLA-A*0207, an allele not found in the British population.

1.2.9 The structure of the HLA class I and class II molecules

Class I molecules consist of a membrane-inserted heavy chain of approximately 42 kd and a non-covalently attached light chain of 12 kd, known as β_2-microglobulin (β_2m). The crystallographic structure illustrates that the class I molecules consists of a peptide binding super-domain supported by two immunoglobulin like domains (Bjorkman *et al.*, 1987a; Bjorkman *et al.*, 1987b). The extracellular portion of the MHC class I heavy chain consists of three domains (α1, α2, α3). The α1 and α2 domains of the heavy chain fold to form a platform composed of an eight-stranded β pleated sheet, topped by two antiparallel α helices which are closed at both ends, creating a groove which is the peptide binding site. In turn, the immunoglobulin-like domains of the α3 and noncovalently associated β_2m, support the heavy chain α1 and α2 domains. Heavy chain polymorphism directly changes the chemical nature of the peptide-binding site of the MHC molecule and therefore influences the selection of antigenic peptides which can be bound. The side chains of the amino acid residues in the class I molecule create a series of pockets within the groove which can constrain or define the possibilities for binding a peptide. The initial analysis of these pockets in the HLA-A*0201 molecule has identified six distinct pockets, designated A-F (Saper *et al.*, 1991). Two of these (A and F) interact with the amino and carboxyl termini of bound peptides, while the remaining B-E pockets interact with the peptide side-chains. The side-chains which are most critical for the high affinity binding of the peptides have been termed anchor residues (Falk *et al.*, 1991), and the structures of MHC class I molecules have shown how these anchor residues interact with defined pockets on the MHC class I molecules. The pockets and their relative importance in peptide binding affinity can vary among HLA alleles (Matsui, 1994; Rohren *et al.*, 1993).

Although MHC class I and II molecules have little sequence homology, these proteins have similar three-dimensional structures (Brown *et al.*, 1993; Brown *et al.*, 1988). MHC class II molecules are also heterodimers, but consisting of two chains α and β of similar size

(about 30 kd), both of which are inserted into the membrane . The N-terminal domains of the α and β chains (α1 and β1) of class II molecules both contribute to the formation of the peptide binding site, generating a platform of β sheets supporting a peptide binding groove similar to the structure formed by class I. Despite these similarities, important differences in the structures of class I and class II molecules can be found in the peptide binding groove. The first part of the α-helix found in the α1 domain of class I molecules appears to be deleted in the α1 domain of class II molecules. This creates the effect of a more open or extended binding groove than in the case of HLA class I molecules.

1.2.10 The influence of HLA class I and class II molecules on T cell immunity

Despite the structural similarity of the HLA class I and class II molecules, they play different roles in the immune response. The HLA class I molecules are involved in presenting peptides derived from endogenous proteins (either viral or derived from other intracellular proteins) which are processed through the cytosolic pathway of antigen presentation. The function of the HLA class I molecules is to present peptides to $CD8^+$ cytotoxic T lymphocytes. In contrast, the HLA class II molecules bind peptides generated via the exogenous pathway (endosomal/lysosomal), which are associated with defences against bacterial and multicellular organisms. Most peptides presented in the context of HLA class II molecules are derived from antigens internalised by antigen presenting cells. HLA class II molecules present peptides to $CD4^+$ helper T lymphocytes. This system of presentation has evolved in this manner so that peptides from different antigenic pools are accessible for functionally different purposes.

1.2.11 Antigen Processing by HLA class I and class II molecules

Class I

Endogenous proteins which are presented by HLA class I molecules include cytosolic self and viral proteins or proteins originating from intracellular pathogenic bacteria that enter the cytosol (Bennik *et al.*, 1982; Townsend, 1986). However, proteins targeted into the ER by signal sequences (Siliciano and Zoloski, 1995), and peptides derived from nuclear (Townsend, 1984) and mitochondrial proteins (Udaka *et al.*, 1993), can also be presented on MHC class I molecules. The degradation of many intracellular proteins is initiated by their modification with the small polypeptide ubiquitin (Ciechanover, 1994). The polyubiquitin chain serves as a molecular tag which marks a protein for rapid degradation by the proteasome (Michalek *et al.*, 1993). The proteasome is a 20S-26S high molecular weight multicatalytic cytosolic protease complex, which has been proposed to be the major site of proteases involved in the processing of cytosolic and nuclear antigens (Gaczynska *et al.*, 1993; Rock *et al.*, 1994). The access to the inner compartment of the proteasome is guarded by a narrow channel, and entry to this central cavity is only afforded to completely unfolded proteins (Wenzel and Baumeister, 1995). The unfolded polypeptide chain is stored in the antechamber and slowly fed through the second narrow constriction into a central chamber which contains the catalytic sites. In the central chamber, the polypeptide is cut into fragments generally four to nine residues long, which are then released through the third and fourth constrictions by as yet unknown mechanisms. The fine specificity of endopeptidase activity is influenced by two proteasome subunits, LMP2 and LMP7, which are encoded in the MHC region (Goldberg and Rock, 1992; Martinez and Monaco, 1991; Monaco, 1992) and their expression is regulated by interferon-γ. However, the exact nature of the influence of LMP2 and 7 on endopeptidase specificity is controversial.

Peptides produced in the cytosol must pass into the lumen of the ER before they can bind to the MHC class I molecules which are assembled in this organelle. Such peptide fragments are transported to the endoplasmic reticulum (ER) through the action of the TAP transporter complex. This complex is encoded by two genes, TAP-1 and TAP-2, located in the MHC region (Spies *et al.*, 1992). Peptide binding to TAP is ATP-independent, although ATP hydrolysis is required for peptide translocation (Lehner and Cresswell, 1996). TAP only transports peptides longer than seven residues, and its efficiency drops off dramatically with peptides longer than about twelve amino aids, but there is no clear upper limit to peptide size (Androlewicz *et al.*, 1993; Momburg *et al.*, 1994; Shepherd *et al.*, 1993): There is also some specificity for the C-terminal residue of the peptide. Some peptides which are expressed in the cytosol of cells lacking TAP transporters are presented by MHC class I (Henderson *et al.*, 1992; Zweerink *et al.*, 1993). This suggests that a TAP-independent mechanism for transferring peptides into the ER exists. However, since there are few class I-presented peptides in TAP deficient cells, mainly signal sequences, it is likely to be a minor pathway.

The assembly of a functional HLA class I complex occurs in the ER. HLA class I heavy chains and β_2m are co-translationally inserted into the ER membrane, and peptides derived from protein degradation are transported into the ER, then they reach the class I heavy chains and bind into the groove. Until all three components of the complex are present (heavy chain, peptide and β_2m), the heavy chain and peptide are retained in the ER (Neefjes and Ploegh, 1988). Although the assembly sequence of class I heavy chain, β_2m and peptide is not clear, it is known that the ER resident chaperone, calnexin is bound to assembled complexes of heavy chain and β_2m, and thus retains empty class I molecules in the ER (Neefjes and Ploegh, 1988; Williams and Watts, 1995). Calnexin dissociates from the heavy chain when the heavy chain/β_2m and peptide complex forms (Nossner and Parham, 1995; Sugita and Brenner, 1994). After assembly, MHC class I complexes transit through the Golgi where N-linked carbohydrates are modified. They are then rapidly

transported by the default exocytic pathway to the plasma membrane, where they display their bound peptides. Not all newly synthesised HLA class I heavy chains and β_2m assemble into stable complexes, and excess free β_2m is secreted from the cell and on to the cell surface (Neefjes and Ploegh, 1988). Most of the empty HLA class I molecules (ie. those lacking peptides) dissociate rapidly and are retained intracellularly (Benjamin *et al.*, 1991). Thus, it is postulated that there are actually very few MHC class I molecules on the cell surface that are empty (Benjamin et al., 1991; Schumacher *et al.*, 1990).

A direct requirement for the association of class I molecules with peptide to obtain stable cell surface expression was first established for the mutant mouse lymphoma RMA-S cell line (Karre *et al.*, 1986) and subsequently for the human mutant T2 cell line (Cerundulo *et al.*, 1990). These cell lines synthesise normal levels of class I heavy and light chain and β_2m, but are deficient in the assembly and transport of class I molecules to the plasma membrane, and seem to retain unfolded heavy chain molecules in the ER (Townsend *et al.*, 1989). Although both cell lines fail to present endogenous peptides to class I restricted CTL, they could be sensitised for CTL killing by the incubation with exogenous synthetic peptides. The T2 cell line and its parental line L721.174, have a large deletion in the class II region of the MHC which includes a region encoding the transporter proteins TAP1 and TAP2 (Spies et al., 1992).

Class II

The HLA class II molecules primarily bind peptides generated by degradation of proteins in the conventional endocytic pathway, which internalises molecules from the cell surface by pinocytosis or receptor mediated endocytosis via clathrin-coated vesicles. These processes target the HLA class II molecules to early endosomes, late endosomes, and ultimately to lysosomes (Brodsky *et al.*, 1996). Following biosynthesis, HLA class II α and β chains assemble into the ER, where they are bound by a chaperone-like molecule, the invariant chain (Cresswell, 1994). This molecule has two functions; to direct the $\alpha\beta$-heterodimer to

a class II loading compartment and to prevent premature peptide loading to class II molecules. Three class II heterodimers form a nonameric complex with the trimeric invariant chain that is targeted to the endocytic pathway (Cresswell, 1994). This endocytic system of vesicles, characterised by a high concentration of endosomal and lysosomal proteases known as cathepsins, is where peptide loading takes place (Sanderson *et al.*, 1994). The proteases cathepsin B and D are involved in the generation of the HLA class II binding peptides (Brodsky, 1991). Class II molecules accumulate in class II-rich pre-lysosomal compartments, before they reach this point, most of the invariant chain is already removed by proteolysis (also dependent on cathepsin B and D-like activities). The HLA-DM molecules that also accumulate in class II-rich compartments (Sanderson et al., 1994), have been shown to catalyse the low pH-dependent removal from the class II peptide binding site of a peptide called class II-associated invariant chain peptide (CLIP), which is derived from cleaved invariant chain (Denzin and Cresswell, 1995; Sloan *et al.*, 1995). CLIP maintains the class II molecules in a relatively unstable, peptide-receptive conformation. Removal of CLIP by DM enhances class II binding of other peptides generated in the endocytic pathway, which can induce class II molecules to fold into a highly stable conformation. The peptides loaded onto class II molecules can be derived not only from endocytosed protein but also from protein endogenous to the cells, particularly membrane bound proteins which have the possibility of co-localising in the class II loading compartment. Finally, the peptide-loaded αβ-heterodimers are translocated to the cell surface, where they display the peptide to T lymphocytes (Krensky, 1997).

As suggested above, although there seems to be a strict separation between the processing pathways for class I and class II, there is strong evidence of considerable cross-over between the two pathways. Peptides derived from cytosolic proteins, for example, can be loaded onto class II molecules (Pinne 1994). Further, peptides derived from phagocytosed proteins can be loaded onto class I molecules, especially if the phagocytosed protein is aggregated (Rock *et al.*, 1993). Such alterations of processing pathways deserve interest because they could be exploited for new strategies of immune intervention.

1.2.12 HLA associated peptides

Since the structures of the HLA class I and class II molecules differ markedly in the peptide binding groove, the characteristics of the peptides bound to these respective molecules also differ. The HLA class I molecule peptide binding groove is closed at both ends, and is generally long enough to accommodate peptides of only 8 or 9 amino acid residues in an extended conformation (Madden *et al.*, 1991). Longer peptides can also fit by bulging partly out of the groove or by zigzagging within the cleft (Madden *et al.*, 1993). Peptide residues with side-chains pointing out of the binding site, and MHC residues along the top of the α1 and α2 domain helices, are thought to form a direct set of interactions with TCR (Bjorkman et al., 1987b). Peptides bound to MHC class I molecules exhibit allele-specific motifs, that is particular amino acids that are preferred in specific sequence positions of the peptide. Peptides bound by MHC class II molecules are longer (13-15 amino acids) and more heterogeneous (Rammensee, 1995; Sinigaglia and Hammer, 1994). They possess a core sequence of about nine amino acids, usually with three anchor residues, and their ends extend beyond the peptide-binding groove (Madden, 1995; Sinigaglia and Hammer, 1994).

Much work has involved the identification of binding motifs for different MHC class I molecules, but the prediction of MHC class II motifs remain under investigation (Nelson *et al.*, 1997). Although the identification of anchor residues is vital, the contribution of the remaining residues present in the peptide may play an important in the determining the affinity of the peptide and T cell interactions as demonstrated for HLA class I molecules (Barber and Parham, 1994).

The understanding of the rules for peptide selection by MHC molecules, has greatly influenced the prediction and determination of the peptides recognised by CTLs, known as "CTL epitopes". These rules have been defined by the following strategies: the analysis of naturally processed peptide pools eluted from MHC molecules (Engelhard, 1994; Joyce and Nathenson, 1994), peptide binding studies (Drijfhout *et al.*, 1995; Kubo *et al.*, 1994),

the analysis of the effect of mutations in sequences of known T cell epitopes on peptide binding to MHC molecules and on T cell responses (Ruppert *et al.*, 1993); and the crystal structure analysis of defined peptide-MHC complexes (Madden, 1995; Madden et al., 1993).

1.2.13 CTL activation and costimulatory molecules

When a CTL comes into contact with an antigen presenting cell which expresses MHC class I molecules presenting endogenously processed peptide antigens, there are several recognition events which must be orchestrated in order to trigger an antigen specific CTL response. These events include the activation of the CTL by the transduction of signals through the TCR, and with this activation, the setting in motion of both secretory and non secretory cytotoxic machinery of the CTL (Berke, 1994).

The activation of mature CD8+ T cells is triggered by the interaction between the α and β chains of the TCR and the antigenic peptide bound to MHC class I molecule. This interaction is reinforced by simultaneous binding of CD8 molecules to the non polymorphic region of MHC class I, which results in enhanced binding and co-signalling through protein kinases (Lck) within the cell. Several lines of evidence suggest that for these interactions to result in functional T cell activation, the TCR and CD8 molecules should interact with the same MHC class I molecule (Anel *et al.*, 1997). The earliest biochemical event during T cell activation is the phosphorylation of tyrosine residues within the immune receptor tyrosine activation motifs of the TCR and CD3. Successful activation of T cells via their TCR results in phosphorylation of the TCR-CD3 complex subunits, activation of protein tyrosine kinases (Lck and Fyn of the Src family), protein phosphorylation, activation of the phosphatidylinositol pathway, increased intracellular CA^{+2} levels and transcriptional activation of several genes, most notably IL-2 (Weiss and Littman, 1994). The above cascade may be effected by minor changes in the peptide bound to the MHC

molecule, which may generate partial signals (Sloan-Lancaster *et al.*, 1993; Sloan-Lancaster *et al.*, 1994). Also some peptides may block proliferation altogether, and induce a state of long-lasting unresponsiveness known as anergy (Sloan-Lancaster et al., 1994).

Activation of naive CTL via the TCR alone is not sufficient for the initiation of the immune response. Co-stimulatory signals are provided by T cell surface molecules CD28 and CTLA-4 which bind to CD80 (B7.1) or CD86 (B7.2) on the surface of an antigen presenting cell (Azuma *et al.*, 1993). Following a TCR-mediated signal, ligation of CD28 results in the upregulation of the Interleukin-2 receptor, increased IL-2 production, cytokine secretion, and ultimately proliferation by TCR-stimulated T cells (Allison, 1994; Chambers *et al.*, 1996; Linsley *et al.*, 1991). Other interactions include the association between the CD2 molecule on the CTL with its ligand LFA-3 on the target cell (Krensky *et al.*, 1984; Shaw *et al.*, 1986), and the interaction of the leukocyte adhesion molecule LFA-1 on the CTL with its ligand ICAM-1 (intracellular adhesion molecule-1) on the target cell surface.

In the presence of such co-stimulatory signals, antigenic stimulation can lead to a productive immune response, characterised by proliferation, differentiation, and clonal expansion of the T cell, accompanied by the induction of effector function, that is target lysis in the case of CTLs. However, in the absence of appropriate costimulation, antigenic stimulation can also result in anergy (Jenkins *et al.*, 1987; Mueller *et al.*, 1989).

The affinity of the TCR for the MHC molecule is low, and the number of MHC molecules expressing the appropriate peptide on the target cell is minimal. Several models have been proposed by which efficient recognition can take place via this apparently suboptimal interaction. The kinetic proof reading hypothesis proposes that the complex series of signal transduction events which takes place during T cell activation, provides a temporal lag, during which lower affinity MHC molecules disengage, leading to incomplete activation, whereas higher affinity MHC molecules provide a full activation signal

(McKeithen, 1995). The serial triggering model proposes that a single MHC complex engages multiple TCR molecules in a serial manner, the peptide-MHC complex then detaches and forms a "contact cap" which transduces signals when a threshold number of TCRs are assembled (Valitutti, 1995).

1.2.14 Cell mediated cytotoxicity

Lymphocyte mediated cytotoxicity by CTLs of infected cells or tumour cells is a complex, multistep mechanism, involving the binding of the lymphocyte to its target cell, the delivery of the lethal hit, pre-lytic fragmentation of the target cell DNA (apoptosis), lysis of the target cell and recycling of the cytotoxic T cell to attack another cell. Two independent mechanisms account for cell mediated cytotoxicity: A major pathway is mediated by the secretion by the cytotoxic cell of pore forming proteins such as perforin. Perforin expression is mainly confined to $CD8^+$ T cells, NK cells and $\gamma\delta$ T cells, however, it has also been reported in a $CD4^+$ T cell clone. Upon interaction with the target cell, the cytotoxic effector cell releases the content of its cytoplasmic granules, including perforin, in a directed manner into the intracellular space between the lymphocyte and the target cell (Berke, 1994; Burrows *et al.*, 1993). There, perforin undergoes a Ca^{2+} induced conformational change, integrates into the target cell membrane, and forms pores, which are similar to the pores formed by the membrane attack complex of the complement system. The permeabilisation of the target cell membrane finally leads to the death of the target cell. The serine proteases, granzymes, which are co-secreted with perforin, are thought to penetrate the target cell through these pores and to induce apoptosis (Kagi *et al.*, 1994). The effector cells recycle, and are able to lyse additional target cells.

The second, non secretory lytic mechanism, involves a surface ligand (Fas-ligand), expressed on the CTL membrane, which cross-links with the death receptor APO-1/Fas (CD95) on the surface of the target cell (Kagi, 1996). APO-1/Fas is a transmembrane

glycoprotein widely expressed in lymphoid and non-lymphoid tissues. Signalling occurs by a cross-linking between the Fas receptor and Fas ligand. This cross-linking triggers a cascade of intracellular protein-protein interactions and proteolytic activities which culminate in apoptosis of the target cell.

1.2.15 Mechanisms of evasion of CTL recognition by viruses

The elaborate mechanisms employed by viruses to evade presentation on MHC class I antigens emphasises the importance of class I restricted CTL in defence against viral infections. A number of recent studies have identified some of the viral genes responsible, and the mechanisms used by viruses to downregulate MHC class I molecules. Class I downregulation often involves the attenuated transcription of genes encoding the class I subunits (Wiertz *et al.*, 1996). Furthermore, some viruses produce proteins that can bind and retain newly synthesised class I molecules in the ER. Such is the case of the adenovirus E3 19k protein, and this mechanism may also apply to murine CMV-infected cells, where retention of class I molecules in the ER has been observed (Del Val *et al.*, 1992). Another mechanism involves the inactivation of TAP peptide transporters, as with the herpes simplex virus US12 gene product, called ICP47, which binds to TAP, preventing delivery of cytosolic peptides to the lumen of the ER, and thus generating an unstable assembly of class I molecules and thereby inhibiting their expression at the cell surface (Fruh *et al.*, 1995; Hill *et al.*, 1995; York *et al.*, 1994).

1.3 Immune responses to CMV

Herpes viruses, represent the prototype of a group of viruses against which the cell mediated immune mechanisms have a protective role. However, other non-specific

mechanisms such as α and β interferon production, NK cells and macrophages, also contribute to host resistance. Together with pre-existing antibodies, these mechanisms determine the early course of herpes virus infections, whereas T cell mediated immune responses determine the later course.

Cytomegalovirus is a typical case of a herpes virus in which the cellular immune response has been demonstrated to play an important role in the control of infection. Under normal conditions the virus and host have reached an equilibrium, where the host's immune system has managed to control the infection. In contrast, infection in immunocompromised individuals is often associated with CMV disease. CMV disease is common in patients who are immunosupressed as a result of immunosuppressive therapy associated with solid organ or bone marrow transplantation, or the effects of HIV infection. Congenital CMV infection also occurs, due to the immature immune system of the foetus. The relative contribution of the various aspects of the immune response in controlling CMV infection are discussed below.

1.3.1 Humoral mediated immunity to CMV

The humoral response to CMV in humans does not prevent infection but seems to make the disease less severe. Also it has been shown that antibodies do not protect against reinfection, particularly in AIDS patients where re-infection with new strains of CMV has been reported. (Spector and Spector, 1982) and renal transplant patients (Grundy *et al.*, 1988b). However, the protective role of the antibody response against CMV has been evident in many types of CMV infection, for example seropositive renal transplant recipients are much less likely than seronegative individuals to present with serious CMV disease (Smiley *et al.*, 1985b); and passively transferred CMV hyperimmune globulin provides protection against CMV-associated interstitial pneumonitis in bone marrow transplant recipients (Huart *et al.*, 1987; Winston *et al.*, 1987). When considering the protective antibody response to CMV, the predominant interest is in neutralising

antibodies. A lack of CMV-specific antibodies has been associated with severe disease in cardiac transplant recipients (Rasmussen *et al.*, 1982); Maternal immunity before conception can reduce the damage caused by foetal infection, and lower the rate of maternal/foetal transmission (Fowler *et al.*, 1992; Stagno *et al.*, 1986). Increased levels of neutralising antibodies slows down the progression of CMV retinitis in AIDS patients (Boppana *et al.*, 1995), and a deficiency in gH-specific antibody in AIDS patients was associated with an increased severity of CMV retinitis (Rasmussen *et al.*, 1994).

These observations, together with an increasing understanding of the viral antibody responses which are important for protective immunity, have provided the basis for the design of vaccines against CMV. The CMV envelope glycoproteins gB and gH are the main candidates for the generation of subunit vaccines, as they elicit a strong neutralising antibody response in natural infection (Gonczol and Plotkin, 1990). This point will be discussed further in later sections.

1.3.2 NK cell mediated immunity to CMV

In vitro, CMV-infected fibroblasts have been shown to be targets for NK cells (Borysiewicz *et al.*, 1985). The most convincing evidence *in vivo* for the role of cellular immune response in protection from CMV disease has been provided by studies of mice infected with murine CMV (MCMV), a virus that is genetically distinct from human CMV, but which causes an infection in mice biologically similar to human CMV infection. Using the severe combined immunodeficiency mouse, which has no acquired immunity due to a defect in the rearrangement of antigen specific receptors, it has been shown that mice were not able to control MCMV replication, and succumbed to disease, and that the depletion of NK cells accelerated the development of lethal disease (Welsh *et al.*, 1991). Immunocompetent mice which were also depleted of NK cells by antibody treatment were more susceptible to MCMV infection, and the adoptive transfer of NK cells induced

resistance to the virus in susceptible mice (Bukowski *et al.*, 1983; Bukowski *et al.*, 1984). In the human there is evidence to support a role for NK cells in defence against CMW infection/disease in transplant recipients (Quinnan and Burns, 1982).

1.3.3 CMV-specific CD4⁺ T cell responses

Several CMV proteins have been identified as target antigens for lymphoproliferative and T helper cell responses during CMV infection. These include the regulatory proteins IE1 (pUL123) (Alp *et al.*, 1991; Davignon *et al.*, 1995) and IE2 (pUL122), the MHC class I homologue (ppUL18), the upper matrix protein pp71 (pUL82) (He 1995) and gB (Liu *et al.*, 1991). Likewise, the matrix protein pp65 (ppUL83) has been shown to induce lymphocyte proliferative responses (Forman *et al.*, 1985). In a comparative analysis of fourteen CMV proteins, the latter represented the dominant antigen recognised by $CD4^+$ T cells (Beninga *et al.*, 1995).

The fine mapping of $CD4^+$ T cell epitopes has been performed for IE1 and gB. Within IE1, six $CD4^+$ T cell epitopes were localised (Alp et al., 1991), four of these epitopes were in gB, and the restricting HLA class II alleles for their presentation have been determined (Liu *et al.*, 1993). Inhibition studies with monoclonal antibodies to HLA class I or class II have revealed a class II restricted response to two pp65 derived peptides. The restriction elements were HLA-DR11 for one peptide and HLA-DR3 for the other (Khattab *et al.*, 1997).

Although the primary role of $CD4^+$ T cells in CMV infection has been suggested to be the augmentation of the $CD8^+$ CTL and B lymphocyte responses to CMV, a more direct role of $CD4^+$ T cells in mediating viral clearance may be due to MHC class II restricted cytolysis of infected cells, or to the direct antiviral effects of the Th1 cytokines released by CMV-activated $CD4^+$ T cells (Davignon *et al.*, 1996; Lindsley *et al.*, 1986; Lucin *et al.*, 1992).

1.3.4 CD8⁺ CMV-specific CTL responses

In the murine model, adoptive transfer of MCMV-specific CD8+ T cells in absence of $CD4^+$ T cells, completely protected immunosupressed mice from lethal primary challenge with MCMV, and mice with established MCMV infection, whilst the $CD4^+$ subset did not confer protection when transferred alone (Reddehase *et al.*, 1988; Reddehase *et al.*, 1987; Reddehase *et al.*, 1985). However, $CD4^+$ T cells appear to exert a compensatory function in situations where the $CD8^+$ CTL immune response is absent (Jonjic *et al.*, 1990; Polic *et al.*, 1995). In BALB/c mice, up to 50% of the MCMV-specific CTL precursors are specific for the IE1 gene product (pp89) (Koszinowski *et al.*, 1986; Koszinowski *et al.*, 1987; Reddehase *et al.*, 1984) and immunisation with this protein alone could protect mice from lethal virus challenge (Jonjic *et al.*, 1988). A single nonameric peptide from this protein was identified as the dominant epitope, conferring protection against lethal MCMV disease when administered as a recombinant vaccine (Del Val *et al.*, 1991).

In the human, the initial evidence for the potential role of $CD8^+$ CTLs in the control of CMV infection, was the observation that in the peripheral blood of normal asymptomatic CMV seropositive individuals, CTLs specific for CMV were present with a relatively high frequency (Borysiewicz *et al.*, 1988b). Further evidence has come from studies in bone marrow transplant recipients.

In man immunosuppressive therapy is known to influence the development of CMV disease. Immunosuppressants which dramatically depress the number of circulating T lymphocytes, such as antithymocyte globulin and OKT3 (anti-CD3 monoclonal antibody), are risk factors for disease (Portela *et al.*, 1995). In one study, during the first 100 days post allogeneic bone marrow transplantation, half of the recipients were persistently deficient in $CD8^+$ cytotoxic T lymphocytes specific for CMV. In another study, interstitial pneumonitis was observed only in patients with undetectable levels of CD8+ CMV-specific

CTL (Reusser *et al.*, 1991). Furthermore, in post BMT patients, the recovery of CD8+ cytotoxic T lymphocytes (CTL) responses to CMV has been correlated with an improved outcome from CMV disease, and also with protection from the subsequent development of CMV disease (Quinnan *et al.*, 1981; Reddehase et al., 1985; Reusser et al., 1991). The development of an efficient $CD8^+$ CTL response in these patients has been suggested to depend upon the presence of adequate $CD4^+$ helper function (Reusser et al., 1991).

1.3.5 Antigen specificity of $CD8^+$ CTL responses to CMV

The importance of $CD8^+$ CTL responses in providing protective immunity in CMV-seropositive individuals has fostered studies to elucidate the specificity of this response for selected CMV antigens, with the objective of developing methods to augment CTL responses in immunocompromised hosts (McLauglin-Taylor et al., 1994). However, the human CMV genome is complex and may encode 200 proteins, providing a large number of potential immunodominant viral antigens for the CTL response to CMV in humans. The target antigens recognised by the $CD8^+$ class I MHC restricted CTLs have only recently begun to be identified.

Borysiewicz *et al*, demonstrated the presence of CMV-specific cytotoxic T cells precursors in the peripheral blood lymphocytes (PBL) of seropositive individuals, and that most of these CTL precursors were directed against non-structural immediate early viral antigens (Borysiewicz *et al.*, 1983). Further studies using recombinant vaccinia viruses to express the non-structural 72 kd CMV immediate early protein and the envelope glycoprotein, gB, demonstrated that, in two CMV seropositive individuals, a subset of CTL recognised the immediate early protein and a minor subset recognised gB (Borysiewicz et al., 1988b). However, the majority of CMV-specific CTL in these individuals recognised other undefined viral antigens (Borysiewicz et al., 1988b).

An alternative approach which takes advantage of metabolic inhibitors, has been used to determine whether antigens expressed at immediate early, early, or late stages of the replicative cycle were dominant target antigens for $CD8^+$ CMV-specific CTLs. CTL lines were generated by the stimulation of PBL from CMV seropositive donors by autologous CMV-infected fibroblasts. The CTL lines which were generated lysed autologous cells infected with CMV in the presence of a sequential cycloheximide-actinomycin D blockade, which only allowed the expression of immediate early proteins. These CTL lines were unable to lyse cells infected with a vaccinia recombinant virus encoding the 72 kd major immediately early CMV protein (Riddell *et al.*, 1991a). In addition, such cells also efficiently lysed cells infected with CMV in the presence of an actinomycin D blockade, in which no CMV gene expression was detectable, and the only antigens available for presentation would be the structural virion proteins introduced into the cell after viral entry (Riddell et al., 1991a). Lysis by these CTL clones was completely abrogated by pre-treatment of the CMV-infected target cells with brefeldin A, demonstrating a requirement for intracellular processing of the target viral antigens (Riddell et al., 1991a). Taken together, these results show that structural viral proteins delivered to the cell in the incoming virions could be presented by HLA class I molecules, and thus sensitise the cell for $CD8^+$ CTL recognition before the onset of viral protein synthesis. This pathway provides an opportunity for the immune system to attack CMV-infected cells before the onset of viral replication. However, the efficacy of this pathway is directly related to the multiplicity of infection, as it relies on sufficient quantities of virions entering the cell upon infection, and this may be lower *in vivo* than in *in vitro* models. CTL specific for structural viral proteins constituted the dominant response to CMV in immunocompetent CMV seropositive individuals (Boppana and Britt, 1996; Li *et al.*, 1994; Riddell and Greenberg, 1994). Thus, the putative viral target antigens for CTL may include those proteins from the envelope, tegument and capsid of the virion. The most abundant constituents of the virion are the tegument proteins pp65 and pp150, and the host CTL response in normal seropositive individuals and allogeneic BMT recipients has been shown

to be predominantly focused on these structural proteins (Li et al., 1994; McLauglin-Taylor et al., 1994; Riddell and Greenberg, 1994). Both proteins are efficiently presented in the absence of *de novo* protein synthesis, unlike other CTL targets, such as gB and the CMV immediate early antigen. Polyclonal CMV-specific CTL lines, generated by the stimulation of PBL with autologous CMV-infected fibroblasts, recognised cells expressing gH or gB very poorly, suggesting low CTL precursor frequencies specific for these proteins (Borysiewicz *et al.*, 1988a; Borysiewicz et al., 1988b). These results suggest a limited role for CTLs specific for viral glycoproteins in CMV infection. The precursor frequency for CMV immediate early specific clones has also been confirmed to be low (Gilbert *et al.*, 1993).

The identification of specific CTL targets is important for the design of therapeutic strategies aimed at restoring the components of the host immune response essential for protective immunity against CMV in immunocompromised patients.

1.4 The effect of CMV on the host immune response

Like all herpes viruses, CMV becomes a permanent resident of its' host following primary infection. CMV maintains an intriguing and complex relationship with its' host. It replicates and establishes latency in cells involved in immune responses, particularly bone marrow progenitors, endothelial cells, polymorphonuclear leukocytes and monocytes (Taylor-Wiedeman *et al.*, 1991). This implies that the virus must not only escape immune responses aimed at its elimination, but that it must also have devised ways to control its own level of replication and expression, in order to stay in the host organism without being recognised. The multiple strategies that CMV seems to have adopted to modify immune

responses and their influence in the establishment of latency of this virus will be discussed below.

1.4.1 The induction of immunosupression by CMV

The contact between CMV and the host cell has immediate repercussions, even before the onset of viral gene expression (Bodaghi *et al.*, 1995). Host cell protein synthesis is dramatically reduced, and cellular proteins undergo phosphorylation due to protein phosphatases carried into the cell by the virus (Michelson *et al.*, 1996). Host cell metabolism is then greatly modified by the expression of CMV immediate early and/or early proteins, whether or not full viral replication occurs. One of the most marked effects of CMV-infected monocytes is their immunosuppressive effects on lymphocyte proliferative responses. During acute CMV infection, lymphocytes from mononucleosis patients do not respond to concanavalin A stimulation or stimulation via their T cell receptor (Carney and Hirsch, 1981), nor do lymphocytes from congenitally infected children (Timon *et al.*, 1993). When monocytes from seropositive donors are infected *in vitro* with CMV and added to autologous lymphocytes, there is no lymphoproliferation in response to concanavalin A stimulation (Carney and Hirsch, 1981). Furthermore, lymphocyte proliferative responses measured *in vitro* upon stimulation with recall antigens, mitogens and allogeneic stimuli, are decreased during CMV infection (Hirsh and Felsenstein, 1984; Roenhorst *et al.*, 1985; Timon et al., 1993).

1.4.2 The effect of CMV on the induction of cytokines and chemokines, and the upregulation of adhesion molecules

Several studies have confirmed the ability of CMV to increase the production of inflammatory cytokines such as IL-2 (Geist *et al.*, 1992) and TNF-α. The production of TNF-α has been demonstrated after CMV infection of monocytes and macrophages and

after transfection of CMV immediate early genes into T cells and monocyte cell lines (Geist *et al.*, 1994; Smith *et al.*, 1992). Also the production of transforming growth factor-β (TGF-β) is enhanced by CMV infection (Michelson *et al.*, 1994). Furthermore, TNF-α and TGF-β have also been implicated in increasing the efficiency of CMV replication, and may contribute not only to pathogenesis, but also to the persistence of the virus (Laegreid *et al.*, 1994; Michelson et al., 1994). Interferon β, is the main form of type I interferon released from infected cells (Rodriguez *et al.*, 1987), and it has been shown that it can upregulate the expression of MHC class I molecules on the non infected cells that interact with the infected cell (Grundy *et al.*, 1988a). Upregulation of the IL-1β has been reported following CMV infection in monocytes (Moses and Garnett, 1990). Another cytokine induced by CMV infection of fibroblasts and endothelial cells is interleukin-6 (St.Jeor *et al.*, 1993), a cytokine which induces multiple effects, including the induction of T cell proliferation and cytotoxic T cell differentiation. In addition, IL-6, like interferon-β is an endogenous pyrogen, and the viral induction of both of these cytokines could contribute to the high fevers observed during episodes of CMV infection in transplant recipients (Lui *et al.*, 1992).

Chemokines are a family of cytokines which also have chemotactic activity (Baggiolini, 1993). It has been shown that CMV infection of endothelial cells (Grundy *et al.*, 1998) and fibroblasts (Craigen and Grundy, 1996; Craigen *et al.*, 1997), induces enhanced production of C-X-C chemokines, such as IL-8 which is predominantly a chemotactic factor for neutrophils and T cells. The relevance of these findings is that CMV-induced neutrophil recruitment might enhance CMV dissemination. Furthermore, the attraction of T cells and neutrophils to sites of CMV infection could contribute to inflammation and potentially contribute to pathology (Craigen et al., 1997).

CMV infection also modulates the expression of a number of cell surface molecules. It has been shown that CMV infection in fibroblasts causes an increased expression of ICAM-1 and LFA-3 (Craigen and Grundy, 1996; Grundy and Downes, 1993). This increased

expression of adhesion molecules was accompanied by an increase in the adhesion of $CD2^+$ lymphocytes to the infected fibroblasts (Grundy *et al.*, 1993). Increased adhesion molecule expression and increased leukocyte binding would both be expected to contribute to inflammation and in the case of an infected allograft, to possibly provoke rejection processes (Grundy, 1998).

1.4.3 The evasion of cell mediated T cell recognition by CMV

Both human (Beersma *et al.*, 1993; Warren *et al.*, 1994) and murine CMV (Del Val *et al.*, 1989) cause MHC class I downregulation, due to a failure of class I molecules to leave the ER. In the case of human CMV, early evidence of MHC class I downregulation was given by Barnes & Grundy, who demonstrated a dramatic reduction in the cell surface level of class I molecules on infected cells (Barnes and Grundy, 1992). Since then several CMV genes have been shown to be responsible for that effect. Experiments using a series of CMV deletion mutants identified a 7 kb region, encoding 10 genes, that is required for class I downregulation in CMV-infected cells (Hengel *et al.*, 1996; Jones *et al.*, 1995). Three of the genes encoded in this region, US2, US3 and US11, were able to reduce class I expression at the cell surface when expressed individually (Ahn *et al.*, 1996; Jones *et al.*, 1996; Wiertz *et al.*, 1996a). The US2 and US11 gene products decrease the surface expression of MHC class I molecules by causing a rapid translocation of newly synthesised class I heavy chains from the ER to the cytosol where they are exposed to proteases and proteosomes and degraded. In the case of the US2 gene product, it has been shown to be capable of relocating the nascent ER class I chain back into the cytosol (Wiertz *et al.*, 1996b). The US3 protein destabilises the maturation and transport to the cell surface of class I heavy chains. The US3 gene product forms a complex with β_2m-associated class I heavy chain, which then accumulates in the ER. Thus, class I molecules are retained but not degraded during the immediate early period of the infection (Ahn et al., 1996; Jones et

al., 1996). The mechanism of class I downregulation used by the US6 gene product is to bind directly to the TAP complex, and thereby inhibiting peptide translocation from the cytosol to the ER lumen (Hengel et al., 1996; Lehner *et al.*, 1997).

The observation that even though the 72 kd immediate early protein from CMV is abundantly expressed in the immediate early phase of the infection, it is relatively poorly recognised by CMV-specific T cells, led to the identification of another mechanism of evasion of the T cell response. As demonstrated in a vaccinia virus expression system, recognition of the 72 kd immediate early protein by CMV-specific CTLs was selectively abrogated by the co-expression of pp65, a protein which possesses an associated kinase activity (Gilbert *et al.*, 1996). The interpretation of this finding was that the phosphorylation of the 72 kd substrate by pp65 would limit the access of the 72 kd protein to the class I processing machinery.

Although interference with class I cell surface expression would seem a simple way for any virus to escape immune surveillance, the down regulation of class I molecules could potentially lead to improved recognition by NK cells (Karre et al., 1986). An increasing amount of evidence suggests that NK cells recognise and destroy cells that no longer express MHC class I molecules, the 'missing self hypothesis' (Ljunggren and Karre, 1990). Therefore, any virus infected cell that has reduced or lost the expression of MHC class I molecules in order to avoid CTL attack could be susceptible to attack by NK cells.

Interestingly, CMV seems to have evolved an extra strategy to avoid being recognised by the host's immune system. The CMV UL18 gene encodes a protein with homology to the class I heavy chain (Beck and Barrell, 1988; Browne *et al.*, 1990) which binds to β_2m and is also able to bind peptides (Fahnestock *et al.*, 1995). In one study, cells transfected with UL18 became resistant to NK cell lysis (Reyburn *et al.*, 1997). However, this finding has recently been disputed (Leong *et al.*, 1998), and the function of UL18 awaits further

classification. The reason why CMV might have to have had to acquire these complementary strategies in order to avoid immune recognition, might be because of its prolonged replication period, which means that the virus has an extended exposure to immune recognition and attack.

1.5 Pathogenicity of CMV infection

There are several clinical situations in which individuals are incapable of mounting an effective immune response to certain viruses. In these type of situations CMV infections represent an important cause of severe and sometimes fatal disease. Before transplantation became a common procedure, congenital infection of the immunologically immature foetus was the most important clinical manifestation of CMV infection in humans (Weller, 1971). However, since the emergence of solid organ and bone marrow transplantation, which require long-term immunosuppression to prevent allograft rejection or graft-versus host disease, CMV infection has been associated with a high incidence of morbidity and mortality (Drew *et al.*, 1984; Riddell and Greenberg, 1995a; Rubin *et al.*, 1985). The appearance of the acquired immunodeficiency syndrome (AIDS) generated an additional population of immunocompromised patients at high risk from CMV disease.

The pathology of CMV disease in general can be caused by two different mechanisms, the first one being direct viral damage of infected cells, which is common in severely immunocompromised hosts, such as AIDS patients or in congenital infection, where the absence of an immune response allows high levels of viral replication and systemic disease. The second mechanism, involves pathology caused by the host immune response, and may be a contributory factor in the development of conditions associated with CMV infection in allogeneic transplant recipients (Grundy, 1993).

1.5.1 Congenital infection by CMV

Congenital infection by CMV of the immature foetus occurs in up to 2% of live births (Stagno et al., 1982). The main concern with this type of infection is the ability of CMV to damage the central nervous system, resulting in impaired development, mental retardation, neuromuscular abnormalities, deafness and blindness (Stagno et al., 1991; Weller, 1971).

Identification of the exact time during pregnancy when infection occurs can be difficult, since in normal adults disease is usually subclinical. In contrast, rubella virus infections are often associated with a distinct rash that alerts both the patient and physician to the possibility of a virus infection (Gilstrap *et al.*, 1994).

The severity of infection is undoubtedly related to the level of foetal development up to the time of virus exposure, and to whether the infection was a primary exposure or caused by the reactivation of latent virus (Stagno et al., 1986). Infection that results from reactivation or reinfection has less severe consequences for the foetus than primary infection (Stagno et al., 1986). The presence of CMV in an infant is not invariably associated with congenital defects. When children in a hospital nursery were examined for viruria, as many as 1% were shedding CMV with no apparent congenital malformations (Wigglesworth and Singer, 1991). Persistently infected children appear to mount a normal antibody response but are unable to eradicate the virus. This may be caused by a defect in T cell immunity, or by immunological tolerance caused by infection occurring early in immunologic development and through clonal selection consequently eliminating specific T cell clones (Oldstone, 1990).

1.5.2 CMV disease in HIV infected individuals

CMV retinitis is the most common sight-threatening infection in HIV infected individuals, developing in up to 30% of long lived AIDS patients (Jabs *et al.*, 1989). Direct damage of

the retina is usually responsible for the pathology of the disease. Organs of the gastrointestinal tract are the second most common target for CMV infection, with severe enteric disease occurring in 2% to 12% of HIV infected hosts (Jacobson and Mills, 1988). CMV pneumonitis is much less common in AIDS patients than in other immunocompromised patients. Although CMV is recovered from 30 to 50% of cultures of bronchoalveolar lavage from AIDS patients, several large studies have discarded the importance of CMV as a cause of pneumonia in AIDS patients (Miles *et al.*, 1990; Millar *et al.*, 1990). These observations support the idea that the pathogenesis of CMV pneumonia does not occur solely as a result of viral replication, but involves components of the host immune response which may be absent in AIDS patients (Grundy *et al.*, 1987).

It is not clear whether the proposed association between the rapid progression of HIV disease and CMV infection is due to a severely compromised cellular immune function allowing the development of CMV invasive disease, or whether CMV is a cofactor in the pathogenesis of AIDS (Webster *et al.*, 1989; Webster *et al.*, 1992).

The interaction between CMV and HIV at the cellular and molecular level results in the enhancement of HIV replication, and could potentially speed the progression to AIDS in HIV infected patients. Among other mechanisms for the enhancement of HIV infectivity, CMV has been shown to transactivate the HIV long terminal repeat (Webster, 1991). Furthermore, the induction of TNFα by CMV infection has been shown to induce HIV replication. It has also recently been proposed that CMV infection results in the expression of a superantigen which activates a subset of T cells expressing particular Vβ genes, which form a reservoir for HIV throughout the course of the disease (Dobrescu *et al.*, 1995)

1.5.3 The pathogenesis of CMV infection in recipients of solid organ transplants

In transplant recipients CMV infection can originate from one of three sources: 1) primary infection, where a seronegative donor becomes infected with latent virus carried in the allograft, or virus carried in blood products; 2) reactivation, where the seropositive transplant recipient undergoes reactivation of endogenous virus from latency; and 3) reinfection, where CMV carried in the transplanted organ or transfused blood products is reactivated from latency, and the seropositive recipient is infected with a new strain of CMV. The patients at highest risk of primary infection are CMV seronegative recipients receiving an organ from seropositive donors (Ho *et al.*, 1975; Singh *et al.*, 1988). Reactivation disease tends to be less severe than primary infection in some patient groups. Pre-existing immunity is important in limiting the severity of CMV disease, although the effect of this diminishes with increased immunosuppression (Smiley *et al.*, 1985a).

CMV infection has been demonstrated to impair both the patient and the graft survival in renal transplantation (Rubin and Tolkoff-Rubin, 1983), and an association between either acute or chronic rejection and CMV infection has been reported in several series of renal (Lopez *et al.*, 1974; von Willebrand *et al.*, 1986); liver (O'Grady *et al.*, 1988); and heart (Grattan *et al.*, 1989) transplants. Interestingly, CMV infection appears to target the transplanted organ, particularly in liver, heart, or lung transplant recipients (Gonwa *et al.*, 1989; Stratta *et al.*, 1989). The latter suggests that either allografts are particularly vulnerable to infection, or that there is local reactivation of CMV in the latently infected allograft. CMV disease at sites other than the transplanted organ is less common in liver and lung transplant recipients. This may suggest that the host immune response can control infection in tissues other than the transplanted organ, but cannot do so in the allograft due to reduced CTL recognition of the infected cells because of HLA mismatches (Arnold *et al.*, 1992; Smyth *et al.*, 1991b). In renal transplantation, CMV has been

suggested to cause graft glomerulopathy association with CMV viremia (Richardson *et al.*, 1981), and to trigger the immune mechanisms of acute rejection in kidney transplant patients (Lopez et al., 1974; von Willebrand et al., 1986). It has been suggested that CMV is able to trigger graft rejection by increasing the expression of class II in the graft (von Willebrand et al., 1986). This is thought to be mediated via interferon-γ which can be produced in large amounts during viral infection. In cardiac transplantation, CMV infection has been associated with chronic rejection. This condition, also known as cardiac allograft vasculopathy, is characterised by arteriosclerotic changes, similar to those seen in classic atherosclerosis (Everett *et al.*, 1992). In these patients CMV infection has been correlated with an increased incidence of graft loss, and an increased incidence of death, both due to graft atherosclerosis (Grattan et al., 1989). Liver allografts have been described as being a susceptible target organ in primary CMV infection in liver transplant recipients (Stratta et al., 1989). In addition to a variety of other CMV associated clinical manifestations, CMV hepatitis is a common finding in liver transplant patients (Paya et al., 1989). An association between CMV infection and chronic liver allograft rejection has also been reported (O'Grady et al., 1988). Inter-dependent cofactors for the chronic rejection of liver allografts, which manifests as the 'vanishing bile duct syndrome', have been reported to be CMV infection and HLA-antigen mismatches (O'Grady et al., 1988). It would appear that the bile ducts are important in the process of chronic rejection, although the exact mechanism involved has not yet been identified. The above clinical findings suggest that either CMV causes direct damage to the cells, or it activates the immune response against the infected cells, or that the immune response against the virus induces various cytokines, which leads to the increased expression of HLA antigens and adhesion molecules, and triggers alloreactivity. Finally CMV infection is associated with a decreased survival of heart-lung transplant recipients (Duncan *et al.*, 1991; Smyth et al., 1991a). Obliterative bronchiolitis is the most significant long term complication following pulmonary transplantation, and is thought to be a manifestation of chronic rejection. CMV

seropositivity was found to be a risk factor for the development of this condition post transplantation (Sharples *et al.*, 1996). The underlying mechanisms by which CMV is associated with allograft rejection in these patients are not clear, but virus induced inflammation of the endothelium and the vascular wall, mediated by the increased expression of MHC antigens and adhesion molecules might be contributing factors.

1.5.4 The pathogenicity of CMV infection in allogeneic BMT recipients

In the case of bone marrow transplant (BMT) recipients, the major clinical problems associated with CMV infection currently are as a result of reactivation of latent endogenous virus in seropositive recipients. This is due to the increase in the use of screened seronegative blood products, in order to avoid re-infection or primary infections (Bowden and Meyers, 1990). After allogeneic BMT, CMV infection occurs in approximately 70% to 80% of patients who are CMV seropositive before transplantation, and it is the principal infectious cause of death in these patients (Meyers *et al.*, 1986; Reusser *et al.*, 1990). Established CMV disease includes: oesophagitis, gastritis, hepatitis, encephalitis, vasculitis and CMV-associated syndrome (fever and cytopenia with CMV viremia). However, a fatal outcome from CMV infection is primarily related to the occurrence of interstitial pneumonitis, which develops in one third of infected patients after allogeneic BMT (Meyers et al., 1986). Currently, several studies have shown that despite the current treatments available, the mortality rate of patients surviving an episode of CMV pneumonitis is still 60 to 70% at 12 months post transplantation, as reported from several centres in the USA and Europe (Emanuel, 1993; Ljungman *et al.*, 1992) CMV pneumonitis thus remains a major determinant in the outcome of allogeneic BMT.

1.5.5 Pathology of interstitial pneumonitis

Several clinical observations have led to the hypothesis that the pathogenesis of CMV pneumonitis involves tissue damage caused by the immune system. There is a poor correlation between the titres of infectious virus in the lung tissue and bronchoalveolar lavage fluids and the severity and outcome of disease (Churchill *et al.*, 1987; Slavin *et al.*, 1994). CMV can be detected in bronchoalveolar lavage fluids at day 35 post bone marrow transplantation, but the peak of the onset of pneumonitis occurs between 70 and 120 days post transplantation, suggesting that a further stimulus is required to initiate disease (Schmidt *et al.*, 1991). The incidence of CMV pneumonitis differs markedly among the different types of bone marrow transplants. In the case of allogeneic bone marrow transplants, the incidence of CMV pneumonitis was reported to be 17 to 19 %. However, in recipients of autologous or syngeneic bone marrow transplant, CMV pneumonitis is much less common. Furthermore, it has been demonstrated that in allogeneic bone marrow recipients, CMV pneumonitis is associated with the occurrence and increased severity of graft versus host disease (GvHD) (Meyers et al., 1986). This could mean that CMV has no pathogenic potential in syngeneic BMT, and that in order to induce pneumonitis it requires cofactors, such as cytokines, delivered by immune cells during the inflammatory process of a graft-versus-host (GvH) reaction. Vice versa, GvHD could potentially be exacerbated by CMV induced cytokines. A synergy between GvHD and CMV infection has been postulated (Cray and Levy, 1993; Grundy *et al.*, 1985), and the progression of interstitial CMV pneumonia after antiviral therapy is indicative of an immunopathological component in CMV disease (Forman, 1991; Grundy et al., 1987). Alternatively, CMV could have a pathogenic potential of its own, which causes lethal viral disease, unless infection is controlled by antiviral mechanisms. The low incidence of CMV pneumonitis in AIDS patients has been discussed in a preceding section, and it is thought to be due to the absence of an alloreactive immune response in these patients. These observations plus the fact that antiviral chemotherapy on its own fails to prevent death from CMV pneumonitis in the

BMT recipients, despite reducing viral titres in the lung to almost undetectable levels (Shepp *et al.*, 1985), supports the hypothesis of an immunological component in the pathogenesis of CMV pneumonitis in allogeneic transplant recipients.

1.5.6 Antiviral agents used in the treatment of CMV infection

The current antiviral agents licensed specifically for the treatment of CMV infection are ganciclovir 9-(2-hydroxy-1(hydroxymethyl)ethoxymethyl)guanine and foscarnet. These agents act by inhibition of the viral DNA polymerase, and thus prevent viral DNA replication and the formation of CMV late proteins and the generation of new virions (Matthews and Boehme, 1988). These two compounds, although exerting similar effects on the viral replication cycle, do so by different mechanisms. Intravenous immunoglobulin is also licensed for the prophylaxis of CMV infection, and appears to ameliorate the symptoms of CMV disease, but the mechanism for the latter effect is not known (Meyers *et al.*, 1983; Snydman *et al.*, 1993).

1.5.7 Clinical strategies for the control of CMV disease in allogeneic bone marrow transplant recipients

There are four clinical strategies which have been used for the control of CMV disease in allogeneic bone marrow transplantation (Prentice and Kho, 1997): (1) Prophylaxis; in which antiviral drugs are given to every patient at risk of developing CMV disease, when either the donor or the recipient are CMV seropositive. (2) Suppression; in which drugs are given after bone marrow engraftment when CMV was detected from peripheral sites such as urine or throat washings but before the development of any symptoms. (3) Pre-emptive therapy; whereby drugs were also administered after engraftment but only when CMV is detected systemically, either in blood or bronchoalveolar lavage, and before the

development of symptoms. (4) Treatment; where antiviral drugs were given when symptoms of an established CMV disease are present.

The results from clinical trials employing these strategies suggest that preventive alternatives are the most efficient ways of reducing the incidence of CMV disease. To date, treatment has been relatively unsuccessful, despite the fact that the antiviral therapy seems to control viral replication, the mortality rate was still high (Prentice and Kho, 1997).

Attempts have been made in the past to treat established CMV infections with interferon (Meyers *et al.*, 1980) or a combination of vidarabine and interferon (Meyers *et al.*, 1982), but these met with little success. Even acyclovir, 9-(2-hydroxyethoxymethyl) guanine, a nucleoside analogue related to ganciclovir, which is highly effective against *herpes simplex* virus (HSV) and varicella zoster virus, was also shown to be ineffective (Wade *et al.*, 1983). Although ganciclovir has proved effective in the treatment of several types of CMV infection such as CMV retinitis and CMV hepatitis, in the case of treatment of gastrointestinal disease in bone marrow recipients it has had only marginal effects (Reed *et al.*, 1990). The treatment of primary CMV pneumonitis with ganciclovir has been most successful in renal-transplants, however similar treatment of recipients of bone marrow transplants has been unsuccessful (Shepp et al., 1985) or of only limited success. Recent data showed that a combination of ganciclovir and immunoglobulin reduced the mortality rates of patients with CMV pneumonia to 48% (Scmiddt, 1988). For CMV infections that are resistant to ganciclovir, foscarnet is an alternative drug. However, foscarnet has substantial renal and metabolic toxicity, causing renal failure, hypocalcemia, and seizures. Cidofovir, another DNA polymerase inhibitor, effectively inhibits CMV replication in tissue culture, but its oral bioavailability is very poor and it is nephrotoxic.

The relative failure of antiviral therapy may be linked to the fact that CMV induces many proinflammatory activities, most of which are due to immediate early and early gene products (Alcami *et al.*, 1991; Crump *et al.*, 1992; Iwamoto *et al.*, 1990). The drugs used

for therapy of CMV infection are in general inhibitors of DNA synthesis, which inhibit the production of late viral proteins, but do not have any effect in early and immediate early proteins which are transcribed from incoming viral DNA (Wagstaff and Bryson, 1994; Wagstaff *et al.*, 1994). Thus in the case of treating an established disease in which the viral load can be very high, these antiviral agents could fail to inhibit many of the pro-inflammatory effects of the virus, which might contribute to the pathology of CMV pneumonitis, and may even be linked to an acute or chronic rejection processes.

Suppressive and pre-emptive treatment are not the best strategy to prevent CMV disease after bone marrow allograft, since CMV disease has been reported in 13-29% of the patients in which there was no previous detection of the virus (Prentice and Kho, 1997). Prophylaxis potentially confers protection to all patients, including those with a low risk of developing CMV disease. It does, of course involve drug administration to all patients, including those who might never suffer from CMV disease. The disadvantage of exposing these patients to long term intravenous administrations of potentially toxic drugs argues against prophylaxis for all patients. In the case of ganciclovir, its main disadvantage is its myelotoxicity, which has been associated with the occurrence of a severe neutropenia in 30% of the patients, and which can predispose the patient to increased risk of bacterial and fungal infections and to immunosupression (Metzger *et al.*, 1994). Another drawback of the use of prophylactic and pre-emptive therapy is a delay in the development of the $CD8^+$ CTL response, which has led to the appearance of late CMV disease (more than 100 days after transplantation) in BMT and lung transplant recipients after the cessation of therapy (Goodrich *et al.*, 1991; Li et al., 1994). Therefore, it appears that there must be a balance between allowing sufficient CMV replication to stimulate a protective host response, while preventing the virus from inducing immunopathology, a situation which may be difficult to achieve in the immunosuppressed transplant recipient.

Thus the development of alternative strategies has proven to be essential to limit the increase of CMV disease particularly after BMT. The use of new orally administered antiviral drugs with lower toxicity may allow long term prophylaxis which could perhaps protect the patient until the host immune response can control the virus. There is now an oral formulation of ganciclovir which is already in clinical trials (Gane *et al.*, 1997). The use of novel antiviral drugs which act early in the viral replication cycle, might control CMV replication and also suppress proinflammatory activities. Alternatively, other approaches aiming to reconstitute the cellular immune response to CMV, might be the key to the control of CMV infections.

1.5.8 T cell adoptive immunotherapy for CMV infection

In bone marrow transplantation, the ability to manipulate the immune system of the marrow donor before transplantation offers opportunities not available in other transplant situations. The adoptive immunotherapy approach has been initiated by Riddell and Greenberg's group in Seattle, and it has already been used in clinical trials, where it has proven to be successful in the reconstitution of protective immune responses. The strategy involves the use of $CD8^+$ CMV-specific T cell clones derived from the bone marrow donor, these cells are propagated by *in vitro* culture, and returned to the bone marrow transplant recipient during the period when they are at maximum risk of CMV infection (Riddell *et al.*, 1991b). It has been reported that this therapy was not associated with any toxicity, and that it resulted in the selective and persistent reconstitution of CMV-specific CTL responses in all treated patients (Riddell *et al.*, 1992b). A major drawback in the implementation of this approach as the therapy of choice is the requirement of large number of cells (from 10^8 to 10^9 cells in four doses over 4 weeks). This requires the expansion of cells *in vitro* to such an extent that one may encounter certain technical obstacles.

1.5.9 Development of CMV Vaccines

The use of live, killed, recombinant, or subunit CMV vaccines given to patient or donor or to both before transplantation is an alternative approach which has been tested in renal allograft recipients (Plotkin *et al.*, 1994; Plotkin *et al.*, 1991). In the latter case the vaccine was a live Towne strain virus preparation attenuated by passage in cell culture. This vaccine has been reported to give a level of protection to seronegative transplant recipients, comparable to that given by previous infection with CMV. However, concerns about the potential for latency, reactivation and the induction of chronic disease by such live virus vaccines have focused the interest in virion subunits which mimic the immunogenic capacity of live whole virus (Plotkin, 1991).

Based on the fact that gB plays an important role in the humoral immune response, it has been considered to be an important candidate for a CMV subunit vaccine (Britt, 1991; Plotkin, 1991; Spaete *et al.*, 1988). A gB subunit vaccine has been tested in animals. These studies demonstrated the presence of neutralising anti-gB antibodies which increased after a booster dose in the immunised animals (Plotkin, 1991). In man, proliferative assays have shown that, in seropositive individuals, the T cell proliferative response to gB was present in only 74% of the tested population (He *et al.*, 1995). Furthermore, studies by others have shown that 22% of seropositive individuals failed to respond to purified gB preparations, despite having detectable anti-gB antibody levels post vaccination. Thus, a single gB subunit vaccine might not be effective in all individuals vaccinated (He et al., 1995). Additionally, since MHC class I mainly presents peptides generated upon processing of newly synthesised proteins, vaccination with killed virus, or purified viral proteins, leading mainly to presentation by MHC class II, is considered a far less promising way of inducing a $CD8^+$ cytotoxic immune response (Raychaudhuri *et al.*, 1992; Vitello *et al.*, 1995; Zhou and Huang, 1993). Therefore, alternative strategies for the vaccination or prevention of CMV disease are required.

1.6 Aims of the thesis

The $CD8^+$ CTL response to CMV infection appears to be biased towards the recognition of the CMV structural protein pp65. Hence it is clinically relevant to study this protein, and to identify the immunogenic peptide epitopes in pp65 which are able to elicit a CTL response against CMV. The knowledge obtained from such studies might help in the development of new strategies to reconstitute the immune response to CMV in immunocompromised individuals, or to induce protective immunity in the population at large.

The overall aim of the work presented in this thesis was to identify the cytotoxic T cell epitopes derived from the CMV tegument protein pp65 which could be presented in the context of HLA-A2, the most common HLA allele found in the Caucasian population. To achieve this overall aim the following specific questions were addressed:

i) Which pp65-derived peptides have the binding motifs for the HLA-A2 molecule?

ii) Which of these peptides show appropriate binding affinity to HLA-A2 molecule?

iii) Can these pp65 - HLA-A2 binding peptides generate an *in vitro* CTL response?

iv) Can pp65-peptide-specific CTLs recognise endogenously processed pp65 peptides?

v) Were any pp65 CTL epitopes thus defined, conserved among wild type isolates of CMV?

2. Chapter 2 . Materials and Methods

2.1 Mammalian cell culture

2.1.1 Origin of cell lines used

Human embryonic Lung (HEL) fibroblasts were isolated from the lung tissue of 12-17 week gestation foetuses which were obtained from the MRC tissue bank (Hammersmith Hospital, London)

The human foetal lung fibroblast cell line (MRC-5) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, Witshire, UK).

The 293 cell line, a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad5) DNA was obtained from the American Type Culture collection (ATCC, Rockville, Maryland, USA)

The T2 cell line (174CEMT2) was originally obtained from Dr. Peter Cresswell (Dept. Immunology, Yale Medical School, New Haven, CT). This cell line was produced as a hybrid of the B-lymphoblastoid cell line (B-LCL) .174 (Cerundulo et al., 1990) and the T-lymphoblastoid cell line CEM^R. Both copies of chromosome 6 derived from CEM^R have been lost from 174CEM.T2. Like .174, this cell line encodes HLA-A2 and -B5, but expresses only small amounts of the A2 antigen, and expression of the undetectable B5

antigen at the cell surface due to a mutation inactivating a trans-acting regulatory gene encoded within the MHC class II region (Salter, 1986).

The JY B-lymphoblastoid cell line (B-LCL), was obtained from the International HLA Workshop Cell Panel.

2.1.2 Culture of cells in suspension

B-lymphoblastoid cell lines and other cells which grow in suspension were cultured in Roswell Park Memorial Institute hydrogen-carbonate-buffered medium (RPMI 1640) containing 2 mM L-Glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Gibco/Life Technologies Ltd, Paisley, UK), and supplemented with 10% (v/v) foetal calf serum (FCS) Serum Supreme, (BioWhittaker, Walkersville, Maryland), which was heat inactivated at 56 °C for 20 min. Cells were grown in tissue culture flasks (Falcon) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Half of the volume of media was changed every three days. Cell viability and cell density were estimated by staining an aliquot of cells with an equal volume of 4% trypan blue (Sigma) in phosphate buffered saline (PBS, 171 mM NaCl, 3.4 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) followed by counting in a haemocytometer.

2.1.3 Culture of adherent cells

Adherent cell lines were grown in Modified Eagles medium with Earle's salts (MEM, Gibco) containing 2 mM L-Glutamine penicillin (100 IU/ml) and streptomycin (100 µg/ml), and supplemented with 10% heat inactivated FCS. In the case of the MRC-5 and 293 cell lines, the growth medium was supplemented with 1% non-essential amino acids (Gibco). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C and were passaged twice weekly prior to reaching confluency. Passaging was performed by rinsing

the cell monolayers with Hanks Balanced salts without calcium or magnesium (HBSS, Gibco), this was decanted and 5 ml of trypsin (0.05%)/EDTA (0.01%) solution (Gibco) was added for 3 min until the cells had become detached from the surface of the flask and could be loosened by gentle agitation. The cells were then quickly resuspended in 10 ml MEM/10% FCS and centrifuged at 1500 rpm for 5 min to remove the trypsin/EDTA. The cells were either resuspended in MEM/10% FCS and seeded out at 2-4 x 10^4 cells/cm^2 for propagation, or frozen down for future use as described below in section 2.1.6.

2.1.4 Separation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were obtained by centrifugation of whole blood on a ficoll density gradient. Human blood samples or buffy coats obtained from the North London Blood Transfusion Centre were diluted in an equal volume of RPMI 1640. Each 15 ml of diluted blood was underlayered with 10 ml Lymphoprep (Nycomed Pharma AS, Oslo, Norway), then centrifuged for 20 min at 2400 rpm (998 g) in a Heraeus Megafuge 2.0. PBMC were removed from the Lymphoprep interface, and transferred to a 50 ml conical tube (Falcon). This was diluted with 40 ml of RPMI 1640, mixed and then centrifuged at 1800 rpm for 10 min to pellet the PBMC, 2 further washes with RPMI were performed at 1500 rpm for 5 min. The PBMC were then resuspended in RPMI/10% FCS and mixed with trypan blue solution (0.4%, Sigma) at a 1:1 ratio, counted in an improved Neubauer counting chamber using phase microscopy to distinguish live from dead cells.

2.1.5 Production of B-LCL transformed cells using Epstein Barr Virus (EBV)

B Lymphoblastoid cell lines (B-LCL) were generated by inoculating $1x10^6$ PBMC with a 0.5 ml aliquot of supernatant from the EBV infected marmoset cell line B95.8 in RPMI/20% FCS with the addition of 2 mg/ml PHA (Sigma) and 250 ng/ml Cyclosporin A (Sandoz, UK). Cells were placed in a polystyrene tube with a lid (2052 Falcon), and incubated at 37 °C in a CO_2 incubator until growth was apparent. They were then transferred into 24 well plates, and fresh medium was added to each well. Transformed B cells grew out from these cultures in approximately 20 days.

2.1.6 Generation of PHA blasts

PBMC were incubated at $1x10^6$ cells/ml in 1 ml of RPMI/10% AB human serum (AB) in the presence of 2 µg/ml of PHA in a 12 well plate. On day 3, 500 µl of medium was removed, and the cultures were supplemented with 25 IU/ml IL-2 and 2 µg/ml PHA in 500 µl of medium. On day 6, a further 25 IU/ml of IL-2 was added to the cultures.

2.1.7 Cryopreservation of cells

Cells were cryopreserved one day post changing of the medium. The cells were centrifuged at 1500 rpm for 5 min, and the cell pellet was resuspended at a concentration of 10^7 cells/ml in a freezing solution consisting of 90% FCS and 10% dimethylsulphoxide (DMSO, BDH Laboratory Supplies, London, UK), which had been cooled to 4 °C. The cell suspension was then transferred to freezing vials (Nunc, Inc) in 1 ml aliquots. The vials were placed inside small insulated polystyrene boxes which were then placed at

-70 °C, followed by transfer to liquid nitrogen the following day. Stocks of cells were kept in liquid nitrogen at -192 °C until they were required.

Prior to use, the cells were thawed rapidly in a 37 °C water bath, until only a small particle of ice remained. In order to avoid DMSO toxicity the cells were immediately diluted with a 10 fold volume of serum rich medium, centrifuged at 1500 rpm /min and the cell pellet was resuspended at the desired concentration in the appropriate medium.

All cell lines were screened for Mycoplasma and confirmed to be negative using the Mycoplasma TCII test kit (Gene-probe).

2.2 Generation and screening of T cell lines

2.2.1 Analysis of lymphocyte phenotypes by flow cytometry

Cells wells were counted and $2x10^5$ cells were dispensed into Falcon 2052 polystyrene tubes and washed once in PBS containing 0.02% sodium azide (PBSA). The cell pellet was resuspended in 50 µl of the appropriate monoclonal antibody and incubated for 30 min at 4 °C. The cells were washed twice and resuspended in 50 µl of a 1/50 dilution of FITC- (fluorescein isothyocyanate) or PE- (phycoerythrin) conjugated F(ab')2 fragment of a rabbit anti-mouse IgG (Dako), and incubated at 4 °C for a further 30 min. In the case of double staining, directly conjugated primary antibodies were used in parallel, one being FITC-conjugated and the second conjugated to PE. The antibodies used for the staining of lymphocytes are shown in Table I. Stained cells were fixed in 1% paraformaldehyde. Directly conjugated purified mouse immunoglobulins of the appropriate isotope were used at identical concentrations to the primary antibodies as negative controls. Samples were analysed by flow cytometry using a FACScan (Becton Dickinson, Oxford, UK), 5000 events were acquired and the results were analysed using Cellquest software. A gate was placed around the lymphocyte population based on

forward and side light scatter characteristics prior to analysis. The data given is expressed as the percentage of lymphocytes positive for a particular surface antigen.

2.2.2 Tissue Typing

Cell lines and PBMC were typed by serology using standard microtiter typing techniques by the Tissue Typing laboratory of the Anthony Nolan Bone Marrow Trust. HLA-A2 subtyping was performed by Iain Scott using sequence specific oligonucleotide probing (SSOP) as described elsewhere (Tiercy *et al.*, 1994).

2.2.3 Preparation of stimulator cells

PHA blasts from HLA-A*0201 and CMV seropositive individuals, or T2 cells, used as stimulators in the generation of CTLs, were washed and resuspended at $1x10^6$ cells/ml in RPMI/10% AB serum, pulsed with 50 µg/ml of the appropriate CMV peptide and incubated at 37 °C in a humidified atmosphere of 5% CO_2 . After overnight incubation, peptide-pulsed cells were gamma-irradiated in a Gammacel® 3000 ELAN irradiator (Nordion International, Inc), which emits 503 RAd per minute (5.03 Gy per min). PHA blasted cells received 6000 Rads while T2 cells received 9000 Rads. Cells were then washed, counted and then viability estimated as described in section 2.1.4 prior to their use as stimulators for PBMC.

2.2.4 In vitro induction of cytotoxic T lymphocytes (CTL's)

2.2.4.1 Stimulation of CTL's using peptide-pulsed T2 cells.

Peripheral blood mononuclear cells from a CMV seropositive and HLA-A*0201 positive individual I (HLA A2, A3; B7, B60) were stimulated with 5000 irradiated T2 cells pulsed with peptides AE41, AE42 or AE44. Cultures were incubated at a stimulator to responder ratio of 1:2.5 in RPMI with 10% AB serum in 96 well round bottom plates. One 96 well plate was set up for each peptide. Recombinant interleukin-2 (IL-2) (Boehringer) was added on days 3 and 6 at a concentration of 20 IU/ml prior to a secondary re-stimulation on day 10 which consisted of the addition of 5 000 irradiated T2 cells pulsed with peptide and $1x10^4$ irradiated autologous PBMC as feeder cells plus 120 IU/ml IL-2 in a final volume of 100 µl/well. Responding T cells were expanded by weekly re-stimulations following the same protocol.

2.2.4.2 Stimulation of CTL using peptide-pulsed PHA blast cells

Peripheral blood mononuclear cells from CMV seropositive and HLA-A*0201 positive individuals ((I) HLA A*0201, A3; B7, B60 and (II) HLA A*0201, A19, B7, B27) were stimulated with 5000 irradiated autologous PHA blasts pulsed with peptide. Cultures incubated at various stimulator to responder ratios (1:10, 1:5, 1:2, 1:0.4) in RPMI/1640 with 10% AB serum in 96 well round bottom plates. On days 3 and 6 the medium was supplemented with 5 IU/ml recombinant IL-2. On day 10, each well received $5x10^3$ irradiated autologous PHA blast pulsed with peptide and $1x10^4$ irradiated (3000 Rads) autologous PBMCs as feeder cells plus 10 IU/ml IL-2. Cell were re-stimulated using the same protocol after a week, and subsequent weekly re-stimulations were performed by substituting the peptide-pulsed PHA blasts for peptide-pulsed T2 cells as stimulator cells and increasing the concentration of IL-2 to 20 IU/ml.

2.2.5 Depletion of the $CD4^+$ T cell population

Depletion of the $CD4^+$ T cell subset from the responding cell population obtained in the previous section 2.2.5, was performed by the use of Dynabeads M-450 CD4 (Dynal, UK. Ltd.). Prior to their use the beads were washed in HBSS/2% AB serum, the supernatant was discarded, and the beads were resuspended at a final concentration of approximately $1x10^7$ beads/ml and cooled to 4 °C. 84 µl of anti-$CD4^+$ Dynabeads was added to $3x10^6$ cells, and incubated in polystyrene tubes at 4 °C for 30 min with slow tilting and rotation. Following the addition of 500 µl HBSS/2% AB serum, the tubes were placed on a magnet for 2 min. The $CD4^+$ cell population remained attached to the magnet, while the remaining cell suspension was recovered from the supernatant and transferred to a fresh tube. The cells were counted, and resuspended at a concentration of $5x10^5$/ml in RPMI/10% AB serum.

2.2.6 Cytotoxicity Assays

Cytotoxic activity was measured by standard ^{51}Cr-release assays. Target cells comprised either of peptide (50 µg/ml) pulsed T2 cells or fibroblasts infected with CMV or recombinant adenovirus were labelled with 100 µCi of $^{51}NaCrO_4$ (Amersham International) for 1 h at 37 °C. Cells were washed twice with RPMI, resuspended in RPMI/10% AB serum and $2.5x10^3$ labelled target cells were incubated with varying numbers of effector cells (E:T ratios varied between 5:1 and 50:1). In experiments where the cold target lysis inhibition method was used, a 10 fold excess of unlabelled target cells was also added. At least 8 replicates of spontaneous and total release were prepared. The spontaneous release was calculated from wells in which no effector cells were added to the target cells, and total release was determined from wells in which 1% Triton-X-100 had been added to the target cells. After a 4 h incubation at 37 °C, the plates were centrifuged to pellet the cells, and

50 µl of supernatant was harvested and used for the measurement of gamma radioactive emission in a 1450 liquid scintillation counter (Wallac, UK).

The percentage of specific lysis was calculated as:

$$\text{Specific Lysis} = \frac{\text{Measured release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

Spontaneous release controls were normally less than 25% of the total release. The data from the ^{51}Cr-release assays shown in Chapter 5 are representative of three similar experiments.

2.3 Peptide binding assays

2.3.1 Synthetic peptides

CMV pp65 and control peptides were synthesised at the Department of Biochemistry, Nottingham University using Fmoc chemistry on solid phase, and were purified by Reverse Phase High performance liquid chromatography (RP-HPLC). Peptides were dissolved in DMSO at a concentration of 10 mg/ml and stored at -70 °C prior to use. The peptide FLPSDYFPSV (Sette *et al.*, 1994) was synthesised and fluorescein (FL)-labelled at the Department of Immunohematology and Blood Bank, University Hospital Leiden, The Netherlands. This peptide was synthesised as a derivative in which a Tyrosine was substituted with a Cysteine in order to tag a fluorescein group to the peptide FLPSDC(FL)FPSV. Labelling was performed with 4-(iodoacetamido) fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (NA-phosphate in water:acetonitrile, 1:1).

The labelled peptide was desalted over Sephadex G-10, and further purified by C18 RP-HPLC and then characterised by MALDI-MS (Lasermat, Finnigan, UK).

2.3.2 T2-stabilization assay

The peptide-induced stabilisation assay of the HLA-A2 class I molecule expressed by T2 cell line was performed using a modification of the method described elsewhere (Elvin *et al.*, 1993). Briefly, $5x10^5$ T2 cells were incubated in the presence of 100 μM of peptide in either RPMI supplemented with 10% FCS or the serum free medium X-vivo-10 (Biowhittaker, Walkersville, Maryland) for 18 h at 37 °C. In some experiments 20 µg/ml of β_2m was added in parallel to the addition of peptide. Prior to staining, cells were washed twice in PBS to remove excess unbound peptide. Fully conformed cell surface HLA class I molecules were detected by indirect immunofluorescence using the antibody W6/32 (Barnstable, 1978; Parham *et al.*, 1979). After 30 min incubation at 4 °C, cells were washed twice in PBS and stained with FITC-conjugated sheep-anti-mouse IgG as the secondary antibody, incubated as previously, and washed twice with PBS. Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Oxford, UK) as described previously in section 2.2.2 . The median channel values of fluorescence (MCF) of the cells incubated in the presence of peptides were obtained following subtraction of the background binding levels of W6/32 to T2 cells in the absence of peptide.

The percentage increase in fluorescence intensity was calculated as:

$$\frac{(\text{MCF test} - \text{MCF control})}{23.1} \times 100$$

Where 23.1 is the number of channels corresponding to a doubling of fluorescence in the FACScan used. All samples were assayed in triplicate to determine the intra-assay variation.

The monoclonal antibody W6/32 specific for HLA Class I molecules complexed with β_2m and peptide was obtained from hybridoma cells (ECACC) grown in an *in vitro* hollow-fibre bioreactor culture system (Tecnomouse™, Integra Biosciences, UK.) and purified by HPLC.

2.3.3 Peptide-binding competition assay

In order to determine the affinity of putative peptides for stable HLA-A*0201 molecules on the cell surface, all peptides were screened using the protocol described by Burg *et al.* (Burg *et al.*, 1995; Burg *et al.*, 1996). Briefly, (1x 10^7) JY cells were washed twice with PBS and placed on ice for 5 min, and then resuspended in 2 ml of ice-cold citric acid-Na_2HPO_4 buffer (a mixture of an equal volume of 0.263 M citric acid and 0.123 M NA_2HPO_4), pH=3.2 for 90 seconds (Burg et al., 1995; Sugawara *et al.*, 1987). The cells were immediately buffered with 13 ml of cold Iscove's modified Dulbecco's medium (IMDM), washed once, and resuspended at 7x10^5 cells/ml in IMDM in the presence of 1.5 mg/ml β_2m. The acid treated JY cells were aliquoted into 96 well "V" bottom plates (Costar, Cambridge, MA) at 100 µl/well, into wells containing 25 µl of a FL-labelled reference peptide (see section 2.3.1) at a final concentration of 150 nM, plus 25 µl of competitor peptide at a range of concentrations from 0.5 µg/ml-32 µg/ml. The plates were incubated for 12 h at 4 °C, washed twice with PBS, and the cell pellets were resuspended in PBS containing 0.5 % paraformaldehyde. The cells were analysed by flow cytometry, and the mean-fluorescence (MF) values obtained. The MF values obtained in the presence of FL-labelled peptide but in the absence of competitor peptide was taken as the maximal binding and equated to 0% inhibition. The MF value obtained without FL-labelled peptide was considered to represent 100 % inhibition. 4 replicates were assayed for the control wells and triplicates for all test wells.

The inhibition of binding was calculated using the following formula:

% inhibition =

$$\frac{\text{MF 150nM labelled peptide + competitor - MF Control without labelled peptide}}{\text{MF150 nM labelled - MF Control without labelled peptide}} \times 100\%$$

The relative binding affinity of a given test peptide was expressed as the peptide concentration needed to inhibit 50% of the binding of the reference peptide (IC_{50}). The binder's affinity was categorised as follows: High $IC_{50} < 5$ μM; Intermediate $IC_{50} \geq 5 <$ 15 μM, or Low $IC_{50} > 15$ μM.

2.3.4 Prediction of CTL peptide epitopes based on computer algorithm

The way in which this computer algorithm scores values for each possible peptide in a determined sequence is as follows: The initial (running) score is set to 1.0. For each residue position, the program examines which amino acid appears at that position. The running score is then multiplied by the pre-calculated coefficient for that amino acid type, at that particular position (Parker *et al.*, 1994). The idea behind this method is the assumption that, to the first approximation, each amino acid in the peptide contributes independently to binding to the class I molecule. Dominant anchor residues, which are critical for binding, have coefficients in the tables that are significantly different than 1. Highly favourable amino acids have coefficients substantially greater than 1, and unfavourable amino acids have positive coefficients that are less than 1. Auxiliary anchor residues have coefficients that are different from 1 but smaller in magnitude than dominant anchor residues. Using 9-mers, nine multiplications are performed. Using 10-mers, nine multiplications are again performed, because the residue lying at the fifth position is skipped. The resulting running score is multiplied by a final constant to yield an estimate of the half time of disassociation.

The final multiplication yields the score which indicates the theoretical half life of β_2m dissociation for each peptide.

2.4 Generation and titration of viral stocks

2.4.1 Preparation of virus inoculum

The following strains of CMV were used: laboratory strains AD169 (passage 94), Davis (passage 76), Towne (passage 132), and low passage clinical isolates Toledo (passage 12), C1F (passage 8), C1FE (8 passages in fibroblasts plus 7 passages in endothelial cells), R7 (passage 4-7) and CRV (passage 5). The AD169, Davis and Towne strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and the Toledo strain was a gift from Dr. Stuart Starr, Philadelphia, USA. C1F, R7 and CRV are clinical isolates which have been passaged through fibroblasts, whilst C1FE was derived from C1F by subsequent passage through endothelial cells and has enhanced pathogenicity for the latter cell type. Virus was propagated by serial passaging through confluent monolayers of human embryonic lung fibroblasts, in MEM supplemented with 4% FCS. Virus stocks were harvested from the supernatant medium from infected fibroblasts on days 5-7 post infection, and clarified by centrifugation at 500 g for 30 min in order to prepare a working stock. Virus stocks were screened for Mycoplasma and confirmed to be negative using the Mycoplasma TCII test kit (Gene-probe).

2.4.2 Titration of CMV stocks by plaque assay

HEL fibroblasts were seeded into 48 well plates (Falcon) at $5x10^4$ cells/well. The virus was serially diluted in log and half log dilutions in MEM/2% FCS and 100 µl/well was

inoculated on to the subconfluent monolayers. All samples were analysed in triplicate. After incubation for 1 h at 37°C in 5% CO_2, the monolayers were washed twice with MEM/2% FCS. Cells were overlaid with 0.5 ml of methyl cellulose overlay and incubated at 37°C in 5% CO_2. On day 10 following inoculation, the fibroblasts were fixed with 0.5 ml formal saline (50 ml formalin, 4 g NaCl and 450 ml distilled water) for 30 min at room temperature, followed by ten washes with tap water, before staining with methylene blue (Sigma) (0.03% in water) for 1 h at room temperature. The plates were further washed in water and allowed to dry.

Plaques appeared as dark blue areas, and the wells which contained approximately 30-60 plaques were selected and the number of plaques counted under a light microscope. The number of plaque forming units per ml (pfu/ml) was calculated by counting the total number of plaques in these wells and applying the equation:

pfu/ ml of virus stock = Mean number of plaques/well x virus dilution factor x 10

2.4.3 Use of defective adenovirus recombinant vectors

The adenovirus serotypes 2 (Ad2) and 5 (Ad5) have been studied extensively as vectors. These viruses contain a linear genome of 36 kb, inverted terminal repeats and DNA sequences near the termini contain the replication origins and packaging signals (Nienhuis, 1993). Wild-type adenovirus are able to package a maximum of 2 kb of foreign DNA into their genome. To permit packaging of larger DNA inserts, adenovirus mutants with deletions in the early region (such as E1) have been used to create space for exogenous DNA insertion (Berkner, 1988; Berkner, 1992). The human 293 cell line contains an insertion of 14% of the total Ad5 viral genome, including the E1 region which can complement viruses that lack E1 (Graham *et al.*, 1977). Recombinant viruses with

deletions in the E1 region are termed conditional or defective, that is, conditional for propagation only in 293 cells (Berkner, 1992).

The recombinant adenovirus RAd-35 which contains a lac Z expression gene was kindly donated by Dr. Gavin Wilkinson (Cardiff University, Wales, U.K). This vector was used in the generation of a pp65 recombinant adenovirus, RAd-pp65, which was constructed and kindly donated to us by Benham Zan, Dr. Christina Baboonian and Dr. Jim Booth (St. George's Medical School, London).

2.4.4 Preparation of adenovirus stocks

Recombinant adenoviruses (RAds) were propagated in 293 cells in MEM/5% FCS. Viral stocks were prepared from a total of eight 175 cm^2 flasks showing gross cytopathology following infection. Cells were detached from the flask by gently scraping and pelleted by centrifugation at 3000 rpm, they were then resuspended and pooled in 7 ml of PBS. An equal volume of the fluorocarbon Arklon-P was added, the suspension was mixed by vortexing thoroughly for 2 min, then centrifuged at 3000 rpm for 5 min. The resultant upper phase (virus) was decanted into a polystyrene universal tube, and the lower phase was re-extracted. The virus preparations were pooled, adjusted to 20 ml with PBS, aliquoted and stored at -70 °C.

2.4.5 Titration of recombinant adenovirus stocks

Serial 10 fold dilutions (neat to 10^{-10}) of adenovirus in MEM/5% FCS were inoculated into 8 replicate wells (100 µl/well) of subconfluent 293 cells in a 96 well plate. After 1 h of adsorption, the virus was removed. 100 µl of medium was added and the plates incubated at 37 °C for 5 days. The method of Reed & Muench was used to calculate $TCID_{50,}$ which is the dose of virus required to induce cytopathic effect in 50% of the wells.

2.5 *Detection of CMV antigens and antibodies*

2.5.1 Determination of the percentage of CMV infection by flow cytometric analysis

In order to determine the level of infection in each particular experiment, infected cells were removed from the plate at 18-24 h post infection by trypsinisation, and an aliquot of $2x10^5$ cells was stained for the CMV immediate early antigen using the monoclonal antibody E13 (Clonatech Biosoft, Paris, France). Trypsinised cells were washed 3 times with 1 ml PBS, and fixed at 4°C for 15 min in 66% acetone/34% PBS. The cells were then washed 3 times in PBS, and a final concentration of 10 μg/ml of monoclonal antibody E13 diluted in PBS/1% BSA was added and incubated for 1 h at 37 °C. Cells were washed twice in PBS/1% BSA, and 50 μl of a 1/50 dilution of FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG (Dako) was added followed by incubation at 4 °C for a further 30 min. Following incubation the cells were washed again with PBS/1%BSA and fixed in 1% paraformaldehyde. Flow cytometric analysis was performed as previously detailed (section 2.2.2)

2.5.2 Examination of stained cells by fluorescence microscopy

In order to obtain visual confirmation of the staining pattern of cells analysed by flow cytometry, an aliquot of cells used for the FACS analysis was applied to coated multispot glass microscope slides (C.A. Henley-Essex. Ltd). Stained cells were allowed to air dry onto the slide before mounting in Citifluor® (Citifluor, Canterbury, UK) (PBS-glycerol

with p-phenylendiamine to retard fading) for examination using an Olympus BH2 fluorescence microscope.

2.5.3 Detection of CMV pp65 expression by immunofluorescence

a) Preparation of Slides: Fibroblasts for immunofluorescence staining were either grown to confluency in 8 well slide chambers (Nunc, Inc. Naperville. Ill) in which $2x10^4$ cells/well were seeded, or cytospin slides were prepared from cell suspensions. For cytospins, cells were resuspended at $2x10^5$ cells/ml and 100 µl of this suspension was used per slide ($2x10^4$ cells). Cytospin slides were prepared using a Centurion centrifuge; cells were centrifuged onto the slides at 800 rpm for 3 min, and the resultant cell spots allowed to air dry for 30 min.

b) Fixation and staining: Cells either as cytospins or in slide chambers were fixed with 5 % paraformaldehyde - 2% sucrose in PBS. The cells were washed twice with PBS/1% FCS and permeabilised with 0.5 % NP-40, 10% sucrose, 1% FCS in PBS for 5 min, and then washed again with PBS/1% FCS. Prior to staining, the cytospin spot was delineated with a polyoxilene pen. The slides were stained for the presence of CMV pp65 antigen using 10 µl of a pp65-specific monoclonal antibody. Two monoclonal antibodies against pp65 were used (see Table 2.1), the Monofluo Kit CMV antibody[1] was used neat and the Clone 14 anti-pp65 ascitic fluid[2] was diluted 1:500 in PBS-1% BSA. The slides were incubated with primary antibody in a moist chamber at 37 °C for 60 min, and then washed 3 times in PBS for 3 min. The slides were drained and excess PBS removed (without allowing the cytospins spots to dry), and 20 µl of a 1:20 dilution of FITC-conjugated rabbit anti-mouse IgG antibody was added. The slides were incubated in a moist chamber for a further 30 min at 37 °C, washed 3 times with PBS, mounted with Citifluor, and viewed with an

Olympus BH2 fluorescent microscope. Only cells with bright nuclear fluorescence were considered positive.

2.5.4 Detection of anti-CMV antibodies by ELISA

The CMV status of the donors of blood samples and buffy coats was determined using an indirect enzyme-linked immuno sorbent assay (ELISA) (Biotest, UK, Ltd). In this ELISA, purified and inactivated nucleocapsid antigens are bound to the solid phase (the wells of a microtest plate). In order to exclude reactions caused by non-specific binding of the antibody, a control antigen isolated from non-infected cells was also used. Serum or plasma samples were diluted 1:2 with diluent reagent, and 25 µl of this dilution was added to the antigen coated wells. Negative and positive controls were treated equivalently, 100 µl of sample diluent was added to each well, and the plates were incubated for 30 min at 40 °C in a water bath. This incubation was followed by 3 washes with PBS/1%BSA (washing buffer) in an automated plate washer to remove any non-specific antibodies. Peroxidase-conjugated monoclonal anti–human IgG (100 µl) was added to each well, and incubated for 30 min at 40 °C as previously. The plates were then washed four times in washing buffer, before the addition of H_2O_2 together with 100 µl of chromogene (ortho-phenylendiamine, OPD) per well, and incubated at room temperature in the dark. The reaction was stopped after 10 min incubation by the addition of 100 µl of 1% H_2SO_4 into each well, and absorbance was measured within 2 hours in a spectrophotometer at 492 nm. The colourless chromogene is converted to a visible yellow colour in proportion to the amount of human IgG antibody to CMV-specific present in the sample. The optical density value for the control antigen was subtracted from the values of the test wells. All samples with a difference exceeding 0.2 optical density units were considered positive for IgG antibodies to CMV.

2.6 Molecular biological techniques

2.6.1 Extraction of DNA from CMV stock supernatants

Viral DNA was obtained by extraction from a cell free stock of CMV. From the CMV stock, 6 ml of supernatant was lysed with an equal volume of lysis buffer, and RNAse A was added at a final concentration of 10 µg/ml. This was incubated at 37 °C for 30 min, Proteinase K was added at 50 µg/ml and incubation continued for 1h. The DNA was then purified by phenol-chloroform extraction and ethanol precipitation. Alternatively, the extraction of DNA was performed employing the kit Qiaquick (Qiagen) following the manufacturers instructions.

2.6.2 Amplification of the CMV pp65 gene by PCR

CMV DNA (from the laboratory strains AD169, Towne and Davis or from the clinical isolates Toledo, C1F, CRV, C1FE, or R7) was used as a template for PCR amplification reactions using primers specific for the pp65 gene (Table 2.2). Controls included reactions without primers, and reactions without added DNA. PCR synthesis was performed in a total volume of 100 µl in a mixture containing 200 µM of dNTP (deoxynucleotides tri-phosphate), 5 µM of 5' and 3' primers containing the sites for Kpn I and Bam HI respectively (Table 3.2), 5 µl of CMV DNA at 20-50 ng/µl, 10 µl (2.5 U) of 10x Cloned *Pfu* DNA polymerase (Stratagene Ltd, La Jolla, CA). In initial experiments, the enzyme Taq polymerase (Gibco) was used instead of Cloned *Pfu* DNA polymerase, the conditions were the same as described above, with the addition of 10 µl of 2mM $MgCl_2$.

The reactions were performed in a Perkin Elmer thermal cycler using the following program:

1 denaturation cycle for 5 min at 95 °C followed by 30 cycles of 1 min of denaturing at 94 °C, annealing for 1 min at 65 °C, and an extension cycle for 2 min at 72 °C. The final cycle was a 10 min extension at 72 °C. When the PCR program was completed, 5 µl of each PCR mixture was removed and mixed with 1 µl of gel loading buffer. These aliquots were run on a 1% agarose gel in TBE with a reference size marker, e.g. Hind III/Eco RI digest of λ DNA. The remaining amplified product was purified by phenol-chloroform and ethanol precipitation.

The pp65 PCR product obtained from the CMV strain AD169 was cloned into the bacteriophage M13mp19, sequenced manually, and subcloned into mammalian expression vectors. The pp65 PCR products obtained from the remaining CMV strains used was sequenced automatically. These various procedures will be described in the following sections.

2.6.3 Cloning of pp65 DNA into the Bacteriophage M13mp19 vector.

2.6.3.1 Restriction enzyme digestion

The vector M13mp19 (10 µg), and the pp65 PCR product were digested using a 5 fold excess of the enzymes Kpn I and Bam HI. These enzymes differ in their salt requirements for optimal activity. Kpn I requires a medium salt buffer and Bam HI a high salt buffer. In order to satisfy their requirements, a double digestion was performed in two stages. Firstly, the enzyme Kpn I was used in a 50 mM NaCl digestion buffer, and incubated at 37 °C for 2 h. Bam HI was then added, the salt concentration was adjusted with digestion

buffer 3 to 100 nM NaCl, and the incubation continued for a further 2 h. The digested product was purified by phenol-chloroform and ethanol precipitation. The efficiency of DNA digestion was evaluated by agarose gel electrophoresis.

2.6.3.2 Dephosphorylation of cloning vectors

The 5' phosphate groups of the linearised vector were removed by treatment with calf intestinal alkaline phosphatase (CIAP, Gibco BRL) in phosphatase buffer (50 mM Tris.HCl pH 8, 0.1mM EDTA). An excess of the required amount of vector for subsequent ligations (1μg) was incubated for 30 min at 37 °C with 25 fold excess of CIAP (25 U/ μg of DNA). Following incubation, the CIAP was inactivated at 65 °C for 30 min in the presence of 5 mM proteinase K, 5 mM SDS and 5 mM EDTA, followed by phenol purification and ethanol precipitation. The recovered vector DNA was quantified by agarose gel electrophoresis.

2.6.3.3 DNA ligations

The molar ratio of M13mp19 to pp65 DNA insert in the ligation reaction was 3:1. 200 ng of dephosphorylated M13 vector and 140 ng of DNA insert restriction fragment were ligated with 1 U of T4 DNA ligase (Gibco) in a total volume of 20 μl. The reaction mix was incubated overnight at room temperature. An aliquot of this ligation mix was diluted 5 fold, and 5 μl from this dilution was used to transform competent bacteria as described below. In order to test for self-ligation, dephosphorylated M13 vector was used as a control.

2.6.3.4 Transformation of competent bacteria with the Bacteriophage M13 DNA

DH5αF' competent bacterial cells (MAX efficiency DH5αF', Gibco), were used for transformation. 100 µl of bacterial cell suspension was aliquoted into chilled polypropylene tubes (Falcon 2059) and mixed with 5 µl of the ligation mixture described above. The cells plus DNA mix was left on ice for 30 min and then heat shocked at 42 °C for 45 sec, and returned to ice for 2 min. The transformed bacteria were diluted with 0.9 ml of S.O.C. medium (see Appendix), and 200 µl was added to 3 ml of 2 YT top agar (see Appendix) (maintained at 45 °C) containing 50 µl of 2% X-gal, 50 µl of 100 mM IPTG and 200 µl of DH5αF' cells. The bacteria were plated on 2 YT bottom agar plates, by pouring the top agar mix onto the plate and spreading gently. The agar was left to solidify and the plates were inverted and incubated overnight at 37° C.

2.6.4 Identification and analysis of recombinants by direct gel electrophoresis

Vectors of the mp series are derived from a recombinant filamentous bacteriophage M13mp1, which carries a short segment of *E. coli* DNA. (Messing *et al.*, 1977). The presence of this segment, which contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (lac Z), has allowed the development of a simple colorimetric test to distinguish between vectors that carry a segment of foreign DNA and those that do not. The F' plasmid of the bacterial host cell carries a defective β-D-galactosidase gene that encodes for an enzymatically inactive polypeptide lacking amino acids 11-41. Unmodified vectors plated on host cells carrying the appropriate F' episome will be able to complement to form an enzymatically efficient protein. These vectors will form blue plaques when the medium contains IPTG (isopropylthio-β-D-galactosidase, a gratuitous inducer of β-galactosidase) and X-gal (5-

bromo-4-chloro-3indolyl-β-D-galactosidase, a chromogenic substrate). Insertion of foreign DNA into the lac Z region usually gives rise to recombinants that form pale blue or colourless plaques (Gronenborn and Messing, 1978). Recombinants carrying sequences of foreign DNA longer than 200-300 nucleotides can be easily detected by agarose gel electrophoresis of DNA prepared from recombinant plaques, when compared to non recombinant plaques.

2.6.5 Preparing stocks of bacteriophage M13 from single plaques

Stocks from putative recombinant bacteriophages were prepared from single colourless plaques, and from several non recombinant blue plaques which were used as controls. The plaques were selected from single isolated colonies, and transferred to 15 ml tubes (Falcon 2059), containing 2 ml of 2 YT media. These colonies were incubated for 12 h at 37 °C with constant shaking. Following this, 1 ml of culture was transferred to a sterile microfuge tube, and centrifuged at 12000 g for 5 min at room temperature in a microfuge. The supernatant was retained as a stock preparation, and transferred to a fresh, sterile microfuge tube and stored at -70 °C. The remaining 1 ml of the expanded bacterial culture could be used to prepare single-stranded DNA, or double-stranded replicative DNA (see sections 2.6.6).

2.6.6 Small-scale preparation of single-stranded phage DNA

Expanded cultures of recombinant clones were prepared as described previously (section 2.6.5). 1.5 ml of the infected culture was transferred to microcentrifuge tubes and the phage was harvested by centrifugation for 5 min at 12000 rpm. The supernatant was decanted into a clean microcentrifuge tube, to which 300 µl of 20% polyethylene glycol (PEG 800) in 2.5 M NaCl was added and mixed by vortexing. The phage particles were

allowed to precipitate at room temperature for 15 min, and then pelleted by centrifugation at 12000 rpm for 10 min. The supernatant was removed by aspiration, and the pellet resuspended in 100 µl of TE buffer (pH 8.0). The phage proteins were removed by phenol extraction and the DNA was precipitated with ethanol. Finally, the DNA obtained was resuspended in 50 µl of TE and electrophoresed in an agarose gel to confirm by altered mobility that the insert had been incorporated into the vector.

2.6.7 Large scale preparations of the replicative form of the bacteriophage M13 DNA

Large scale double-stranded DNA preparations were performed using an inoculum from the original bacterial stocks prepared as described in section 2.6.5. 2.5 ml of DH5αF' non-competent bacteria were transferred to a sterile tube (Falcon, 13 x 100 mm), and 0.2 ml of the bacteriophage stock was added. The mixture was allowed to stand for 5 min at room temperature, and then decanted into a 2 litre flask containing 250 ml of fresh LB medium prewarmed to 37 °C. The cultures were incubated for 12 h at 37 °C with constant shaking. The expanded bacteria were harvested by centrifugation at 4000 rpm for 15 min at 4 °C, after which the supernatant was removed and stored at 4 °C to be used for the large scale preparation of single-stranded bacteriophage M13 DNA. The bacterial pellet was resuspended in 20 ml of ice-cold STE buffer (0.1 M NaCl, 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0) and centrifuged at 4000 rpm for 15 min at 4 °C. Bacteriophage M13 replicative form, double-stranded DNA was isolated by the alkaline lysis method, and purified using MAXIPREP Qiagen tip columns (Quiagen) using the method recommended by the manufacturers.

2.6.8 Large scale preparations of single-stranded Bacteriophage M13 DNA.

In order to prepare single-stranded M13 DNA, the stocks stored after the growth of M13 infected bacteria in section 2.6.7 were used. This supernatant was mixed with a solution of 20% polyethyleneglycol (PEG800) in 2.5 M NaCl (3 parts of supernatant : 1 part of PEG), and aliquoted in 50 ml tubes. The phage particles were left to precipitate for 15 min at room temperature, then centrifuged at 4000 rpm for 30 min. The supernatant was removed and the pelleted DNA resuspended in 600 µl of TE buffer and transferred to 1.5 ml microfuge tubes. The phage proteins were purified by phenol-chloroform followed by ethanol precipitation. The resultant DNA was resuspended in 200 µl of TE buffer. The concentration of DNA and the presence of an insert was assessed by agarose gel electrophoresis. The DNA obtained by this procedure was used in the sequencing reactions described in section 2.6.9 .

2.6.9 Sequencing of single-stranded M13 DNA containing the pp65 gene

2.6.9.1 Sequencing Reactions

All the sequencing reactions were performed using the dideoxy chain termination method with the T7 sequencing Kit from Pharmacia Biotech according to the manufacturers recommendation. 7 sequencing primers were used: one was the universal M13mp19 primer, and the remaining 6 were pp65-specific oligonucleotides. The first step involved in sequencing is the annealing, in which the primers bind to the DNA. During the course of the labelling reaction, extension of the annealed primer is initiated in the presence of limiting amounts of all four deoxynucleotides (dNTP's), one of which is radiolabelled. The reaction mixture is equally divided into four separate tubes for competition of the

termination reaction. Each tube contains a single different ddNTP in an excess of all four dNTPs. This two step reaction is required, as the T7 polymerase enzyme uses ddNTPs very efficiently. By excluding the ddNTPs during the labelling reaction, and introducing them during the termination reactions, the synthesis of long chain-terminated fragments is achieved.

a) Annealing of primer to single-stranded template: DNA was used at a concentration of 1.5-2 μg and primers at 1 μM. In the annealing reactions, 10 μl of DNA template was mixed with 2 μl of primer and 2 μl of annealing buffer. The annealing reaction preparations were mixed by vortexing, and incubated at 60 °C for 10 min, followed by a 20 min incubation at room temperature.

Deaza G/A ^{T7}Sequencing™ mixes were used to eliminate the ambiguities which can occur in dideoxy sequencing using T7 DNA polymerase. These reagents are used to resolve compressions caused by G-C base paring and purine base stacking, by the substitution of c^7dGTP for dGTP and c^7dATP for dATP respectively. Both analogues are suitable substrates for T7 DNA polymerase and thus were used for routine DNA sequencing.

b) Labelling Reaction: 3 μl of dATP-labelling mix, 1 μl of α ^{35}S-dATP and 2 μl of T7 DNA polymerase (dilution 1:5 in diluent buffer), was added to the annealed template/primer mix. The reaction mixture was incubated at room temperature for 5 min. During this incubation step, 2.5 μl of dNTP mixes (dATP, dCTP, dGTP and dTTP), were placed into separate wells of a microtiter plate and warmed in a water bath at 37 °C for 1 min in preparation for the termination reactions.

c) Termination reaction: 4.5 μl of the labelling reaction which had been incubated for 5 min at 37 °C, was added to each of the four pre-warmed sequencing mixes. The components were mixed by gentle pipetting, and incubated at 37 °C for a further 5 min,

followed by the addition of 5 µl of Formamide stop solution to each well. Prior to loading aliquots of these reactions on to a sequencing gel, the samples were denatured by heating at 85 °C for 5 min and then placed on ice. 3 µl of each denatured reaction was loaded and run on a sequencing gel. The remaining material was stored at -20 °C.

2.6.9.2 Gel electrophoresis

The DNA from the sequencing reaction was electrophoresed in an 8% polyacrylamide denaturing gel (410 x 330 mm x 0.4 mm thick), in 1x TBE buffer. The gels were cast using Hydro-Link "Long-Ranger" gel solution (AT Biochem), which is a 50% chemically modified acrylamide monomer with a novel cross-linker. The gels were prepared using 31.5 g urea (Sigma Chemical Co), 7.5 ml Long-Ranger, 9 ml 10X TBE and 20 ml of distilled H_2O. These reagents were mixed, and the urea was dissolved by warming the solution whilst stirring. The volume was adjusted to 75 ml with distilled H_2O, and 75 mg of ammonium persulfate (Sigma Chemical Co.) was added. Prior to pouring, 90 µl of TEMED (BDH Laboratory supplies, Ltd.) was added, and the gel was then allowed to polymerise for 2 h at room temperature (or left at room temperature overnight). Prior to loading the samples, the gel was warmed up by running at 55 W for at least 30 min. Immediately following this pre-run, the samples were loaded, and electrophoresis was continued at 60 W for two h (for short gels) or four h (for long gels).

2.6.9.3 Autoradiography

Following electrophoresis, the gels were dried at 80 °C in a vacuum drier (Bio Rad) for 2 h, and exposed to X-ray film in cassettes with intensifying screens (Kodak) for 24 to 48 h at room temperature. DNA sequences on the autoradiograms were read manually, and entered onto a computer using the Gene Jockey program for subsequent DNA sequence-matching analysis, and for amino acid translation.

2.6.10 Fluorescence-based cycle sequencing of pp65 DNA

The pp65 PCR products obtained from the CMV laboratory strains AD169, Towne and Davis and the clinical isolates Toledo, C1F, C1C, C1FE, or R7 were purified prior to sequencing using the Quiaquick DNA purification kit (Quiagen), following the protocol recommended by the manufacturers. DNA was sequenced using Big Dye Terminators, a set of dye terminators labelled with high-sensitivity dyes, which contain a fluorescein donor linked to a dichlororhodamine (dRhodamine) acceptor dye. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor. Each of the four nucleotides (terminators) A,C,G or T has a different fluorescein donor, which would emit a particular colour (A=green; C= red; G=blue and T =yellow) when it is stimulated by the argon ion laser in the PE Applied Biosystems DNA sequencing instruments.

2.6.10.1 Preparation of sequencing reactions

Sequencing reactions were performed using the ABI Prism™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit, which contains Ampli Taq DNA polymerase and the dye terminators plus deoxynucleotide triphosphates, magnesium chloride and buffer, all premixed into a single tube. To this terminator reaction mix, 30 ng of pp65 DNA and 3.2 pmol of primer were added together with water to obtain a final volume of 20 µl.

Cycle sequencing was performed in a PE Applied Biosystems thermal cycler. The following cycle was repeated for 25 cycles:

A rapid thermal ramp to 96 °C, followed by 96 °C for 10 sec. A second rapid thermal ramp to 50 °C followed by 50 °C for 5 sec, and a third rapid ramp to 60 °C followed by 60 °C for 1 min.

Samples were electrophoresed in an ABI 377 automatic sequencer (PE Applied Biosystems).

2.7 Cloning of pp65 into mammalian expression DNA vectors

2.7.1 Mammalian expression vectors

2.7.1.1 pS vector

This is a hybrid vector which was constructed by Dr. Salim Kakhoo at the Anthony Nolan Research Institute. The vectors pREP8 and pMEP4 (Invitrogen), were digested with the enzyme Sal I and ligated in order to obtain this hybrid vector pS. The resultant pS vector contained Epstein Barr virus origin of replication (oriP) and nuclear antigen (EBNA-1) coding sequences which allows it to replicate stably in primate (including human) cell lines, and the hygromycin cassette from pMEP4 plus a Simian virus 40 (SV40) polyadenylation site, a polycloning site and the Rous sarcoma virus long teminal repeat (RSV LTR) promoter from pREP8 (see Appendix).

2.7.1.2 pEGFP-N2 vector

This vector encodes a red-shifted variant of wild type green fluorescence protein (EGFP) (Chalfie *et al.*, 1994; Prasher *et al.*, 1992), which has been optimised for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 448 nm; emission maximum = 507 nm). The MCS in pEGFP-N2 is between the immediate early promoter of CMV ($P_{CMV\ IE}$) and the EGFP coding sequences (see Appendix). Genes cloned into the MCS will be expressed as fusions to the N-terminus of green fluorescent protein if they are in the same reading frame as green fluorescent protein and there are no

intervening stop codons. SV40 polyadenylation signals downstream of the green fluorescent protein gene direct proper processing of the 3' end of the green fluorescent protein mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin-resistance cassette (neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpex simplex thymidin kinase gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette (P_{amp}) expresses kanamycin resistance in *Escherichia coli.* (*E.coli*) The pEGFP-N2 backbone provides also a pUC19 origin of replication for propagation in *E.coli*, and an f1 origin for single-stranded DNA production.

2.7.2 Restriction enzyme digestion

The recombinant M13mp19-pp65 DNA was digested with the appropriate restriction enzyme(s) in order to excise the pp65 DNA fragment from the M13mp19 DNA and to sub-clone it into a number of plasmid vectors. The enzymes Bam HI and Kpn I (Gibco) were selected for digestion when cloning into the plasmid pS, whilst the enzyme Eco RI (Gibco) was used for digestion when cloning in pEGFP-N2. The digests were performed in a total volume of 20 µl containing 50 µg of DNA and a five fold excess of the required restriction enzyme, and incubated at 37 °C for 2 h (see section 2.6.3.1). Utilising the same strategy, 5 µg of the appropriate vector DNA was digested with the same enzyme(s). Following completion of the restriction enzyme(s) digestion, the vector was dephosphorylated as described previously (section 2.6.3.2).

The digested DNA was separated into its component fragments by electrophoresis on a 0.8% agarose gel at 80 V. The relevant fragment encoding pp65 was excised directly from the agarose gel for extraction with the QIAEX II Kit (Qiagen Ltd), following the manufacturers instructions. The pp65 DNA fragment was then ready for ligation.

2.7.3 DNA ligation

In order to clone pp65 into the vector pS, 98 ng of the digested pp65 gene was ligated into 140 ng of digested-dephosphorylated vector pS. A reaction was set up in parallel consisting of the digested-dephosphorylated vector alone as a control. The T4 DNA ligase (1 U, Gibco) and 2 µl of 10x ligase buffer were added to the reaction mixtures, the volume was adjusted to 20 µl with ddH_2O, and the reaction incubated at room temperature for 18 h. Cloning of pp65 into pEGFP-N2 was performed as above using 289 ng of pp65 DNA ligated at an insert:vector ratio of 4:1 to 200 ng of pEGFP-N2 vector. The ligated products were then used to transform competent DH5α bacteria.

The insert to vector ratio was calculated according to the following formula:

$$\frac{\text{Insert length (bp)}}{\text{Vector length (bp)}} \times \text{ng vector/ligation} \times \text{ratio} \frac{\text{insert}}{\text{vector}} = \text{ng insert vector}$$

2.7.4 Transformation of competent bacteria with plasmid DNA

The ligation mixtures were diluted 5 fold, and 5 µl of this dilution was used to transform competent DH5α *E. coli* (Gibco). From 1-10 ng of the ligation reactions, or 0.2 ng of control circular plasmid were incubated in 100 µl of competent *E .Coli* DH5α bacteria, and incubated at 4 °C for 10 min. The bacterial cells were then heat-shocked for 45 s at 42 °C and placed on ice for 2 min. Transformed bacteria were then diluted with 0.9 ml of S.O.C. medium (see Appendix A) and incubated for 1 h at 37 °C with shaking. A 100 µl aliquot of a 10 fold dilution and 100 µl of neat culture were plated onto separate 2 YT agar plates containing the appropriate antibiotic. Recombinant bacteria transformed with the vector pS were plated in 2 YT agar plates containing 100 µg/ml of ampicillin/methicillin, whilst recombinant bacteria transformed with the vector pEGFP-N2 were plated in agar containing 30 µg/ml of Kanamycin, for the selection of transformed

colonies. The plates were incubated overnight at 37 °C, and individual colonies were then selected and expanded for preparation of DNA according the method described in section 2.7.5.

2.7.5 Small scale preparations of ligated recombinant plasmid DNA

2.7.5.1 Harvesting

Single bacterial colonies were selected and transferred into 2 ml of LB (Luria Bertrani) medium (see Appendix A) containing the appropriate antibiotic in a loosely capped 15-ml tube, and incubated overnight at 37 °C with vigorous shaking. 1.5 ml of this culture was transferred to a microfuge tube and centrifuged at 12000 rpm for 5 min. The supernatant was removed by aspiration, and plasmid DNA was extracted from the bacterial pellet by alkaline lysis as described below. The remainder of the culture was retained as a stock and mixed with an equal volume of glycerol for storage at -20 °C.

2.7.5.2 Alkaline lysis

The bacterial pellet obtained above was resuspended by vigorous vortexing in 100 µl of ice cold solution I, before the addition of 200 µl of solution II and mixing by inversion. The tubes were placed on ice for 5 min. Solution III (150 µl) was added, mixed by gentle vortexing, and incubated for 10 min on ice for Solutions I, II, and III, see Appendix A. The mixture was centrifuged at 12000 rpm for 5 min, the supernatant transferred to a fresh tube, and the DNA was extracted using phenol-chloroform. Double-stranded DNA was ethanol precipitated, and the resultant pellet resuspended in 50 µl of TE buffer. The presence of an insert was analysed by restriction enzyme digestion followed by agarose gel electrophoresis.

2.7.6 Large scale preparation of recombinant plasmid DNA

A 30 ml culture from the positive colonies determined in section 2.7.5 was grown to late log phase (i.e. OD 600 = 0.6) in LB medium at 37 °C with shaking. 500 ml of 2 YT medium containing the appropriate antibiotic was inoculated with 500 µl of stock from the desired clone. The bacterial culture was incubated overnight at 37 °C with vigorous shaking and a large scale preparation of double-stranded DNA was prepared using a scaled-up version of the method described in section 2.7.5. The DNA obtained was resuspended in 500 µl of TE, and quantified by agarose gel electrophoresis. An aliquot of this DNA was digested and analysed by enzyme digestion in order to detect the presence of an insert.

2.8 Expression of cloned genes in cultured mammalian cell lines

2.8.1 Transfection of cells by electroporation

Cells to be transfected were replenished with fresh RPMI/10%FCS medium one day prior to electroporation, and their viability, (which should be at least 80% for efficient transfection) was assessed. Cells were harvested by centrifugation, and 50 ml of supernatant was stored at 4 °C (to use as conditioned medium). The cells were washed in 1X HEPES buffered saline (HBSS) and resuspended in the same buffer at a concentration of 1-2 x 10^7 cells/ ml. 800 µl of this cell suspension was transferred into a 0.4 cm Gene Pulser® electroporation cuvette (Bio Rad Laboratories Ltd), and mixed with 20 µg of plasmid DNA. This mixture was incubated on ice for 5 min, prior to electroporation in a Gene Pulser II electroporation system (Bio Rad Laboratories Ltd). Cells were pulsed with an electrical current of 240 V, an infinite resistance, and a capacitance of 960 µF. Following electroporation, the cells were incubated for 10 min on ice, and transferred to

10 ml of RPMI/20% FCS in a 24 well plate and incubated at 37 °C in a CO_2 incubator. After 48 h, the cells were centrifuged and resuspended in RPMI/20% FCS with the addition of the relevant selection antibiotic (1 mg/ml for G418, 125 µg/ml for hygromicin). The cells were plated into 4 wells of a 24 well plate (Costar) for expansion. When cells were confluent, they were transferred to a 12 well plate, and subsequently into a 25 cm^2 flask (Falcon). In instances where cells were slow to expand after the addition of selection medium, 20% of previously stored condition medium was added to the cultures. Mock transfected cells were included as a control, and the expression of transfected genes was analysed when the mock transfected cells were dead, whilst the test cells remained alive. This expansion period ranged from two to three weeks.

2.8.2 Transfection of cells by lipofection

The transfection of the pEGFP-N2/pp65 construct into fibroblasts and 293 cells was performed by the use of lipofection with the Superfect™ Reagent (Qiagen Ltd), according to the manufacturers instructions. Adherent cells were plated on the day prior to transfection into a 60 mm petri dish containing a sterile glass coverslip, at a concentration of $8x10^5$ cells per dish in 5 ml of MEM/10% FCS, and incubated at 37 °C in an atmosphere of 5% CO_2. The seeding density of cells was such that a confluency of between 50-80% was achieved on the day of transfection. 5 µg of plasmid DNA was diluted to 150 µl with MEM without the addition of serum or antibiotics. The DNA was mixed with 30 µl of Superfect Reagent, homogenised by pipetting, and incubated for 10 min at room temperature in order to allow the DNA and liposomes to form complexes. Following incubation, 1ml of MEM (containing 10% of FCS) was added to the reaction tube containing the transfection complexes. The reaction mixture was homogenised by pipetting, and transferred to the cells in the 60 mm dishes, which had been washed once with 4 ml PBS. Cells were incubated with the complexes for 2-3 h at 37 °C, after which

the medium containing the remaining complexes was removed from the cells by gentle aspiration. Cells were washed once with 4 ml PBS, and incubated in fresh MEM/10% FCS for 24 to 48 h, and the levels of expression of the green fluorescent protein was measured as described in the following section.

2.8.3 Detection of green fluorescent protein and variants following transfection

In order to monitor the production of green fluorescent protein after transfection of fibroblasts or 293 cells, the coverslip with adherent cells was removed from the dish, and mounted onto a microscope slide with a drop of Citifluor mountant. The coverslip was then sealed to the slide with glass coverslip sealant, which was applied around the periphery of the coverslip. The sealant was allowed to dry for 30 min, and the slides were examined by fluorescent microscopy.

Table 2.1 Murine monoclonal antibodies specific for CMV antigens

Specificity	Clone	Isotype	Source
CMV IE antigens 1 and 2	E13	IgG1	Biosoft,Paris, France
CMV pp65 antigen	Monofluo Kit[1]	IgG2b	Diagnostics Pasteur, Marnes-la-Coquette, France
CMV pp65 antigen	C14[2]	IgG1	Dr. Jim Booth, St George's Hospital, UK

Table 2.2 Primary Murine monoclonal antibodies used in this study for the phenotyping of lymphocyte subsets

Specificity and fluorochromes	Alternative names	Cell type on which the antigen is expressed	Clone	Isotype	Source
CD3/FITC CD3/PE	T3, Leu-4	T cells	UCHT-1	IgG1	Harlan[1] Serotec[2]
CD4/FITC CD4/PE	T4, Leu ™-3	Helper/inducer T cells, thymocytes, monocytes	SK3 F101-69	IgG1 IgG1	BD[3] Harlan
CD8/PE CD8/FITC	T8, Leu- 2	Cytotoxic/suppresser T cells, thymocytes, NK cells	SK1 DK25	IgG1 IgG1	BD Dako[4]
CD16/PE	FcgRIII Leu 11c	NK cells, granulocytes, macrophages	B73.1	IgG1	BD
CD19/FITC CD19/PE	Leu 12	B cells, dendritic cells, B cell precursors	4G7 SJ25-C1	IgG1 IgG1	BD Harlan[5]
CD56/PE	Leu 19,	NK cells, some neural cells	MY31	IgG1	BD

[1] Harlan Sera-Lab Limited, Sussex, England

[2] Serotec, Ltd. Oxford, England

[3] Becton Dickinson, Oxford, England

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3. Chapter 3. The identification of potential HLA-A*0201 restricted CTL epitopes derived from the CMV pp65 protein

3.1 Introduction

The aim of the work presented in this chapter was to identify immunogenic peptide epitopes derived from the amino acid sequence of the CMV pp65 protein which were able to bind to the HLA-A2 class I molecule, and which could potentially serve in the induction of a CMV-specific T cell response.

T cell epitope determination has been greatly influenced by the understanding of the rules for peptide selection by MHC molecules. One of those rules is the presence of allele-specific motifs, that is particular amino acids that are preferred in specific sequence positions. The amino acids which determine these motifs have been defined as "anchor residues" (or dominant anchor residues), because specific pockets inside the peptide binding groove of the MHC molecule accommodate the side-chain residues of these amino acids on the peptide, and this interaction seems to "anchor" the peptide to the MHC molecule (Silver *et al.*, 1992; Young, 1995). For example, the dominant anchor residues described for peptides which bind to HLA-A2 are Leucine, Valine, Isoleucine or Methionine at position 2 and Valine, Leucine, Isoleucine or Alanine at position 9 or 10 (Falk et al., 1991; Hunt *et al.*, 1992) (Figure 3.1). These residues bind in the pockets B

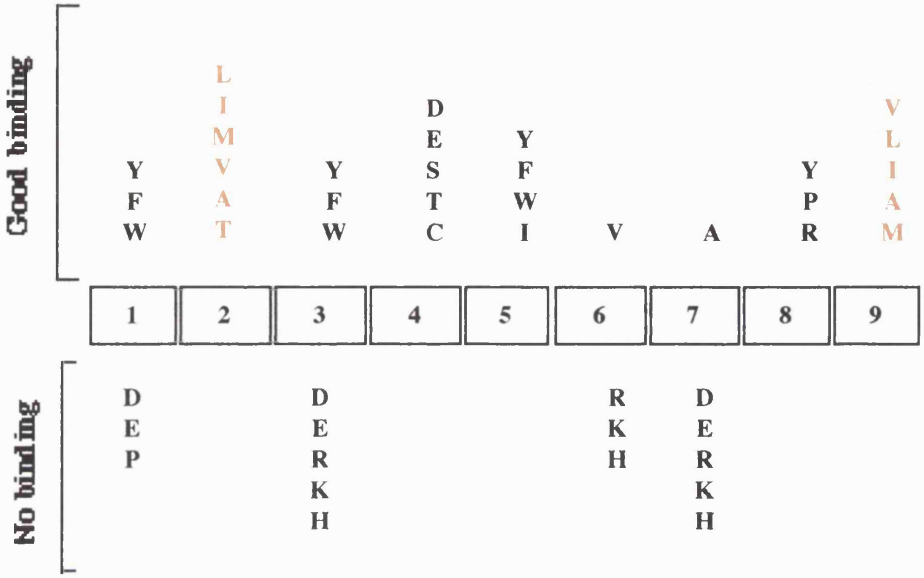
and F respectively on the peptide binding groove of the HLA-A2 molecule (Saper et al., 1991). Some other positions in the peptide sequence have also been identified as being important in the determination of peptide binding to an HLA molecule. These later appear to interact with particular pockets in the peptide binding site, and are enriched for specific amino acids defined as "secondary anchor residues" (Ruppert et al., 1993). Thus, depending on the presence or absence of particular dominant anchor or secondary anchor residues in set positions of the peptide sequences, shown in Figure 3.1 for HLA-A2, the binding affinity of the peptide for a given HLA molecule may either be favoured or reduced (Drijfhout et al., 1995; Ruppert et al., 1993). In several instances these peptide binding motifs, in combination with peptide binding assays, have proven useful in the identification of cytotoxic T cell peptide epitopes from a given protein (Hill *et al.*, 1992; Pamer *et al.*, 1991).

Bearing this in mind, in the present study, the identification of potential CTL peptide epitopes from the CMV pp65 protein was performed using the following strategy: Firstly the pp65 amino acid sequence was screened for peptides containing the anchor binding motifs for HLA-A2. The HLA-A2 molecule was chosen since this molecule is the most common HLA class I allele in the Caucasoid population, and its peptide binding motif is known (Falk et al., 1991; Rammensee *et al.*, 1995; Ruppert et al., 1993). Thus a list of candidate peptides with the potential to bind to HLA-A2 molecules was obtained. The peptides were then synthesised, and peptide binding assays were performed to exclude weak binding peptides. Two peptide binding assays were employed, one of them the T2 stabilisation assay, takes advantage of the HLA-A2 positive mutant cell line T2, which has a reduced capacity to present endogenously processed peptides. The processing deficiency of the T2 cell line is due to a deletion in the MHC class I region, which includes the TAP genes (Cerundulo et al., 1990). Thus, endogenous peptides cannot be translocated from the cytosol into the endoplasmic reticulum, where they could bind and stabilise MHC class I molecules. Consequently T2 cells mainly express unstable, empty HLA-A2 molecules on

their surface. Such molecules can be stabilised by the addition of HLA-A2 binding peptides, leading to a higher density of class I molecules on their surface, which can be monitored by immunofluorescence analysis (Hobohm and Meyerhans, 1993; Stuber *et al.*, 1992). Thus it represents a simple read-out system to study the binding of the pp65 derived peptides to the HLA-A2 molecule (Cerundulo, 1991).

Another method used in this study to evaluate the relative affinity of selected peptides for HLA-A2 molecules was a peptide binding competition assay, which takes advantage of a fluoresceinated reference peptide known to bind with high affinity to HLA-A2 molecules. Peptides are tested for their ability to compete for the binding of the reference peptide to HLA-A2, and thus a measurement of their affinity for this molecule can be made. Thus, while the T2 stabilisation assay gives a qualitative assessment of the affinity of peptides for the HLA-A2 molecule, the peptide-binding competition assay allows the semiquantitative determination of the binding capacity of peptides under investigation (Burg et al., 1995).

a) 9-mer peptides



b) 10-mer peptides

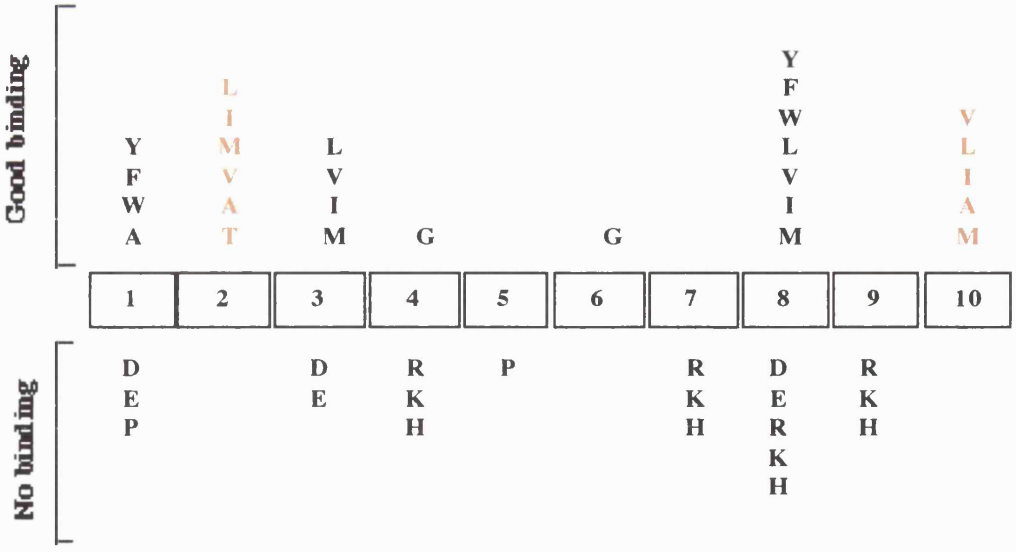


Figure 3.1. Residues which conform to the peptide binding motif for HLA-A2 molecules.

Dominant anchor residues for HLA-A2 molecules in positions 2 and 9 (in nonamers) or 10 (in decamers) are shown in red, whilst amino acids identified as secondary anchor residues are shown in black. The association of the latter with no binding (or poor) or good binding to HLA-A2 is indicated (Adapted from Ruppert et al (Ruppert et al., 1993)).

3.2 Results

3.2.1 Determination of peptides derived from the CMV pp65 protein with the binding motifs for HLA-A2

To identify pp65 derived peptides which would have the capacity to bind to HLA-A2 molecules, the amino acid sequence of the pp65 protein from the CMV strain AD169 (Figure 3.2) was searched for the presence of peptides sharing the consensus binding motifs for HLA-A2, which were first described by Falk *et al.* (Falk et al., 1991). In the first screening, all nonameric and decameric sequences which had either a Leucine, Isoleucine, Methionine, Valine or Alanine in position 2, and a Valine, Leucine, or Isoleucine in positions 9 or 10 were selected. As a result of this screening, 38 CMV pp65 peptides which conformed to this binding motif were identified. In order to limit the number of peptides to be synthesised and tested in peptide binding assays, a second screening was performed. This screening restricted the selection only to those peptides which possessed the most dominant anchor residues previously reported, which are Leucine and Isoleucine in position 2 and Valine, Isoleucine and Leucine in position 9 or 10 (Falk et al., 1991; Ruppert et al., 1993). This second screening identified 17 peptides with the described motifs. Table 3.1 shows the sequence of these 17 pp65 peptides, and their position on the pp65 amino acid sequence. Peptides were placed into groups, based on the presence of the particular anchor residues pairs in positions 2 and 9 or 10, i.e. Leu-Val, Ile-Val, Leu-Ile, Ile-Leu, and Leu-Leu (for amino acid abbreviations see Appendices).

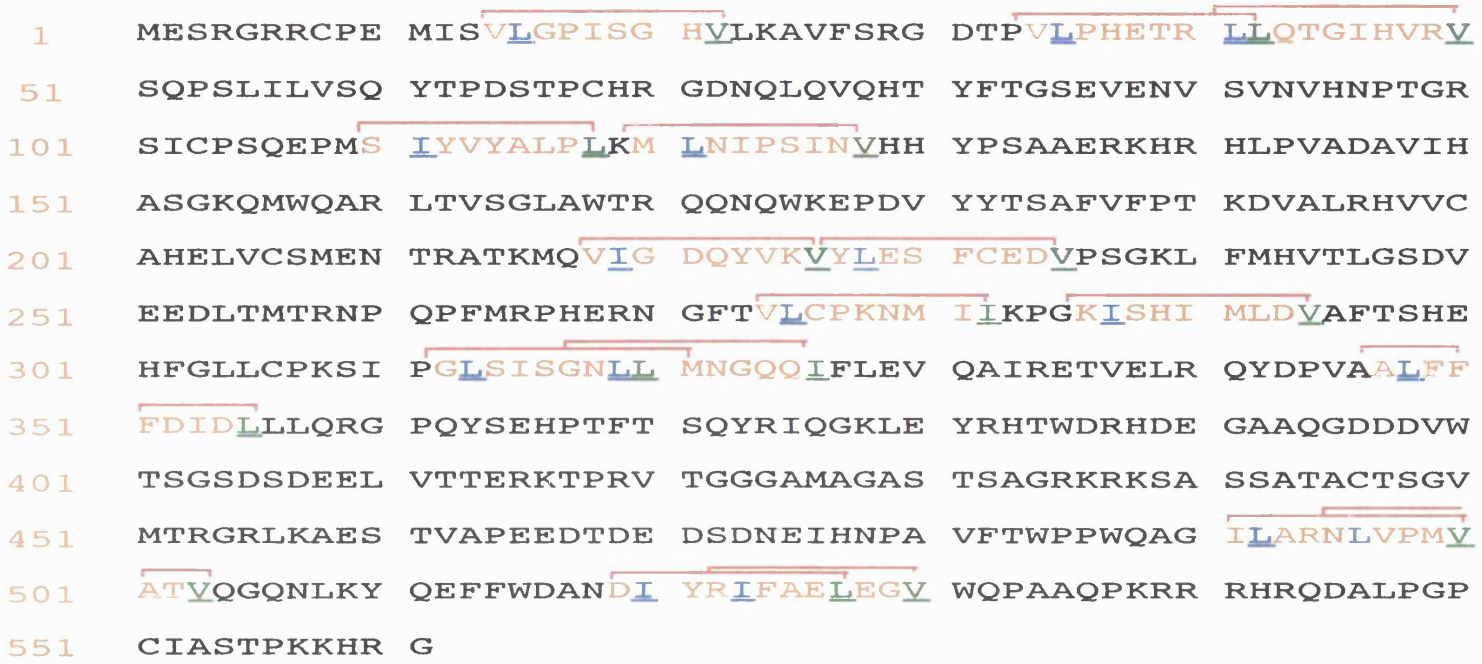


Figure 3.2 The CMV pp65 amino acid sequence illustrating the position of the peptides which contain the binding motifs for HLA-A2.

The above sequences represent nonamers and decamers (in orange) with the dominant anchor residues for preferential binding to HLA-A2 present in the pp65 protein. Peptides which are present in the overlapping sequences are shown with 2 lines. Anchor residues, L or I at position 2 (blue) and V, L or I in positions 9 or 10 in nonamers or decamers respectively (green) are underlined.

Table 3.1. CMV pp65 derived peptides with the HLA-A2 binding motif

Binding motif[a]	peptide	Position[b]	amino acid sequence[c]
L/V	AE42	495-503	NLVPMVATV
	AE44	14-22	VLGPISGHV
	AE45	120-128	MLNIPSINV
	AE47	41-50	LLQTGIHVRV*
	AE48	40-48	RLLQTGIHV
	AE49	491-500	ILARNLVPMV*
	AF88	227-235	YLESFCEDV
I/V	AF83	218-226	VIGDQYVKV
	AF84	286-295	KISHIMLDV
	AF85	522-530	RIFAELEGV
L/I	AF86	274-283	VLCPKNMII
	AF87	318-326	NLLMNGQQI
I/L	AF89	110-118	SIYVYALPL
	AF90	519-527	DIYRIFAEL
L/L	AF91	34-42	VLPHETRLL
	AF92	347-356	ALFFFDIDL
	AF93	312-320	GLSISGNLL

[a] HLA-A2 dominant anchor residues present in the peptide in positions 2 and 9 or 10.

[b] The position of the first and the last amino acid of the peptide in the CMV pp65 protein derived from the virus strain AD169.

[c] The amino acid sequence of potential HLA-A2 binding peptides where anchor residues have been underlined. Peptides with an asterisk * are decamers.

3.2.2 Standardisation of the T2 stabilisation assay

The ability of the pp65 derived peptides identified in the previous section to bind to HLA-A2 molecules was determined by measuring peptide-induced stabilisation of HLA-A2 on the cell surface of T2 cells.

The optimal experimental conditions for the T2 stabilisation assay were first assessed by testing a number of different parameters. In the initial experiments, the stabilisation of HLA-A2 molecules on the cell surface was detected with the monoclonal antibody W6/32 (Barnstable, 1978). This antibody recognises a conformational epitope in the MHC class I molecule which is dependent on the association of the HLA-A, B or C alpha chain with specific peptide and β_2m (Parham et al., 1979). Due to the fact that the antibody W6/32 is not allele specific, a second monoclonal antibody MA2.1, which recognises an antigenic determinant shared by HLA-A2 and B17 (McMichael *et al.*, 1980) was also tested. The results demonstrated that no significant differences were observed between MA2.1 and W6/32 (data not shown). Therefore, W6/32 was used in subsequent experiments as this antibody is produced in house, and a large standard batch could be made for use in subsequent experiments.

3.2.3 The influence of β_2m on the T2 stabilisation assay

There are some reports which describe the T2 stabilisation assay performed in the presence of exogenous β_2m (Nijman *et al.*, 1993), whilst in others exogenous β_2m is omitted (Houbiers *et al.*, 1993; Reali *et al.*, 1996). Therefore it was important to test whether exogenous β_2m had an effect on the stabilisation of HLA-A2 molecules by exogenous peptides. As β_2m is present in foetal calf serum (FCS), for these experiments cells were maintained either in the presence or the absence of FCS, and the addition of exogenous β_2m was also evaluated. Thus, three different conditions were tested: a) culture medium containing FCS, b) serum free medium supplemented with exogenous β_2m (20 μg/ml) and c) serum free medium with no exogenous β_2m added. In these experiments the HLA-A2-binding peptide Flu-MP 58-66, GILGFVTL or the HLA-A2 non-binding peptide Flu-NP 383-392, SRYWAIRTR were used as controls, at three different concentrations. Figure 3.3 shows an increase in the stabilisation of HLA-A2 complexes on the T2 cell surface induced by the positive control peptide Flu-NP 58-66. This peptide-induced stabilisation was not significantly different whether β_2m was present (either in FCS or in its native form added exogenously) or not (Figure 3.3). The same results were observed when different concentrations of β_2m, ranging from 5 to 50 μg/ml, were used (data not shown). The non-binding peptide Flu-NP 383-392 was unable to stabilise class I complexes, either alone, or in the presence of β_2m (Figure 3.3). The addition of exogenous β_2m in the absence of peptide had no effect on the stabilisation of HLA-A2 (Figure 3.3)

As the three conditions tested gave similar results, for subsequent experiments T2 cells were maintained in serum free and β_2m free medium, and the assay was performed in the same conditions. These conditions were preferred over conditions in the presence of

serum in order to avoid the possibility that serum proteases could further degrade the peptides tested, thereby possibly affecting the results.

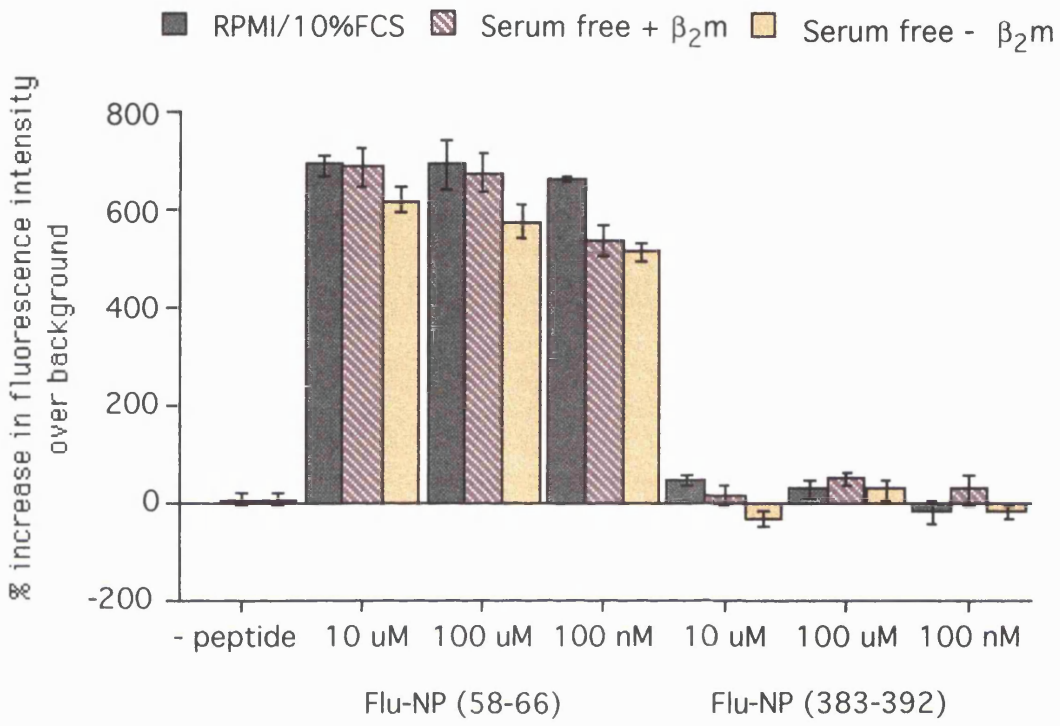


Figure 3.3. The effect of β2m on the peptide-induced stabilisation of HLA class I molecules on T2 cells.

T2 cells were either maintained in serum free medium or in RPMI/10% FCS as indicated. $5x10^5$ cells were incubated either without peptide or with 100 nM, 10 μM or 100 μM of peptides Flu-NP 58-66 or Flu-NP 383-392, for 18 h at 37 °C in the presence or absence of 20 μg/ml of β_2m. All cells were then labelled with the conformational-dependent HLA class I-specific antibody W6/32, and analysed by flow cytometry as described in section 2.3.2. The results are expressed as the percentage increase in fluorescence intensity of cells incubated with peptides after subtraction of the background of W6/32 binding to T2 cells incubated without peptide. This figure shows the mean and standard deviation of triplicate samples.

3.2.4 The identification of CMV pp65 peptides which are able to stabilise HLA-A2 molecules on T2 cells

Following the establishment of optimal conditions for the T2 stabilisation assay, the 17 peptides derived from pp65 bearing the putative binding motif for HLA-A2 (Table 3.1 and Figure 3.4) were screened for their ability to stabilise the HLA-A2 molecules on the surface of the T2 cells (Figure 3.4). While the positive control, peptide Flu-NP 58-66, resulted in a 6 fold increase in the stabilisation of cell surface HLA-A2 molecules (as measured by an increase in fluorescence intensity) the negative control Flu-NP 383-392, HLA-B27-binding peptide showed no such increase. Of the 17 pp65 derived peptides, 6 were able to stabilise HLA-A2 molecules on T2 cells. These peptides showed an increase in fluorescence intensity ranging from 1.5 to 3.5 fold compared with control values of T2 cells without peptide. Interestingly, peptides AE42, AE44 and AE45, bearing the dominant anchor residues Leucine and Valine at positions 2 and 9 respectively, showed an increase in HLA class I stabilisation (2.5, 3 and 3.5 fold respectively), whilst peptides AE47, AE48, AE49 and AF88 showed minimal or no stabilisation, although they possessed the same anchor residues. Another group of peptides which showed some degree of stabilisation were those bearing the dominant anchor residues Isoleucine and Valine (at positions 2 and 9 respectively). This group of peptides, which was comprised of AF83, AF84, and AF85, as well as the peptide AE91 with Leucine in position 2 and 9 as anchor residues, showed an increase in fluorescence intensity of 2 fold above the background.

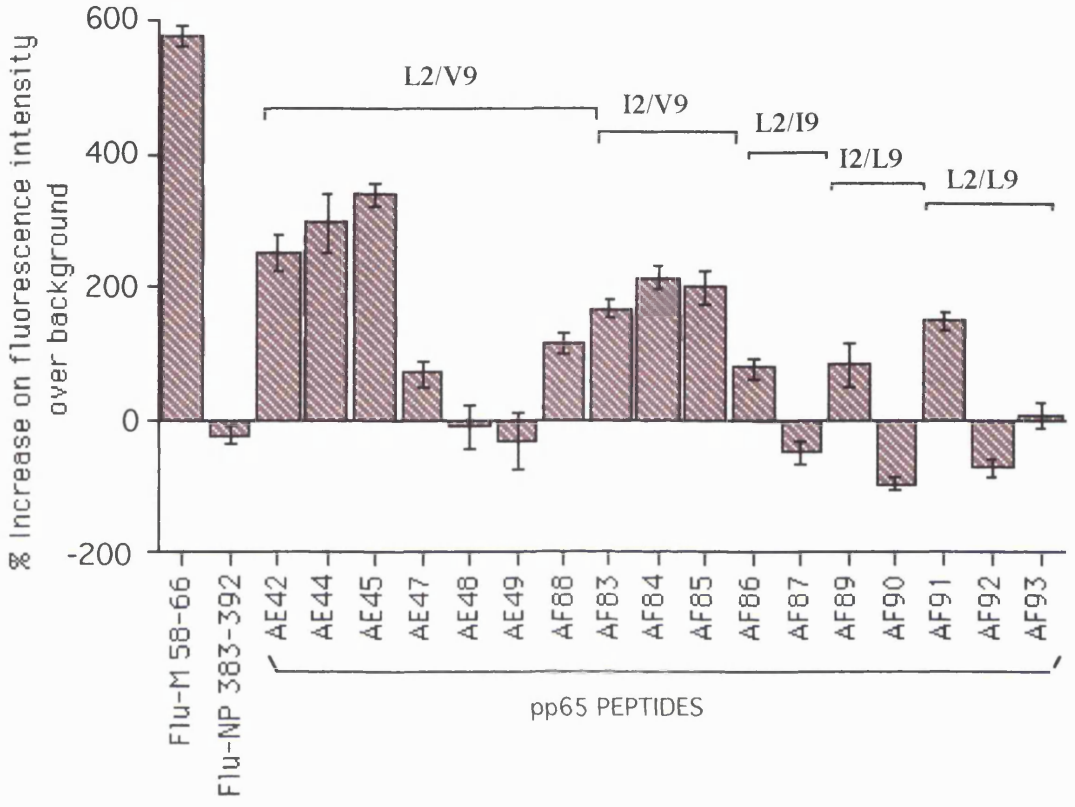


Figure 3.4. The stabilisation of cell surface HLA-A2 molecules on T2 cells induced by CMV pp65 peptides.

T2 cells were maintained in serum free medium and $5x10^5$ cells were incubated with 100 μM peptide for 18 h at 37 °C, before staining with the class I specific antibody W6/32. Stabilisation of cell surface HLA molecules by peptide was detected by indirect immunofluorescence and flow cytometric analysis. The influenza virus derived peptides Flu-M 58-66 and Flu-NP 383-392 were used as positive and negative controls respectively. The results are expressed as the percentage of increase in fluorescence intensity over the background which corresponds to T2 cells incubated without peptide, and was calculated as described in Chapter 2 section 2.3.2. The data shown represents the mean± standard deviation for triplicate samples. The different binding motifs for HLA-A2 that each peptide possesses is shown above the bars using the single letter amino acid code and the number indicates position of the residue in the peptide.

3.2.5 *Standardisation of the peptide-binding competition assay*

The second peptide binding assay employed in this study was the peptide-binding competition assay. This assay allows the semiquantitative determination of the binding capacity of the peptides (Burg et al., 1995) in contrast to the T2 stabilisation assay, which is only a qualitative method.

In this assay, the lymphoblastoid cell line JY, positive for HLA-A2, was acid-treated to remove the peptides present in the HLA molecules on its cell surface. Acid-treated JY cells were then incubated with a mixture of the fluoresceinated reference peptide, and non-labelled test peptides at varying concentrations. The peptide used as a reference was a decamer derived from the hepatitis B virus core protein, corresponding to the amino acid residues 18-27 (FLPSDCFPSV) which has been shown to have a high binding affinity for HLA-A2. Peptides which were capable of binding to the HLA-A2 molecule would compete for binding with the fluoresceinated reference peptide, and the extent of inhibition of the reference peptide provides a measure of the relative affinity of the peptides tested. This affinity is reported as the concentration of peptide necessary to inhibit the binding of the reference labelled peptide by 50% of total binding capacity (IC_{50}).

In order to determine the optimal experimental conditions for the peptide-binding competition assay, and to obtain the IC_{50} values of known high affinity-binding peptides, two known HLA-A2 restricted CTL peptide epitopes were tested in this assay. One of these peptides was derived from the polymerase gene product of human immunodeficiency virus-1 (HIV-1 polymerase 468-476) (Tsomides *et al.*, 1991) and the second was the influenza derived peptide Flu-NP 58-66 (Bednarek *et al.*, 1991), which was also used as a positive control for the T2 stabilisation assay. The HLA-A*0301 restricted CTL peptide epitope, derived from the human papillomavirus protein E6 (IVYRDGNPY), which is known to have a very low affinity for HLA-A2 (Burg et al., 1995) was used as a negative

control. Figure 3.5 shows the ability of the two HLA-A2 restricted peptides to compete for the binding of the fluoresceinated reference peptide to this HLA class I molecule with a high relative affinity, IC_{50} of 8 and 4 μM respectively. In contrast the HLA-A*0301 restricted peptide was unable to compete for the binding to HLA-A2 even at the highest concentration (32.5 μg/ml) tested in this assay (Figure 3.5)

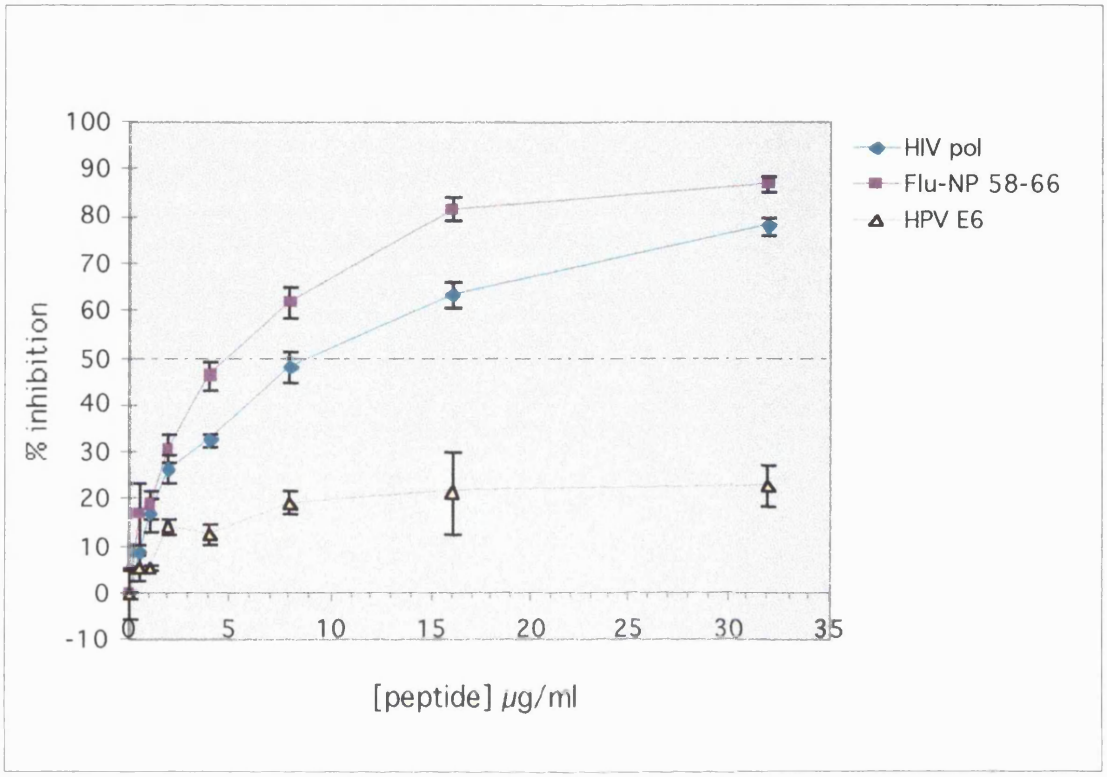


Figure 3.5. Competition of binding of known HLA-A2-restricted CTL peptide epitopes against the fluorescein-labelled reference peptide to empty HLA-A2 molecules.

Acid treated JY cells were incubated with 150 nM of a fluorescein (FL) labelled reference peptide derived from the hepatitis B virus core protein (18-27) FLPSDC(FL)FPSV, and increasing amounts (µg/ml) of the three peptides human immunodeficiency virus-1 polymerase 468-476 (ILKEPVHGV), Flu-NP 58-66 (GILGFVTL) or human papilomavirus protein E6 59-67 (IVYRDGNPY). The inhibition of binding was calculated as previously described in section 2.3.3. The extrapolation of the concentration necessary for each peptide to obtain 50% inhibition (shown by the dotted line), represents the binding capacity of that particular peptide (IC_{50}). The data shown is representative of two experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the triplicates.

3.2.6 Measurements of relative binding affinity of CMV pp65 derived peptides tested by the peptide-binding competition assay

In order to corroborate the results obtained with the T2 binding stabilisation assay described in section 3.2.5, the relative affinity of the group of pp65 peptides bearing the Leucine 2 and Valine 9 motif for the HLA-A2 molecule was further determined by the competition-based HLA class I binding assay described above (Burg et al., 1995). This particular group of peptides was selected for several reasons: firstly the anchor residues L2-V9/10 seem to be predominant observed in high affinity binding peptides in previous studies (Drijfhout et al., 1995), secondly, three of the pp65 derived peptides with these anchor residues showed the highest affinity for HLA-A2 in the T2 stabilisation assay. Furthermore, this group of peptides (with the exception of AE42) had previously been tested in real-time analysis using an optical biosensor system (IASYS™) (Morgan *et al.*, 1998), and therefore, it was convenient to continue working with the same peptides for comparative purposes. Hence, the pp65 derived peptides with the L2-V9/10 anchor residues were tested for competition for the binding to HLA-A2 against the fluoresceinated reference peptide hepatitis B virus core (18-27). As mentioned above, the influenza derived peptide Flu-NP(383-392) was used as a negative control in this assay. As shown in Figure 3.6, six out of the seven pp65 derived peptides tested exhibited either a high, (IC_{50} < 5 μM; (AE45, AE48 and AE47), or intermediate (≤ 15 μM; AE42, AE44, AE49), binding affinity for HLA-A2, whilst peptide AF88 showed only a low binding affinity (> 15 μM). Figure 3.6 also shows a sharp rise in the inhibition of binding induced by high affinity pp65 peptides obtained from 0-10 μg/ml, reaching a plateau of saturation at levels of 20 μg/ml. As expected, the negative control peptide Flu-NP (383-392) showed no competition at the highest concentration tested in this assay (48 μg/ml). (Figure 3.6). Interestingly, peptides AE47 and AE48 which were unable to stabilise HLA-A2 molecules in the T2 stabilisation assay, appeared to have

intermediate and high affinities for HLA-A2 in this peptide-binding competition assay respectively (Table 3.2).

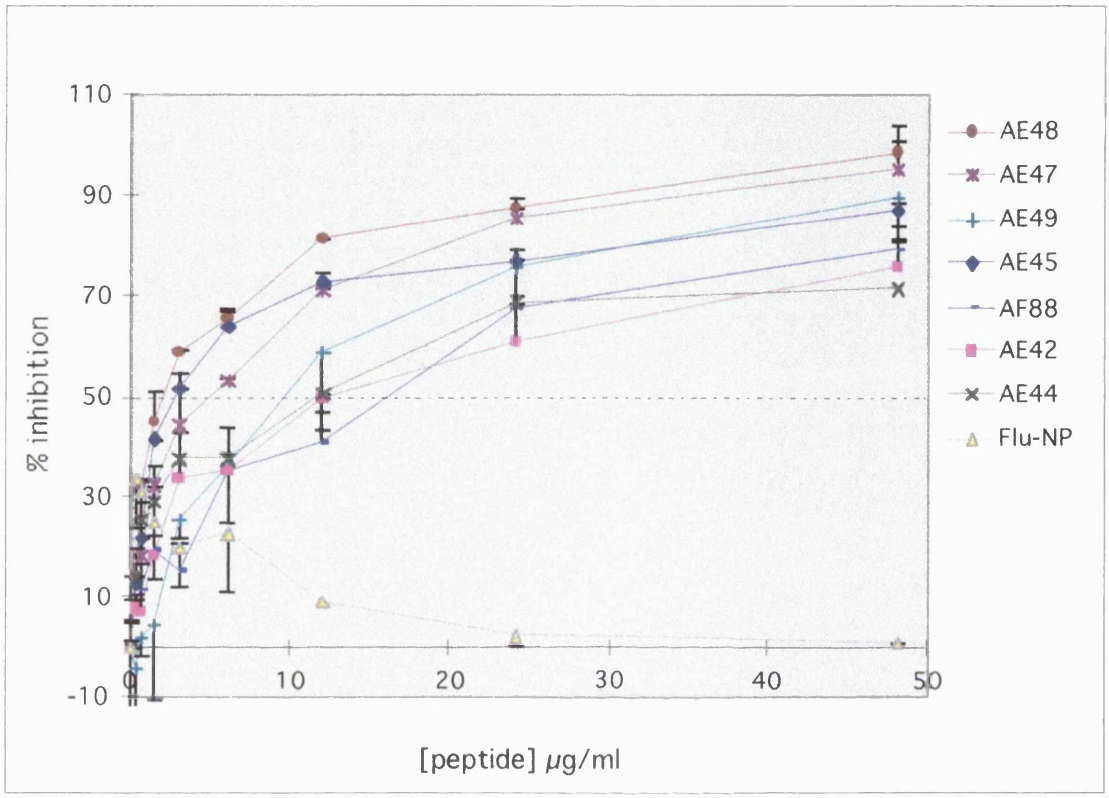


Figure 3.6. Competition of binding of CMV pp65-derived peptides against a fluorescein labelled reference peptide to empty HLA-A2 molecules.

Acid-treated JY cells were incubated with 150 nM of the fluorescein (FL) labelled reference peptide derived from the hepatitis B virus core protein (18-27) FLPSDC(F1)FPSV, and increasing amounts (μg/ml) of pp65 peptides bearing the HLA-A2 binding motif L2-V9/10. Peptide Flu-NP (383-392) was also used as a negative control. Inhibition of binding was calculated as described previously in Figure 2.3.3. The dotted line represents 50% inhibition of binding of the control peptide. The extrapolation of the concentration of each peptide necessary to obtain this level of inhibition represents the binding capacity of that particular peptide (IC_{50}). Samples were assayed in triplicate, and the results shown are representative of two independent experiments. The figure shows the mean and standard deviation of the three triplicates

3.2.7 The analysis of potential pp65 HLA-A2 binding peptides by computer algorithms

The National centre for Biotechnology Information at the National Library of Medicine (www-bimas.dcrt.nih.gov/molbio/hla-bind/), made available a computer program which predicts the number of potential binding peptides for a given MHC molecule, by ranking the potential octamer, nonamer, or decamer peptides, based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker (Parker et al., 1994).

As this algorithm predicting HLA binding peptides was not available at the time when the pp65 peptides bearing the HLA-A2 binding motifs described in section 3.2.1 were identified, it was of interest to determine whether this computer program would find the same peptides as the earlier analysis, and also to see how it would rank the various peptides in their predicted binding affinity to HLA-A2. Therefore, this algorithm was employed to search for all the possible HLA-A2 binding peptides in the amino acid sequence of pp65.

The analysis of the sequence of the pp65 protein using the above program gave the following results: 553 overlapping nonamers (plus 552 overlapping decamers) were predicted as potential binder-peptides. Table 3.2 shows the rank assigned to those peptides that were previously identified as potential HLA-A2 binding peptides in section 3.2.1. The ranking given to the latter peptides was compared with the results obtained by the T2 stabilisation assay and the competition binding assay (sections 3.2.4 and 3.2.6). Table 3.2 lists the rank of each peptide, determined by comparing the theoretical half life of β_2m dissociation to the theoretical half life of β_2m dissociation for all the nonamers that could be generated from pp65 (553). The theoretical half life of β_2m dissociation for each peptide is shown in Table 3.2. The highest ranking peptides found were AE48, AE42,

AF92, AE49, AE45 and AE47. When these results were compared with those obtained in the peptide binding competition assay, it was observed that all these peptides (with the exception of peptide AF92, which was not tested), showed either high or intermediate affinity for HLA-A2 in this binding assay (Table 3.2). However, the results from the T2 binding assay only correlated with the predicted scores for peptides AE42 and AE45 (Table 3.2). Peptide AE44 was assigned a rank of 20th, which was surprising, as this peptide was able to stabilise the HLA class I molecules in the T2 binding assay, and was also able to compete with intermediate affinity in the peptide binding competition assay.

[a]Peptides derived from the CMV pp65 protein and known antigenic peptides derived from the influenza nucleoprotein (Bednarek et al., 1991) or HIV polymerase (Tsomides et al., 1991).

[b]Amino acid sequence of the peptides. Peptides with an asterisk * are decamers the others nonamers.

[c] The rank of peptide determined by computer algorithm, comparing the theoretical half-life of β_2m dissociation to the theoretical half life of β_2m dissociation for all the nonamers and decamers that could be generated from the same protein, as described in section 2.3.4.

[d]Theoretical half life of β_2m dissociation (in min at 37 °C) (Parker et al., 1994).

[e]The capacity of peptides to stabilise HLA-A2 molecules on T2 cells is shown as the percentage increase in fluorescence intensity over background (% FI), as obtained by the T2 stabilisation assay in section 3.2.4.

[f]The results of the peptide binding competition assay (described in section 3.2.6) are shown as the concentration of peptide needed to inhibit binding of the FL-labelled peptide to 50% (IC_{50} values).

Table 3.2 Computer-determined binding capacities to HLA-A2 of 17 pp65-derived peptides

Peptides[a]	Amino Acid[b] Sequence	Theoretical Rank[c]	score[d]	%FI[e]	IC50[f] (μM)
AF92	ALFFFDIDL	2nd	300.35	-72.15	"
AE48	RLLQTGIHV	3rd	257.34	-10.1	2
AE42	NLVPMVATV	4th	159.97	251.08	12.5
AE49	ILARNLVPMV*	4th	271.49	-30.3	10
AE45	MLNIPSINV	6th	118.23	340.55	2.5
AE47	LLQTGIHVRV*	6th	118.23	70.71	5
AF83	VIGDQYVKV	7th	99.98	166.65	ND
AF84	KISHIMLDV	8th	76.98	213.56	"
AF85	RIFAELEGV	12th	39.87	200.58	"
AF87	NLLMNGQQI	13th	38.6	-47.62	"
AF88	YLESFCEDV	14th	38.12	115.44	16
AF93	GLSISGNLL	17th	21.36	7.22	"
AE44	VLGPISGHV	20th	15.37	295.82	12
AF89	SIYVYALPL	24th	9.39	83.69	"
AF86	VLCPKNMII	28th	7.27	77.92	"
AF91	VLPHETRLL	29th	7.26	148	"
AF90	DIYRIFAEL	45th	1.33	-94.91	"
HIV pol	ILKEPVHGV		10	ND	8
Flu-NP 58-66	GILGFVFTL		800	574.67	4

3.3 Discussion

In the present study, a selective approach was adopted in order to rapidly identify CTL peptide epitopes from the CMV protein pp65. Potential antigenic peptides were first selected from the known sequence of pp65 (Ruger et al., 1987), by the presence of HLA-A*0201 anchor residues (Falk et al., 1991), and then a further selection was made by two different methods of their capacity to bind to HLA-A2 molecules.

The results described in this chapter show that, although 17 peptides from the CMV pp65 protein from AD169 carried the appropriate HLA-A2 MHC binding motif, only 7 of them were able to stabilise HLA-A2 molecules as demonstrated by the T2 stabilisation assay. Previous studies had suggested that, besides the dominant anchor residues that comprise a peptide binding motif, the presence of hydrophobic and aromatic residues in secondary anchor positions can be relevant for preferential peptide binding, and that negatively or positively charged residues in the same positions can be associated with no or poor binding (Drijfhout et al., 1995; Ruppert et al., 1993). These latter observations agree with some of the results obtained here, when the pp65 peptides bearing different anchor motifs (L2-V9, I2-L9, L2-I9, L2-L9 or I2-V9) were examined in the T2 stabilisation assay. Peptides AE42, AE44 and AE45 bearing anchor residues Leucine and Valine at positions 2 and 9 respectively, demonstrated particularly good HLA class I stabilisation. These peptides also have several hydrophobic residues in their sequences, such as Valine and Alanine in positions 6 and 7 in peptide AE42. In contrast, peptides AF88, AE47, AE48 and AE49 showed minimal or no HLA-A2 stabilisation, although they possessed the same anchor residues. However, these peptides contain at least one charged residue in secondary positions, which may explain their poor binding. For example, although peptide AF88 has an aromatic residue (Phenylalanine) in position 5, which has been shown to be linked with preferential binding (Drijfhout et al., 1995), its lack of stabilisation of HLA-A2 molecules on T2 cells could be explained by the presence of two negatively charged residues

(Glutamic acid) in positions 3 and 7, since it has been reported that the presence of negatively charged residues at position 3 is common in non-binding peptides (Ruppert et al., 1993).

Certain inconsistencies were found when pp65 derived peptides with the L2-V9 motif were tested in a peptide-binding competition assay. In this assay, all of the peptides, with the exception of peptide AF88, showed either a high or intermediate binding affinity for HLA-A2. Interestingly, the nonamer AE48, and the decamers AE47 and AE49, which did not stabilise cell surface HLA-A2 in the T2 stabilisation assay, appeared to have a high or intermediate relative affinity for the HLA-A2 molecule in the peptide binding competition assay. It is possible that positions 7 and 8, which are secondary residues in HLA-A2 binding decamers, may be influencing peptide binding. For example, AE47 contains a positively charged residue, Histidine, at position 7, and a uncharged Valine residue at position 8. Whilst positively charged residues such as Arginine, Lysine or Histidine are more frequent in HLA-A2 non-binding peptides, uncharged residues at position 8 in decamers have been shown to be associated with high affinity binding to HLA-A2 (Ruppert et al., 1993). Therefore this observation, together with the fact that AE47 possesses preferred anchor residues, may override the detrimental effects of the residue at position 7. In contrast, peptide AE49 contains only one residue in its sequence known to be associated with poor binding, this being an Arginine in position 4 (Ruppert et al., 1993). However, it also possesses an Isoleucine in position 1 which has been associated with good binding (Drijfhout et al., 1995). In the particular case of peptide AE48, no secondary residues were found that would be expected to either decrease or promote the binding of this peptide to HLA-A2. However, when a computer-driven algorithm which analyses the sequence of a given protein in the search for potential T cell peptide epitopes (Parker et al., 1994; Parker *et al.*, 1995), was applied to investigate the possible HLA-A2 binder nonamers or decamers in pp65, peptide AE48 emerged as the

third best binding peptide to HLA-A2. This computer analysis therefore correlated with the results from the peptide-binding competition assay for this peptide.

In general, the pp65 peptides predicted to be good HLA-A2 binding peptides by the computer algorithm, corresponded to peptides which showed a good binding affinity by either of the peptide-binding assays tested in the present study. Two interesting exceptions were observed, peptide AE92 was predicted by Parker's method to be the 2nd best HLA-A2 binding peptide, but this peptide did not show any stabilisation of HLA-A2 molecules when tested in the T2 stabilisation assay. This peptide would be an interesting candidate to test using the peptide binding competition assay, however, due to time limitations it was not tested. In contrast, peptide AE44, which in both the peptide binding assays utilised in the present study showed an intermediate affinity for the HLA-A2 molecule, appeared only at 11th place on the list of the best HLA-A2 binding peptides from the computer algorithm. A similar situation was found with the HIV-derived peptide ILKEPVGH, which is an HLA-A2 restricted CTL epitope. This peptide "theoretically" ranks 45th of the 1007 possible nonamers in the HIV polymerase, which would place it only in the top 5%. Nevertheless, this peptide binds to HLA-A2 much better than expected (Parker et al., 1994), and this peptide was used as a positive control in the peptide-binding competition assay employed in the present study, where it also showed a high binding affinity for HLA-A2. In contrast, the influenza matrix peptide Flu-M (58-66) GILGFVTL, which is a known HLA-A2 CTL peptide epitope and also showed consistently good binding in the assays tested here, ranks first among all possible nonamers from the matrix protein and therefore conforms to the expectations. These observations suggest that the prediction of good binding peptides following either the presence of peptide-binding motifs or algorithms like the one used here, offer a fast and efficient strategy, but may be relatively imprecise.

Inconsistency between the T2 stabilisation assay and the peptide-binding competition assay could possibly be attributed to poor sensitivity of the first method. The latter proposal is supported by a report showing that where the T2 stabilisation assay has been compared to other peptide binding assays that employ either fluoresceinated or radiolabelled peptides to measure affinity, and it was found that they required a lower concentration of peptide to demonstrate a positive effect compared to the T2 stabilisation assay (Zeh III *et al.*, 1994). However, there are other factors to consider when correlating the results obtained with the peptide-binding competition assay and the T2 stabilisation assay, i.e. the fact that two peptide binding assays were performed at different temperatures and in different cell types. This may influence the stability and expression levels of empty HLA class I molecules on the cell surface (Baas *et al.*, 1992; Schumacher et al., 1990)

Discrepancies similar to the ones observed between the T2 binding assay and the competition assay were found when a number of the pp65 derived peptides (AE44, AE45, AE47, AE48 and AE49) tested in this study were analysed in a separate study at the Anthony Nolan Research Institute using an optical biosensor IASYS™ system. The five peptides tested in the latter study significantly increased β_2m exchange kinetics in that system (Morgan et al., 1998) which, although not being a direct measurement of the affinity of peptides for the HLA-A2 molecule, suggested that such peptides facilitated the stabilisation of peptide-MHC-β_2m complexes. These observations highlight the increased sensitivity of the peptide binding competition assay and also the Iasys™ over the T2 stabilisation assay.

In summary, utilising peptide binding motif predictions and the binding assays described here, it was possible to identify a number of peptide sequences from CMV pp65 that stabilised HLA-A2 molecules present on the surface or in the T2 cell line, and which showed either high or intermediate affinity for the HLA-A2 molecule in peptide-binding

competition assays. However, at the present time, the binding predictions are imprecise, and it is known that MHC binding is necessary but not sufficient for a peptide to be immunogenic for $CD8^+$ T cells. It is therefore essential to confirm that the predicted CTL peptide epitopes are able to be generated by endogenous antigen presentation pathways *in vivo*, and that they are also capable of eliciting a CTL response. This point will be crucial if the synthetic peptides in question are to be considered useful as vaccines or for any other form of therapy. Thus it would be useless to generate a CTL response *in vitro* to a predicted peptide fragment of a viral antigen, if that peptide was never generated during a viral infection *in vivo* and did not stimulate a CTL response. Therefore, 5 of the pp65-derived peptides identified in the present study which showed high or intermediate affinity for the HLA-A2 molecule were tested for their ability to generate a CMV-specific CTL response *in vitro*, and this work forms the basis for the results described in Chapter 5.

4. Chapter 4. Construction of pp65 DNA vectors and generation of pp65 expressing cell lines

4.1 Introduction

The classical approach for the generation of a CMV-specific CTL response has been to reactivate CMV-specific CTL precursors by *in vitro* re-stimulation of lymphocytes from CMV-immune donors using CMV-infected fibroblasts as stimulator cells (Borysiewicz et al., 1988b; Reusser et al., 1991; Riddell et al., 1991a). One of the problems which may complicate this type of approach is that CTL specificities reactivated in different individuals are composed of a number of reactive lines restricted through different HLA class I alleles, with each individual allele presenting peptides derived from one or more of the potential viral antigens that are capable of being expressed in the infected cell.

One way to overcome this stimulation of multiple CTL specificities would be the use of a cell which expresses only one CMV protein, such as pp65, in concert with a single HLA class I antigen. A cell line that could be of use in this type of approach is the B lymphoblastoid cell line L721.221, which lacks endogenous HLA class I expression (Kavathas *et al.*, 1980). This cell line has been transfected with single HLA class I alleles, and used in experiments where it has been desirable to consider HLA class I alleles in isolation (Shimizu and DeMars, 1989). Thus, when transfected with a particular HLA class I allele, any peptides derived from endogenously processed antigens in these cells would be presented only in the context of the transfected HLA molecule. Therefore if such

transfected cells were additionally transfected with a gene coding for the pp65 protein, the presentation of peptides derived from the pp65 gene product could be studied in the context of a single class I molecule. As described in chapter 3, the restriction element chosen for the present study was the HLA-A*0201 (HLA-A2) antigen, which is the allele displaying the highest frequency in the Caucasoid population.

Another strategy to generate pp65-specific CTLs would be to stimulate lymphocytes from CMV seropositive individuals using pp65 derived peptides. However, in order to confirm that the peptides used to prime stimulator cells have been able to generate a CMV and pp65-specific CTL response, the CTLs generated must be able to recognise cells in which pp65 was being endogenously produced. Thus a cell line which is HLA-A2 positive and expresses pp65 in the absence of other CMV proteins would be ideal for this strategy.

The aim of the work presented in this chapter was to generate a panel of cell lines expressing the CMV pp65 protein in the context of HLA-A2, which could be useful as a tool in the identification of CMV pp65 derived peptide epitopes. These cells could either be utilised as stimulator cells in the generation of pp65-specific T cells, or as target cells in cytotoxicity assays in order to determine whether CTLs generated against pp65-derived synthetic peptides were able to recognise endogenously processed pp65 protein.

Three alternative vector constructs encoding the pp65 protein were produced in order to generate cell lines which expressed pp65. The first vector used was the episomal expression vector, pS. This vector possesses the long terminal repeat promoter Rous sarcoma virus (RSV LTR). This enhancer-promoter sequence enables the constitutive expression of the gene of interest, which is maintained extrachromosomally in eukaryotic cell lines (Chittenden *et al.*, 1989; Yates *et al.*, 1985). The second mammalian expression vector used was pEGFP-N2. This is a protein fusion vector encoding a green fluorescent protein from the jellyfish *Aequorea victoria* (Chalfie et al., 1994; Prasher et al., 1992). This green fluorescent protein can be used as a marker of the intracellular localisation of

proteins in a variety of cell types (Gerdes and Kaether, 1996; Kaether and Gerdes, 1995; Pines, 1995). The third vector used was a recombinant replication deficient adenovirus vector carrying the CMV pp65 gene, RAd-pp65. This vector has been shown to induce high level expression of pp65 without the expression of adenoviral proteins (Wilkinson and Akrigg, 1992).

4.2 Results

4.2.1 Amplification of the CMV pp65 gene by PCR

In order to obtain the pp65 gene to clone into the mammalian expression vectors mentioned above, pp65 was obtained from whole CMV DNA by PCR amplification. A CMV virus stock was obtained from human fibroblasts infected with the CMV strain AD169. Virus particles were then harvested at day 5 and 6 post infection, and virion DNA was prepared from the cell culture supernatants. This viral DNA was used in PCR amplification reactions using oligonucleotide primers specific for the pp65 gene. These primers, corresponding to the 5' and 3' ends of the nucleotide sequence of pp65, were synthesised with the restriction sites for the enzymes Kpn I and Bam HI respectively. This allowed the resulting amplified DNA to be cloned readily into expression vectors containing the same enzyme restriction sites in their polycloning cassette. A tail of guanine and cytosine bases (GC's) was also included at the 5' end of these primers in order to facilitate anchoring of the restriction enzyme. PCR reactions were performed using Taq DNA polymerase in initial experiments, and a proof-reading DNA polymerase (Pfu, Stratagene™) in subsequent experiments.

4.2.2 The cloning of pp65 into M13mp19

To confirm that the pp65 PCR product obtained in the previous section had the correct DNA sequence previously reported by Ruger *et al.* (Ruger et al., 1987), the pp65 PCR product was cloned into the filamentous *E. coli* bacteriophage M13mp19, and sequenced by single-strand DNA sequencing.

In M13mp19, the region between genes II and IV (see Appendices) was used as the site for insertion of foreign DNA into the vector. It carries a polylinker containing restriction enzyme recognition sites, including the sites for Kpn I and Bam HI, which were employed to clone the pp65 PCR product into this vector. The pp65 PCR product was flanked by a Kpn I restriction site in its 5' end and a Bam HI restriction site in its 3' end. This aided in cloning this fragment into the M13mp19 bacteriophage, as both the vector and the insert were digested with these restriction enzymes. Restricted M13mp19 and pp65 PCR products were purified, and ligated using a molar ratio of M13mp19 (7.5 kb) to pp65 (1.7 kb) of approximately 3:1. Self-ligation of the M13mp19 was prevented by removal of the 5' phosphate groups of the vector with the enzyme calf intestinal alkaline phosphatase. The ligation reactions were used to transform competent DH5αF' *E. coli* bacteria, which were then plated onto agar plates containing IPTG and X-Gal. Under these conditions the recombinant bacteriophages with the insert will appear as colourless plaques, whilst intact bacteriophage vectors will appear as blue plaques (as described in section 2.6.4) In order to confirm that colourless plaques corresponded to recombinant M13mp19 clones containing the pp65 insert, single-stranded DNA was prepared from 16 of these colourless plaques. Recombinant M13mp19 clones were identified by their reduced mobility in agarose gel electrophoresis when compared to single-stranded M13mp19 DNA, or to blue plaques corresponding to the vector without the insert. Fifteen out of the 16 clones from colourless plaques showed a reduced mobility in the agarose gel. This retardation in the

agarose gel was indicative of an increase in DNA size and hence the positive presence of the pp65 insert.

4.2.3 Sequencing of M13mp19-pp65

The presence and identity of pp65 in the recombinant M13mp19 clones identified in the previous section, was performed by single-stranded DNA sequencing on 5 of these clones. DNA sequencing was performed by the dideoxy chain termination method. Oligonucleotides employed for sequencing were designed to obtain overlapping sequences of all the fragments sequenced (Figure 4.1), which spanned a length of approximately 400 bp. The first 10 bp (5' end of pp65) was sequenced using the -40 bp universal M13mp19 primer, whilst the rest of the sequence (1686 bp total) was obtained using 6 pp65- specific oligonucleotides.

The sequences obtained were entered into the Gene Jockey computer analysis program. Using this program the sequence of the pp65 containing clones was verified and compared to the originally reported sequence of pp65 recorded in the Gene Bank (Ruger et al., 1987).

Three non-conserved nucleic acid misincorporations were identified in the clones sequenced initially. It was thought that this result might have been attributable to the Taq polymerase used in the DNA amplification reactions. In order to resolve this problem, PCR amplifications of the pp65 gene were repeated using a proof reading DNA polymerase (pfu DNA polymerase) instead of Taq polymerase. This pfu DNA polymerase not only possesses a 5' to 3' DNA polymerase activity, as does the Taq polymerase, but also a 3' to 5' proof-reading exonuclease activity. Consequently it had a 12-fold higher fidelity of DNA synthesis than Taq DNA polymerase. The new pp65 PCR products were cloned into M13mp19 and used to transform competent DH5αF' *E. coli* bacteria as described above. Sequencing the first 200 bp using a pp65 5' end specific primer, 8 clones were found to be

carrying the pp65 insert. Two of these clones, C1.8 and C1.6 were sequenced in their entirety.

A problem found when sequencing the pp65 gene was the presence of regions in the autoradiogram in which the bands appeared in close proximity to each other, making it impossible to distinguish the number of bands present in that region. These events, known as compressions, are due to the presence of regions rich in the nucleotides dGTP and dCTP, which are found frequently in the pp65 DNA sequence. The compressions are formed by the secondary structure of the DNA, which is not fully denatured during electrophoresis. Sometimes the problem can be resolved by increasing the temperature of the gel during electrophoresis, which would denature these structures, however, this strategy was not successful when used here. Another strategy reported by Sambrook *et al.* (1989) was to use a nucleotide analogue such as dITP (2' deoxyinosine-5' triphosphate), 7-deaza-dGTP (7- deaza-2' deoxyguanosine-5' triphosphate) or 7-deaza-dATP (7- deaza-2' deoxyadenosine-5' triphosphate) in the sequencing reactions. These analogues pair weakly with conventional bases and are good substrates for DNA polymerases. Such an approach was therefore employed, using deaza G/A T7 containing sequencing mixes, which were run in parallel with dATP in all the sequencing reactions. Utilising this strategy, sharper bands were obtained, the compressions were eliminated and, the derived sequences were found to be identical to the published sequence of pp65 (Ruger et al., 1987).

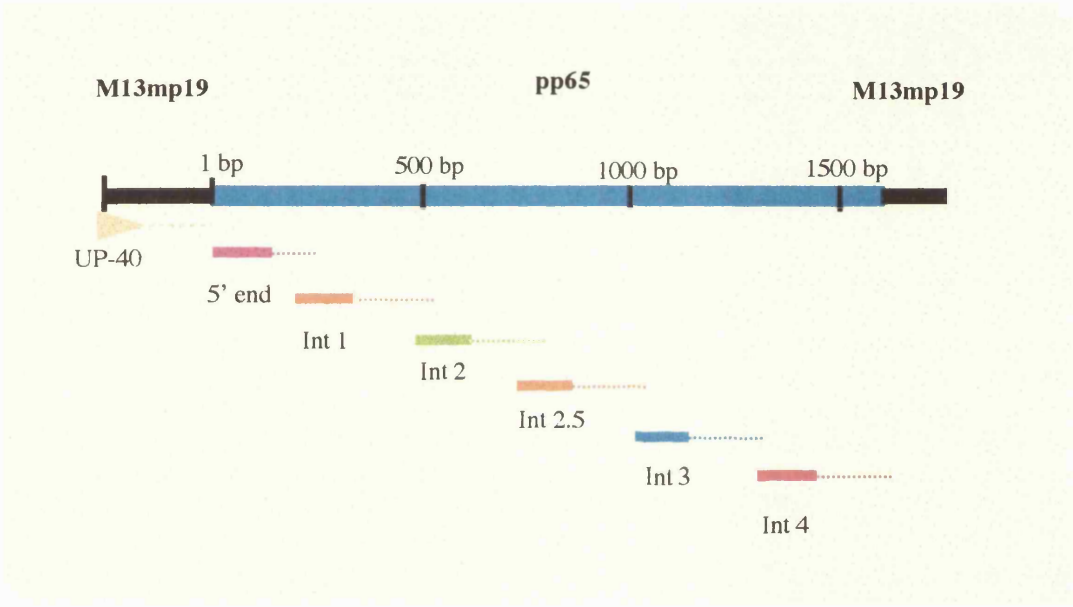


Figure 4.1. The position of oligonucleotides used in the sequencing of the pp65 gene.

CMV pp65 DNA was cloned into M13mp19, sequenced using 6 primers derived from pp65 and a universal primer from M13mp19 (UP-14). The arrow points to the direction of polymerase extension. Dotted lines indicate the approximate length of the fragment sequenced with each oligonucleotide.

4.2.4 The subcloning of pp65 into the mammalian expression vector pS

Large scale preparation of double-stranded DNA was performed from the M13mp19-pp65 recombinant clone, C1.8, and this vector was enzymatically digested to allow the recovery of the pp65 gene for subsequent subcloning into the different mammalian expression vectors. The first vector employed for subcloning pp65 was the expression vector pS, which contains the gene that confers hygromycin resistance when transfected in mammalian cell lines, and an ampicillin resistance cassette which confers resistance to this antibiotic when transfected into bacterial cells (for vector maps see Appendices).

The excision of the pp65 cDNA from M13mp19 of the sequence clone C1.8 was performed with the restriction enzymes Kpn I and Bam HI. This generated a 1.7 kb fragment which was excised from a low melting point agarose gel. The pp65 fragment was purified and ligated into the expression vector pS which had been digested with the same restriction enzymes. The ligation mix was used to transform competent *E. coli* bacteria. The latter were then plated on to ampicillin containing agar plates. Fifteen recombinant ampicillin-resistant colonies were chosen, and plasmid DNA extracted from these. The plasmid DNA was analysed for the presence of the pp65 insert by restriction enzyme analysis using Kpn I and Bam HI. Agarose gel electrophoresis of the digests revealed the presence of the insert in four ampicillin-resistant colonies. One of these colonies was expanded by large scale plasmid preparation, and the DNA digested as above to confirm the presence of the pp65 insert (Figure 4.2). 300 µg of pS-pp65 DNA, determined by spectrophotometry, was recovered and used in subsequent transfection experiments.

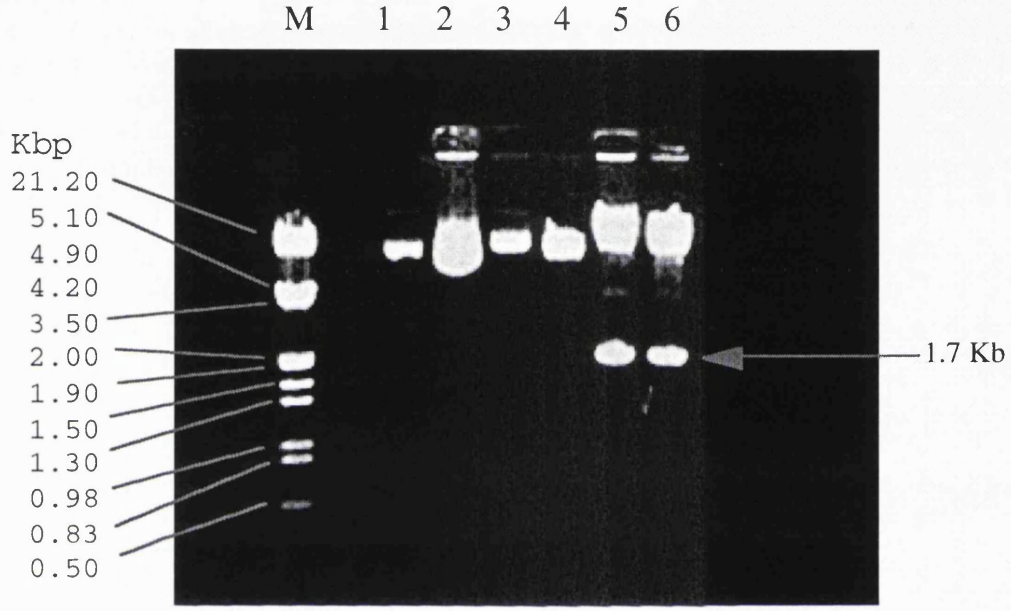


Figure 4.2 Kpn I and Bam HI digestion of the plasmid DNA from the ligation of the pp65 gene with pS.

The figure shows an agarose gel stained with ethidium bromide. The pp65 successfully cloned in the recombinant vector pS is shown by the presence of a DNA band of 1.7 kb in lanes 5 and 6, which correspond to pS-pp65 digested with Kpn I and Bam HI (1.5 µg and 0.5 µg respectively). Lanes 3 and 4 shows vector pS digested with the same enzymes (0.5 and 1.5 µg respectively) and lanes 1 and 2 represent undigested pS and pS-pp65 respectively. The molecular weight markers, Lambda DNA digested with Hind III and Eco RI (0.12-21.2 kb), are shown in lane M.

4.2.5 The generation of the L721.221/HLA-A2 cell line

HLA cDNA clones encoding common Caucasoid HLA alleles, including the HLA-A*0201 allele, were isolated in the Department of Cell Biology, Stanford University, USA. The alleles were cloned from the mRNA encoding the transcript for the gene by synthesis of cDNA from mRNA, and subsequent cloning into the M13 bacteriophage (Parham *et al.*, 1995). DNA's encoding several HLA class I alleles were subsequently cloned into the expression vector pSRαNeo, and transfected individually into the L721.221 B cell line by Miss Euridice Dominguez. (The Anthony Nolan Bone Marrow Trust). L721.221 cells transfected with the HLA-A*0201 gene (designated L721.221/HLA-A2 cells) were employed in the present study. These cells were cultured in RPMI/10% FCS in the presence of the antibiotic G418 (a neomycin analogue), to enable the selective growth of cells carrying the construct. Figure 4.3 shows the level of expression of the HLA molecules on the cell surface of L721.221/HLA-A2 cells, as determined by flow cytometry using the monoclonal antibody W6/32. Mock transfected L721.221 cells were used as a negative control for background levels of fluorescence. After 3 weeks of selection, the population expressing high levels of HLA class I in L721.221/HLA-A2 cells, were separated from the low expressing population using a fluorescence activated cell sorter thereby increasing the percentage of cells positive for HLA class I expression to 90% (data not shown). Subsequently, L721.221 cells expressing high levels of HLA-A*0201 molecules were then additionally transfected by electroporation with the pp65 mammalian expression constructs.

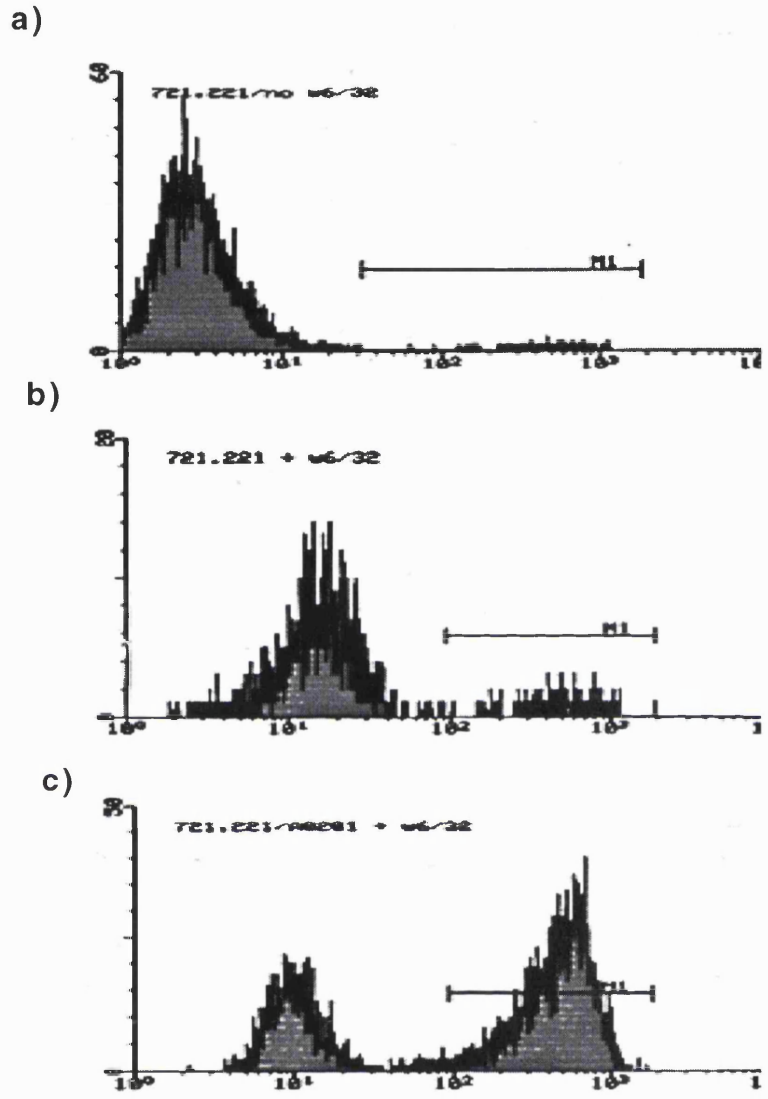


Figure 4.3 The expression of HLA class I molecules in L721.221/HLA-A2 cells.

Cells which were either mock transfected (b) or transfected with the construct pSCRαneo/HLA-A2 (c), were stained with the HLA class I specific monoclonal antibody W6/32 followed by a secondary fluorescein-labelled antibody and analysed by flow cytometry. Background levels of fluorescence were determined by mock transfected L721.221 cells, which were stained with an irrelevant primary antibody (a).

4.2.6 Transfection of pS-pp65 into the L721.221/HLA-A2 cells

L721.221/HLA-A2 cells ($1x10^7$ cells) were further transfected with the pS-pp65 construct or with the pS vector alone. Forty eight hours after transfection, 125 μg/ml of hygromicin was added to the culture media for the selection of cells carrying the vector. Seven days after transfection the mock transfectants had died, and growing cells were clearly visible in wells containing cells transfected either with pS or pS-pp65. Dual-transfectant cells were maintained in medium containing 10% FCS, hygromicin and G418. G418 and hygromycin are aminoglycoside antibiotics produced by streptomycetes. The cytotoxicity of both antibiotics is based on the inhibition of protein synthesis. Both G418 and hygromicin can be inactivated by phosphorylation (Santerre *et al.*, 1984), however, the two phosphotransferase enzymes that act on these antibiotics are quite specific and show no cross-reactivity. Thus, it was possible to perform simultaneous or sequential drug selections employing both resistant markers.

Dual-transfectant cells (designated L721.221/HLA-A2/pp65 cells) were tested 2 weeks after transfection for the intracellular expression of pp65. Cytospin preparations were made using $5x10^4$ transfectant cells per spot, and pp65 was detected by staining with a pp65-specific monoclonal antibody, after fixation and permeabilisation of the cells. pp65 expression was observed in approximately 1% of the pS-pp65 / L721.221/HLA-A2 double-transfected cells, but not in cells transfected with vector pS alone (Figure 4.4). Due to the high concentration of antibiotic used in the selection of these double transfectants, there was a large proportion of dead cells in these cultures, which may have been responsible for the high background fluorescence levels observed in Figure 4.4.

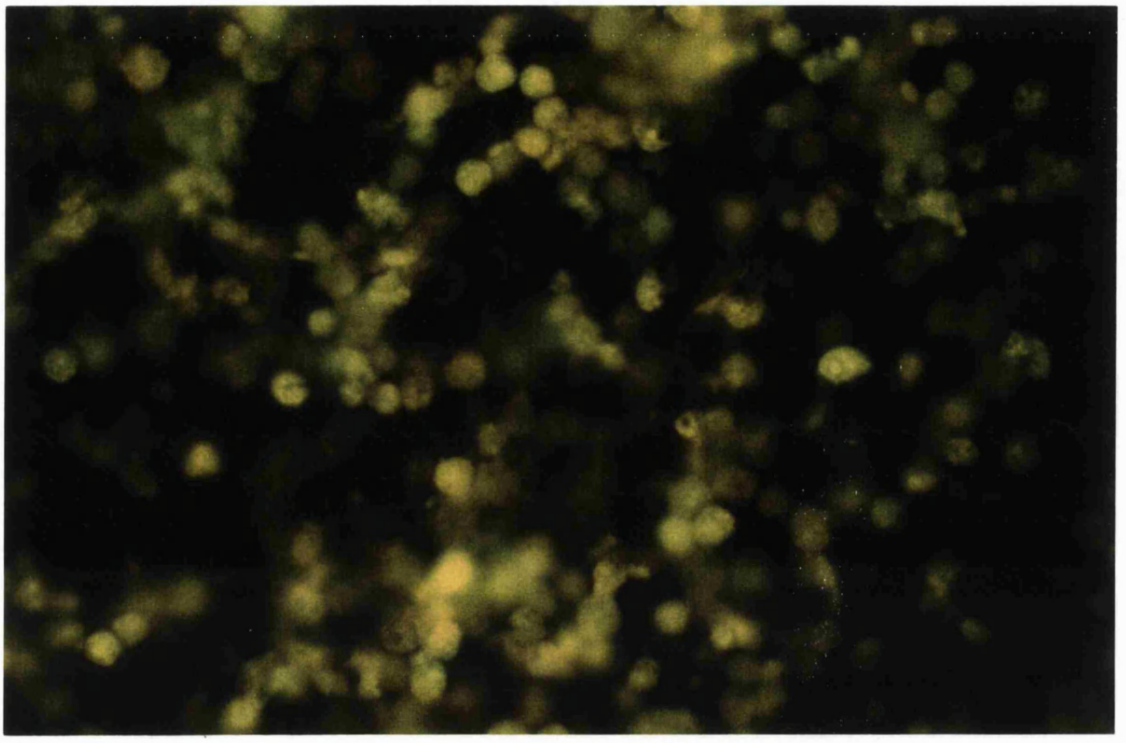
Low efficiencies of transfection are not uncommon, but this problem can be overcome by sorting the population of cells which express the protein of interest. In the case of transfectant cells expressing pp65, such an approach was not possible, since the pp65

protein is predominantly localised in the nucleus (Gallina et al., 1996). pp65 cannot be detected by monoclonal antibodies without disrupting the cell membrane, and therefore it was not possible to select viable pp65 positive cells by cell sorting. In an attempt to isolate a population of cells expressing pp65, the pS-pp65 / L721.221/HLA-A2, double-transfected cells were cloned by limiting dilution at one cell per well. Cells were maintained in serum rich medium supplemented with conditioned medium in 96 well plates. Replicate plates were prepared in the presence or absence of antibiotic selection. Cells were tested for pp65 expression four weeks after cloning. pp65 could not be detected in any of these clones when cultured either in the presence or absence of hygromicin.

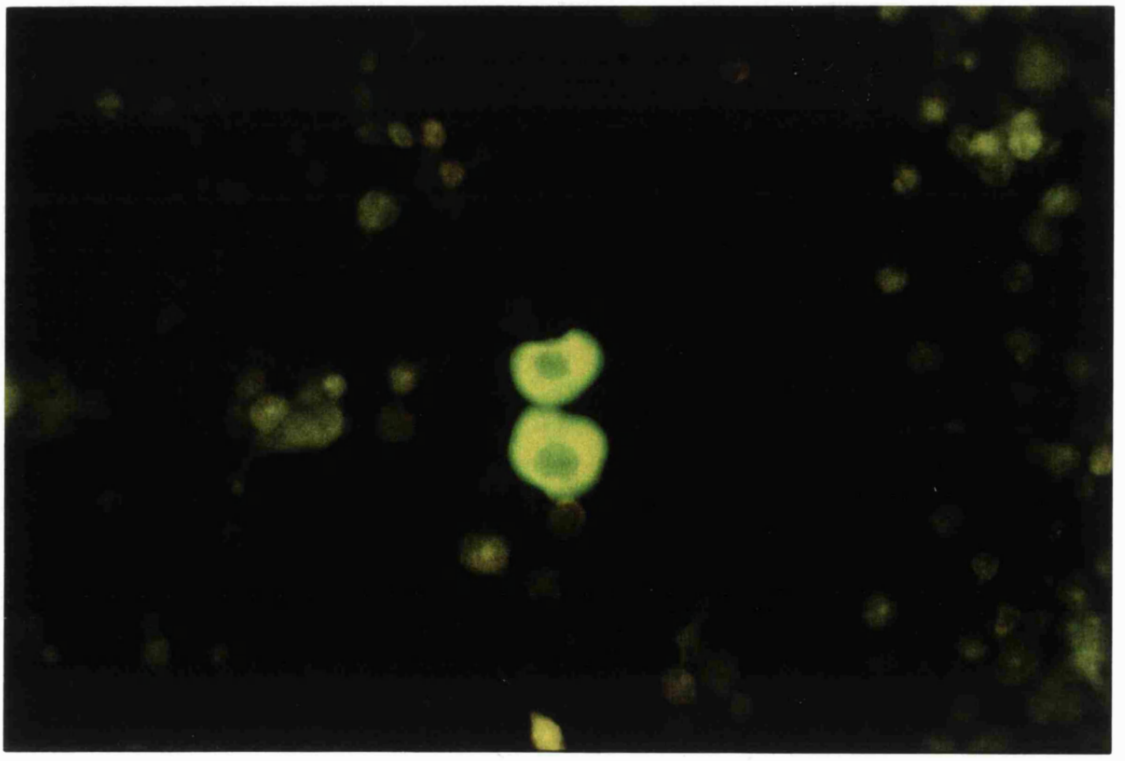
Figure 4.4 The expression of pp65 protein in L721.221/A*0201 cells as detected by immunostaining.

Cytospin preparations of L721.221/HLA-A2 cells transfected with the pS vector alone or transfected with the pS/pp65 construct, were stained with the monoclonal antibody C14, specific for pp65. A fluoresceinated goat anti-mouse antibody was used as a secondary antibody. Cells were observed using an epifluorescence microscope. Magnification x400. In addition, cytospin preparations were stained with appropriate isotype control antibodies as a negative control (data not shown).

a) **L721.221/HLA-A2 cells transfected with the vector pS**



b) **L721.221/HLA-A2 cells transfected with the vector pS/pp65**



4.2.7 Subcloning of pp65 into the pEGFP-N2 vector

To circumvent the problems described in section 4.2.6, an alternative strategy was employed in which transfected cells expressing pp65 could be sorted without disrupting the cells. This approach made use of the reporter vector pEGFP-N2 (see Appendices). Genes cloned into the multi-cloning site of this vector are expressed as fusions to the N-terminus of the green fluorescent protein (EGFP). Fusions to green fluorescent protein retain the fluorescent properties of the native protein, thereby allowing the localisation of the fusion protein in the cell. A neomycin-resistance cassette (neo^r) allows stably transfected eukaryotic cells to be selected using neomycin analogue G418. The target genes can be cloned into one of the following variants of the vector, pEGFP-N1, -N2 or -N3, so that the inserted DNA sequence is in frame with the green fluorescent protein coding sequence.

The pEGFP-N2 variant was selected in order to retain the correct reading frame of green fluorescent protein when pp65 was cloned into the vector. Both the M13mp19/pp65 construct and PEGFP-N2 were digested with the enzyme Eco R1. The Eco R1 digestion of M13mp19/pp65 generated a pp65 DNA fragment which included the initiating ATG codon, and no intervening in-frame stop codons. These requirements are necessary for the successful cloning of this gene into pEGFP-N2 as a fusion protein. The pp65 DNA fragment was purified and ligated at an insert vector ratio of 4:1, into the pEGFP-N2 vector. The ligation reaction product was used to transform DH5 competent bacteria, which were plated on to petri dishes containing agar 30 μg/ml of kanamycin as the selection antibiotic. Twelve kanamycin resistant colonies were selected from which the plasmid DNA was extracted and tested by restriction enzyme analysis for the correct orientation of the insert as described below.

4.2.8 Restriction enzyme analysis of pEGFP-N2/pp65 recombinant colonies

The strategy for cloning of the pp65 into the vector pEGFP-N2 utilised a single restriction enzyme. The pp65 gene could therefore be inserted into the vector in either orientation, 5' to 3' or 3' to 5'. Only the 5' to 3' orientation would allow the appropriate synthesis of the pp65 protein, thus it was important to ensure that the recombinant vector used had the pp65 cloned in the correct orientation. The orientation was determined by restriction enzyme analysis of the recombinant clones with the enzyme Hind III, which cuts in one site of the vector, and in one site of the insert. Digestion with this enzyme produced two fragments of different sizes, depending of the orientation of the insert in the vector. Fragments of 5095 and 1126 bp were expected in those recombinant plasmids carrying the pp65 insert in the correct orientation (5'-3'). In clones where the pp65 insert was cloned in the opposite direction (3'-5'), the sizes of the fragments expected would be a large 5812 bp fragment, and a small fragment of 409 bp, which might not be visible in an agarose gel. Ten recombinant pEGFP-N2-pp65 clones, digested with Hind III, were electrophoresed in an agarose gel, including a non recombinant pEGFP-N2 plasmid DNA as a control. Two out of the ten recombinant plasmids (clones 3 and 14), had incorporated the pp65 insert in the correct orientation, as shown by the expected band sizes in lanes 4 and 9 respectively (Figure 4.5).

Large scale DNA preparation was prepared from the recombinant clone 14, and quantification of the DNA was determined by spectrophotometry. Digestion analysis with the enzymes Eco RI and Hind III was repeated on the DNA obtained to confirm that pp65 was still present, and that it was in the correct orientation (data not shown).

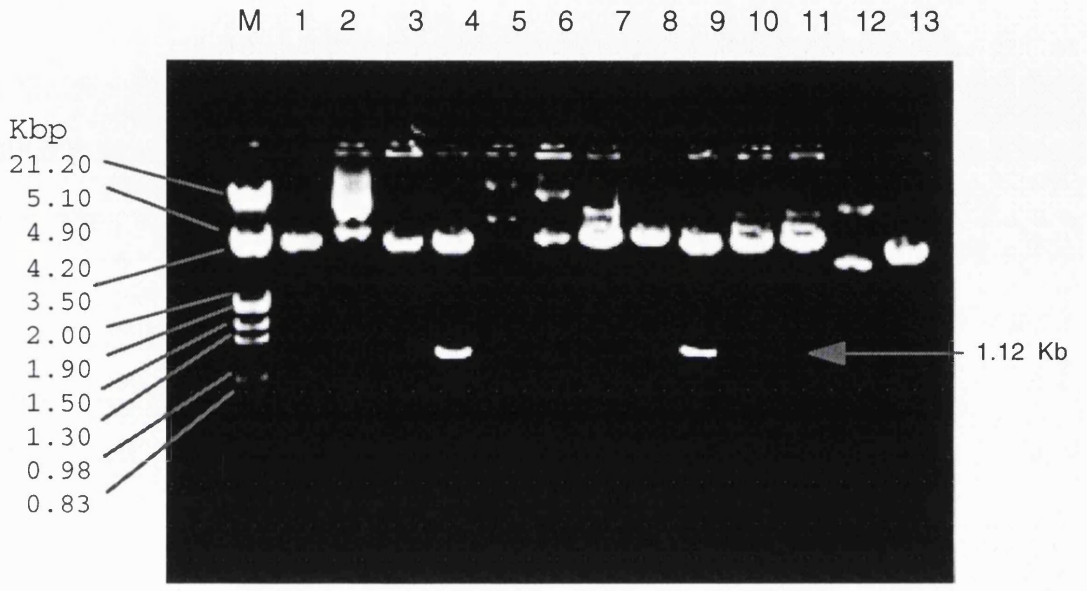


Figure 4.5 Restriction enzyme analysis of recombinant vector pEGFP-N2/pp65.

The figure shows an agarose gel stained with ethidium bromide after electrophoresis of the products obtained after digestion with the restriction enzyme Hind III of pEGFP-N2/pp65 recombinant DNA (lanes 2-11) or pEGFP-N2 (lanes 12-13). In lane 1, pEGFP-N2 undigested DNA is shown as a control. The molecular weight markers, λ DNA digested with the enzymes Hind III and Eco RI, were run in parallel (M). The pp65 gene was successfully cloned in the correct orientation (5'-3') in the clones shown in lanes 4 and 9 (clones 3 and 14 respectively), as demonstrated by the presence of the 120 bp fragment (shown by an arrow).

4.2.9 Transfection of pEGFP-N2-pp65 into 721.221/HLA-A2 cells

L721.221 cells expressing the HLA-A2 allele were transfected with the recombinant vector pEGFP-N2-pp65 by electroporation to obtain expression of both green fluorescent protein and pp65. The recombinant vector pEGFP-N2-pp65 or the intact pEGFP-N2 vector were mixed with the cells and electroporated. Two days after the electroporation, expression of the green fluorescent protein was assessed by flow cytometry, using an aliquot of $2x10^5$ cells. However, no fluorescence was detected either in the cells transfected with pEGFP-N2-pp65 or the pEGFP-N2 vector alone (not shown).

4.2.10 Transfection of pEGFP-N2-pp65 into MRC-5 fibroblasts and 293 transformed kidney cells

It is possible that the lack of green fluorescent protein expression in L721.221 cells transfected with pEGFP-N2-pp65 or the pEGFP-N2 vector alone may have been due to the fact that this cell line was not permissive for the expression of this protein.

One of the goals for the generation of cells expressing pp65 was their use as target cells in the characterisation of CTLs generated against pp65 derived peptides. The main requirement of such target cells was to present endogenous pp65 in the context of HLA-A2. As the successful transfection of 721.221/HLA-A2 cells with pp65 was proving difficult to achieve, an alternative strategy was adopted, this being the use of HLA-A2 positive fibroblasts.

To identify a fibroblast cell lines expressing the HLA-A2 allele, a panel of fibroblasts cell lines derived either from skin biopsies or from human embryo lung biopsies were tissue-typed. One of the cells lines which was found to express the HLA-A2 allele was the

human fibroblast cell line MRC-5, which was used in subsequent experiments. In these experiments, the human transformed primary embryonic kidney cell line, 293, was used as a positive control, since this cell line has been successfully used as a host for transfection and expression of green fluorescent protein using the vector pEGFP (Marshal *et al.*, 1995).

In general, electroporation is not the method of choice for transfection of adherent cells due to its low efficiency in these cell types. Adherent cells are usually transfected via alternative methods which rely on reagents such as liposomes or calcium phosphate.

In this study MRC-5 fibroblasts and 293 cells were transfected with pEGFP-N2-pp65 using the Superfect™ transfection reagent. This is a liposome-like polycationic reagent (Quiagen) which has been reported to yield high transfection efficiencies. The pEGFP-N2-pp65 or pEGFP-N2 plasmid DNA was mixed with the Superfect™ reagent, and then added to a subconfluent monolayer of cells, and incubated at 37 °C for 48 hours. Transient expression of green fluorescent protein was evaluated by viewing cells grown on a coverslip and mounted on a microscope slide under a fluorescent microscope. Alternatively, the cells were detached by trypsinisation and green fluorescent protein expression was analysed by flow cytometry. The results of these experiments showed that similarly to the results for 721.221 cells, no fluorescence was detectable in MRC-5 fibroblasts transfected with pEGFP-N2-pp65 either by microscopy (data not shown) or by flow cytometry (Figure 4.6). Expression of the green fluorescent protein was not detectable when fibroblasts were transfected with intact pEGFP-N2. However, in the case of the 293 cells, fluorescence related to the presence of the green fluorescent protein was detected in approximately 5% of pEGFP-N2 and pEGFP-N2/pp65 transfected cells.

This result suggests that neither fibroblasts nor L721.221 cells were permissive for transfection with this vector, and that they were not appropriate cell lineage's for the

expression of this protein. Alternative experiments using different transfection strategies may have resolved this problem, however due to time constraints such experiments could not be pursued.

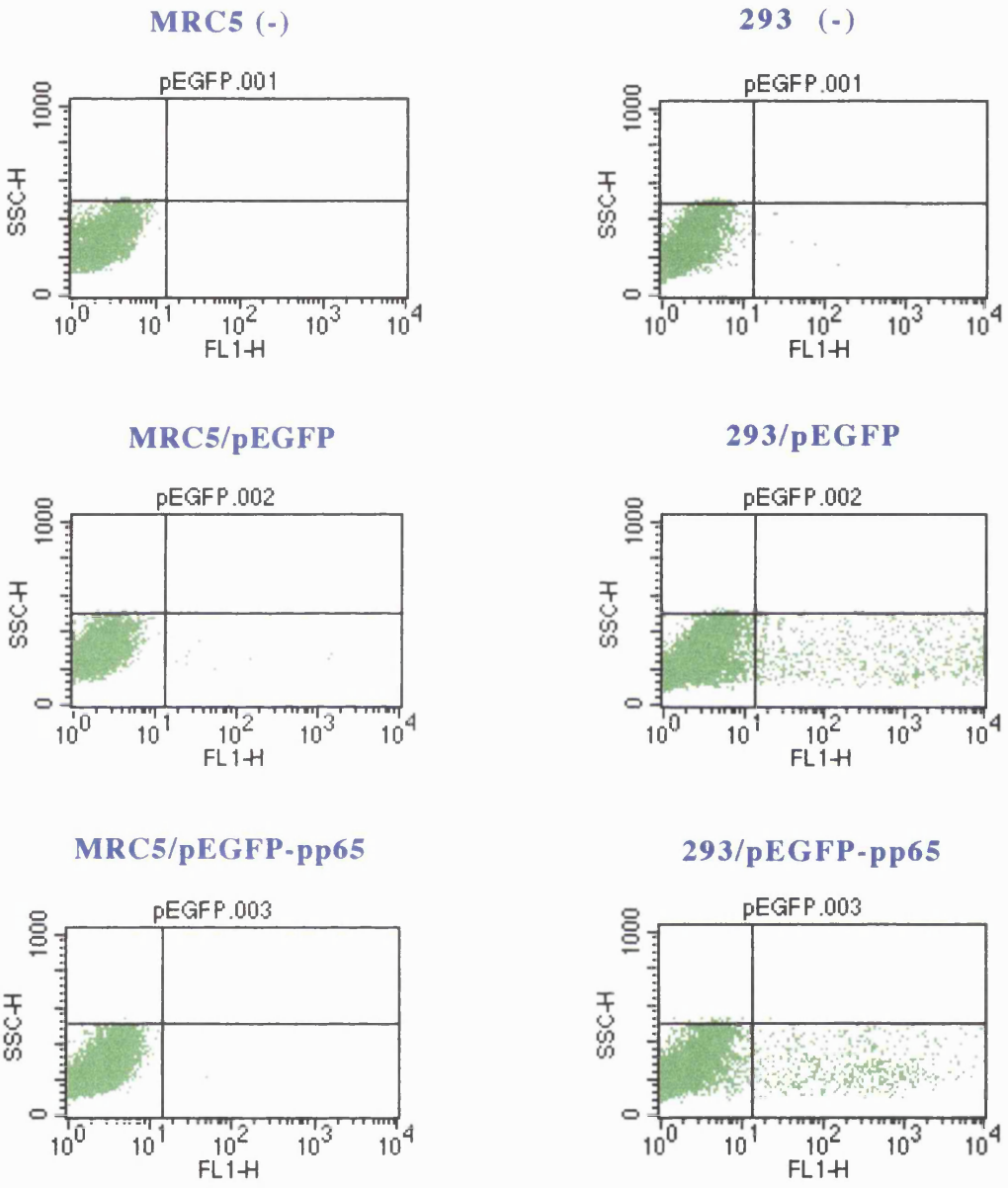


Figure 4.6 Expression of green fluorescent protein in MRC-5 fibroblasts and 293 cells.

MRC-5 fibroblasts and 293 cells were transfected using the Superfect™ reagent with the construct pEGFP-N2, pEGFP-N2-pp65 or mock transfected (-) as shown. Expression of the green fluorescent protein was assessed 48 h post transfection by flow cytometric analysis. In the figure, FL1-H (on the X axes) represents the expression of the green fluorescent protein, whilst SSC-H (on the Y axes) represents the side scatter of the cells.

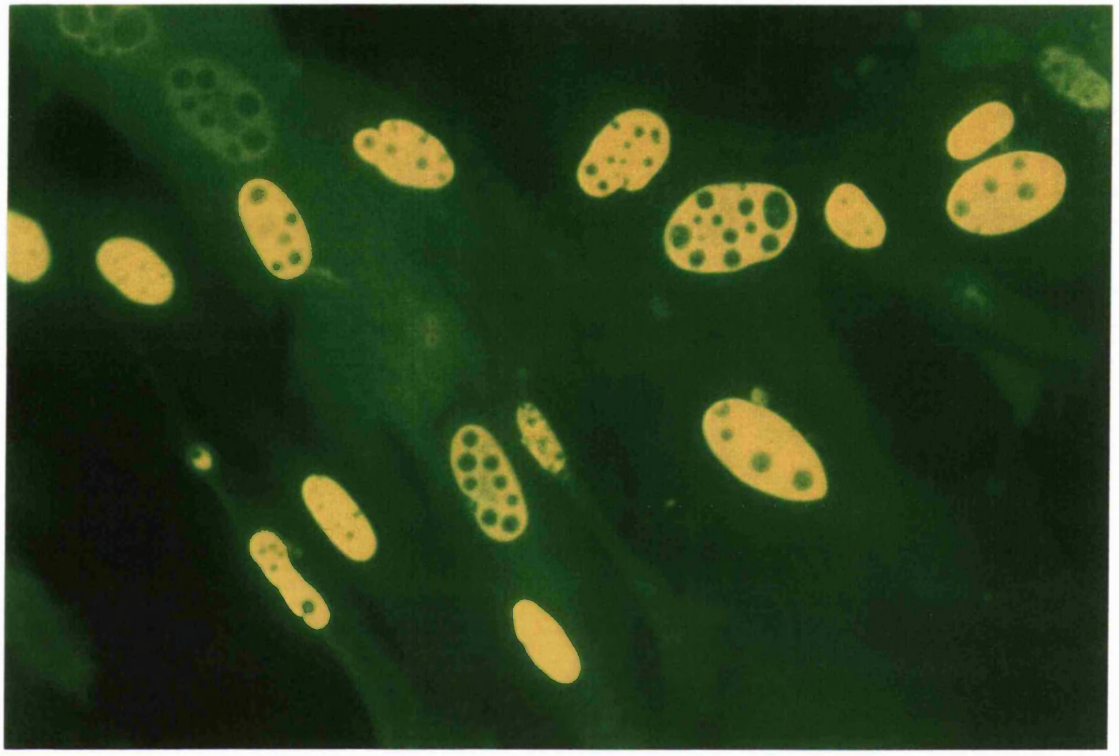
4.2.11 The expression of pp65 in MRC-5 fibroblasts and L721.221 cells after infection with the RAd-pp65

A third strategy pursued to obtain expression of pp65 in fibroblasts and/or L721.221-HLA-A2 cells, used a replication-deficient recombinant adenovirus construct carrying the pp65 gene created by Benham Zan and colleagues at St. George's Hospital School of Medicine, London. To generate this construct, the full coding sequence for ppUL83 was amplified by PCR and cloned into the expression cassette of the plasmid pMV100, under the control of the CMV immediate early promoter. Subsequently, the entire expression cassette from pMV100 was cloned in the shuttle vector, pMV60, to generate pMV60-pp65. The defective adenovirus construct was created by homologous recombination between the shuttle vector pMV60-pp65, the plasmid pJM17 (which contains the entire Ad type 5 *dl*309 genome), and the sequence for the plasmid pBR inserted into the E1a gene early region. These two plasmids were co-transfected into the 293 cell line, which constitutively expresses the Ad E1 protein as a helper function. As a result, the pp65 gene under the control of the immediate early promoter was inserted into the adenovirus genome in place of the E1 sequences (see Appendices). In these experiments, a similar adenovirus construct (RAd-35) expressing an irrelevant gene, β-galactosidase (Wilkinson and Akrigg, 1992), obtained from Dr. Gavin Wilkinson (Cardiff University), was used as a control.

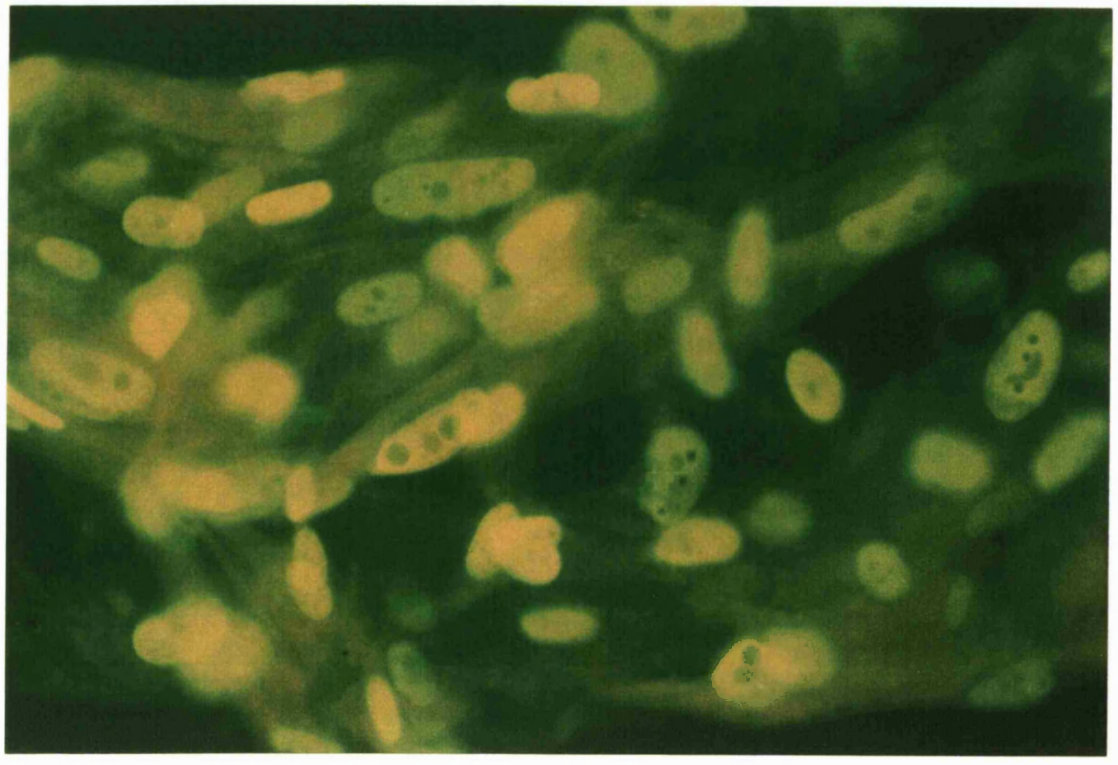
The expression of pp65 was assessed after infection of MRC-5 fibroblasts or L721.221/A2 cells with the recombinant adenovirus/pp65 (RAd-pp65) vector at 100 $TCID_{50}$ units/cell. The cells were stained at 12, 24, 48 and 36 h post infection for the presence of pp65. CMV-infected fibroblasts were used as a positive control. Figure 4.7 shows that fibroblasts infected with RAd-pp665 expressed the pp65 protein at 24 to 48 h post-infection. Approximately 90% of the fibroblasts infected with RAd-pp65 showed characteristic nuclear immunofluorescence following staining with a pp65-specific antibody

(Figure 4.7). Fibroblasts infected with the recombinant adenovirus expressing RAd-35 (β-galactosidase) were used as a control and gave no significant nuclear fluorescence (data not shown). However, when the same experiments were performed using L721.221 cells infected with the recombinant RAd-pp65, no pp65 expression was detectable (data not shown). These experiments were repeated using higher RAd-pp65 virus doses than those used to infect fibroblasts (500 and 1000 $TCID_{50}$ units/cell), but again pp65 was not detected in any case (data not shown).

a) **RAd-pp65 infected fibroblasts (24 h post-infection)**



b) **RAd-pp65 infected fibroblasts (48 h post-infection)**



c) RAd-35 infected fibroblasts (24 h post-infection)

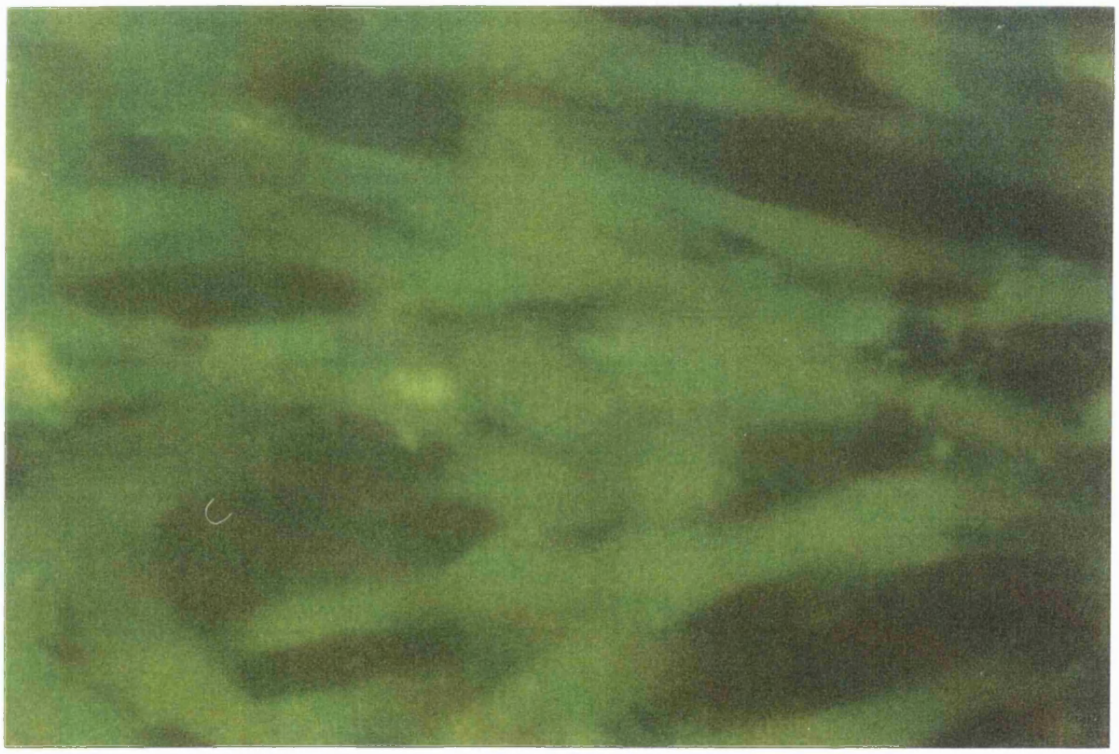


Figure 4.7 Expression of pp65 protein after infection with RAd-pp65.

MRC-5 fibroblasts were seeded into 8 well slide chambers and either exposed to RAd-pp65 or RAd-35 virus preparations at 100 $TCID_{50}$ units/cell. The inoculum was allowed to adsorb for 2h, and then culture medium was added. At 24 h and 48 h post-infection, cells were fixed, permeabilised and stained for expression of pp65, followed by fluorescein labelled anti-mouse immunoglobulin. Magnification x400.
In addition, RAd-pp65 infected fibroblasts were stained with the appropriate isotype control antibody as a negative control (data not shown).

4.3 Discussion

The generation of cells expressing the pp65 protein in isolation from other CMV proteins was of central importance for the overall aims of this project, since they would be used as tools in the characterisation of the pp65-specific CTL response. This chapter describes the cloning of the pp65 gene in expression vectors and the subsequent attempts at the expression of pp65 in mammalian cell lines.

Initial experiments were directed towards the confirmation that the pp65 gene used for the subsequent cloning into mammalian expression vectors was identical to the published pp65 sequence. In order to achieve this, the DNA from a pp65 PCR product obtained from the CMV strain AD169, was sequenced and the sequence compared to the pp65 sequence originally reported (Ruger et al., 1987). During sequencing, several problems were encountered, one of them being the presence of compressions in the bands of the autoradiogram, making the analysis of the results difficult to interpret. This was resolved by the use of nucleotide analogues in the sequencing reactions. A further problem was the presence of nucleotide misincorporations in the M13mp19/pp65 clones sequenced initially, which were attributed to the use of Taq DNA polymerase in the PCR amplification reaction. This enzyme lacks proof reading activity and consequently it promotes a high level of nucleotide misincorporations. The use of the pfu DNA polymerase enzyme (Stratagene™) in the PCR DNA amplifications corrected this problem, and resulted in the generation of clones in which the pp65 sequence was identical to the originally published sequence.

The ability to stimulate PBMCs using a cell line expressing pp65 in isolation from other CMV proteins could potentially allow for the characterisation of the pp65-specific response. In initial experiments, the MHC class I deficient EBV transformed cell line L721.221 transfected with a construct encoding the HLA-A2 allele was employed. Several

constructs expressing pp65 were generated and used to transfect or infect these L721.221/HLA-A2 cells in order to restrict the stimulation of a pp65-specific CTL response to this HLA class I antigen. Similar approaches using this cell line have been used in the search for melanoma (Celis *et al.*, 1994) and hepatitis B virus (Bertoletti *et al.*, 1993) CTL epitopes.

The sequenced pp65 gene was initially cloned in the episomal expression vector pS. This vector was chosen as it contains a cis acting element of EBV, oriP, which permits the maintenance of episomal DNA molecules in cells that carry EBV DNA. This vector also expresses the EBV nuclear antigen 1 (EBNA-1), which should allow the recombinant vector to replicate efficiently and promote low to moderate expression of the protein of interest in EBV transformed cell lines (Chittenden et al., 1989). Therefore, the transfection of the recombinant pS-pp65 vector into EBV transformed L721.221-HLA-A2 cells would theoretically allow the expression of pp65 protein. However, after transfection of the vector pS/pp65 into these cells and selection in hygromicin and G418, the expression of pp65 protein detected was low. This low pp65 expression found after transfection could be due to the large size of the pp65 gene (1.6 kb), as it has been reported that some mammalian expression vectors have strict packaging requirements, and will not accept large pieces of foreign DNA (Sambroock *et al.*, 1989). Previous experience at the Anthony Nolan Institute in the use of this vector for the expression of hepatitis B core antigen in the L721.221 cell line and C1R, an alternative EBV transformed cell line, suggested that the L721.221 cells consistently showed lower expression of the core antigen than the C1R cells. The reasons for this are not clear, however it may be related to the copy number at which the pS construct was maintained in each cell line (Yates et al., 1985).

Unfortunately, due to the presence of nuclear localisation signals in pp65 (Gallina et al., 1996), this protein is found predominantly in the nucleus, therefore, cell sorting of the

pp65 positive population was not a feasible strategy. Cloning of the antibiotic resistant population by limiting dilution was not successful either, as the pp65 positive population was lost after this procedure. One limitation of using episomal vectors is their inability to integrate into the DNA of the host cell, hence the transfected cell has to be grown in the continuous presence of selective media in order to avoid loss of the vector. Furthermore, the need to subject these cells to high concentrations of the selection antibiotic hygromicin during the cloning procedure could have resulted in stress induced growth inhibition or cell death in cells carrying the construct.

Another approach adopted to detect cells transfected with pp65 was to use green fluorescent protein (Prasher et al., 1992) as a reporter gene for pp65 expression. In previous publications, successfully transfected cells could be identified by green fluorescent protein expression, and there was a correlation between the intensity of the observed fluorescence and the relative level of expression of the fusion protein (Marshal et al., 1995). However, when either 721.221-HLA-A2 cells or MRC-5 fibroblasts were transfected with the constructs pEGFP or pEGFP-N2/pp65, no expression of the green fluorescent protein was detected. Cells transfected with the vector alone did not exhibit fluorescence which suggested that the lack of fluorescence in the pEGFP-N2-pp65 transfected cells was not due to the disruption of the coding reading frame of the green fluorescent protein when pp65 was cloned into the vector. Another explanation might be that these cells were not suitable for transfection with this particular vector, since the transcriptional efficiency of a number of commonly used eukaryotic promoters differs in different cell lines (Thomas and Capecchi, 1987). This may well have been the case with the two cell lines used in this study as it was possible to detect green fluorescent protein expression following transfection of pEGFP-N2 into the transformed embryonic kidney cell line 293. This is in agreement with a report in which this cell line had previously been successfully transfected with this vector (Marshal et al., 1995).

An alternative approach to overcome the difficulty of low efficiencies or lack of transfection when plasmid DNA vectors are used, is the use of viral vectors. Vaccinia virus or adenovirus recombinants have been used to obtain expression of proteins of interest in several types of cells. The use of vaccinia virus for the expression of pp65 in EBV transformed cell lines was contemplated in this study. Although vaccinia virus vectors could be used to generate pp65 expressing cells for use as target cells in cytotoxicity assays, their use as stimulator cells to induce a pp65-specific response could present some problems. This is because, in an *in vitro* system, the presence of a replication competent vector, as is the case of vaccinia virus, can itself interfere with an incipient T cell response when it is used to express antigens on stimulator cells (Borysiewicz *et al.*, 1996).

Recombinant adenoviruses (RAd), are replication-deficient, and can provide high levels of expression of a foreign protein, in the absence of detectable expression of proteins derived from the vector (Morgan *et al.*, 1996; Wilkinson and Akrigg, 1992). Therefore, cells infected with RAd-pp65 could be used as stimulator cells in the generation of pp65-specific CTLs, as well as being used as target cells in the characterisation of a pp65-specific CTL response.

A defective adenovirus construct encoding the pp65 gene was tested for the induction of expression of pp65 in L721.221-HLA-A2 cells and also in MRC-5 fibroblasts. Attempts to detect pp65 in L721.221-HLA-A2 cells exposed to RAd-pp65 were not successful, which suggests that these cells are refractory to adenovirus infection. Since the completion of this part of the present study, similar conclusions have been reached by another group (Prince *et al.*, 1998), who found that B and T lymphocytes were relatively resistant to adenovirus-mediated gene transfer. However, a different group (Morgan et al., 1996) reported that PBMC exposed to a RAd expressing the EBV nuclear antigen EBNA3C, were able to elicit a CTL specific response to this protein, suggesting that PBMC were being infected by the virus. Interestingly, in the latter study, staining of the RAd-infected

PBMC showed that detectable EBNA3C expression was limited to only 0.5% of the infected cells (Morgan et al., 1996). In contrast to the results with L721.221-HLA-A2 cells, the recombinant adenovirus construct RAd-pp65 allowed a very high expression of the pp65 protein in MRC-5 fibroblasts, which was detected even at low titres of the virus. This high expression was not surprising, as the recombinant adenovirus vectors employed here were known to be capable of inducing expression of the protein of interest in fibroblasts (Morgan et al., 1996; Wilkinson and Akrigg, 1992; Zahn and Baboonian,).

The high expression of the CMV protein pp65 in the HLA-A2 positive fibroblast cell line provided a tool which could be used in the generation of a pp65-specific CTL response, although this response would not be restricted only to HLA-A2, since these fibroblasts present a full complex of HLA class I antigens. One way to circumvent this problem would be to alternate effector cell stimulations using fibroblasts with different sets of HLA alleles, retaining the common presence of HLA-A2 and thereby restricting the response to this allele.

HLA-A2 positive fibroblasts infected with RAd-pp65 could also provide an ideal target cell for cytotoxicity assays, as they would be expressing high levels of pp65 which would be processed and presented in the context of HLA-A2. When used as target cells they could be tested against responder cells, which have already been primed in the context of a particular HLA class I antigen (HLA-A2), and so the presence of other HLA class I alleles expressed in these target cells would not present a problem. By the time the work described in this chapter had been accomplished and expression of the desired levels of pp65 protein obtained, CTLs generated against synthetic peptides derived from pp65 had been successfully generated (see Chapter 5). Fibroblasts infected with RAd-pp65 were therefore mainly used as targets cells in the characterisation of pp65 peptide-induced CTLs as described in the next chapter.

5. Chapter 5. Induction of CMV-specific cytotoxic T lymphocytes using synthetic peptides derived from the CMV pp65 protein

5.1 Introduction

The cytotoxic T lymphocyte response to CMV is thought to be crucial in the control of CMV in the normal virus carrier. The CMV lower matrix phosphoprotein, pp65, has been suggested to be one of the predominant viral antigens recognised by CMV-specific CTLs after infection (Riddell et al., 1991a; Wills *et al.*, 1996). Currently, several strategies have been used in an attempt to identify the particular peptide epitopes derived from this protein which are responsible for CTL recognition (Diamond *et al.*, 1997; McLauglin-Taylor et al., 1994; Wills et al., 1996). These studies have employed autologous fibroblasts infected with CMV, or cells infected with vaccinia constructs expressing pp65, for the *in vitro* induction and expansion of pp65-specific CTLs (Diamond et al., 1997; Wills et al., 1996). Such stimulation protocols can lead to the activation of polyclonal CTL responses, where effector cells are likely to recognise only the most dominant epitopes derived from the pp65 protein, with the consequent loss of responses to subdominant epitopes (Gammon *et al.*, 1987). This is a potential drawback to such strategies, since immunodominant epitopes are often selected for mutation *in vivo*, thereby hampering their effectiveness as therapeutic targets (Bertoletti *et al.*, 1994; de Campos-Lima *et al.*, 1993; Klenerman *et al.*, 1995; Moskophidis and Zinkernagel, 1995). Alternative protocols of T cell generation could

allow for the triggering of CTL responses with unique specificities, including the selective activation of responses to epitopes which are not detected in polyclonal CTL cultures obtained by conventional stimulation protocols.

In the work described in this chapter, the presence of CMV-specific memory CTL precursors in lymphocyte populations from the blood of CMV seropositive individuals was exploited in order to establish the experimental conditions for the *in vitro* expansion of cytotoxic T cells using synthetic peptides derived from the CMV pp65 protein. The HLA-A2 binding peptides from pp65 identified in Chapter 3 using the T2 stabilisation assay and by a peptide-binding competition assay were tested for their ability to induce cytotoxic T lymphocytes. The present chapter further describes the characterisation of such peptide induced CTLs with respect to their ability to recognise endogenously synthesised peptides.

5.2 Results

5.2.1 The generation of cytotoxic T lymphocytes using peptide-pulsed T2 cells as antigen presenting cells

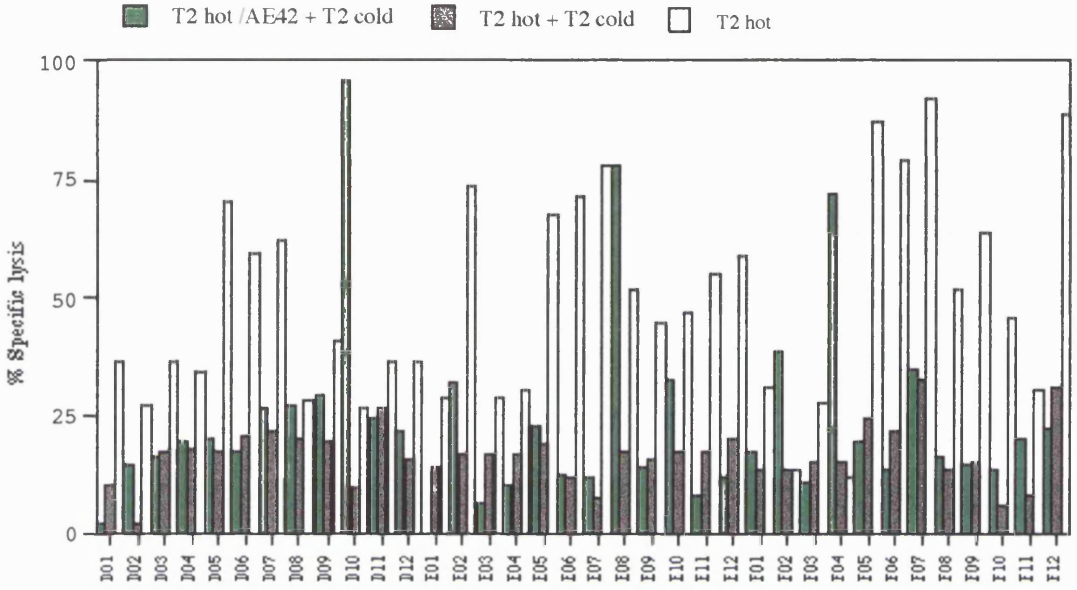
In order to establish the optimal conditions required to induce a pp65- and CMV-specific CTL response using synthetic peptides, several *in vitro* protocols were compared. PBMC from a normal CMV seropositive and HLA-A*0201 positive individual designated as donor I (HLA A*0201, 3; B7, 60), were isolated, and used as "responder cells". Peptides AE42 and AE44 derived from pp65, which had previously shown affinity for the HLA-A2 molecule (Chapter 3), were used in these experiments. The influenza matrix peptide Flu-M 58-66 was also used as a positive control, since this peptide has previously been shown to be the dominant HLA-A2 restricted CTL epitope in the influenza matrix protein (Bednarek et al., 1991; Gotch *et al.*, 1987).

In initial experiments, the induction of peptide-specific CTLs was performed using the antigen processing deficient cell line T2, pulsed with the synthetic peptides. The T2 cell line was chosen as it can be efficiently loaded with exogenously added peptides, and as shown in Chapter 3, the addition of CMV pp65 peptides AE42, AE44 or Flu-M 58-66 induces an increased level of HLA class I molecules on the surface of these cells. Therefore the possibility existed that the use of peptide-pulsed T2 cells as stimulator cells might propitiate the presentation of the appropriate peptide to CTLs.

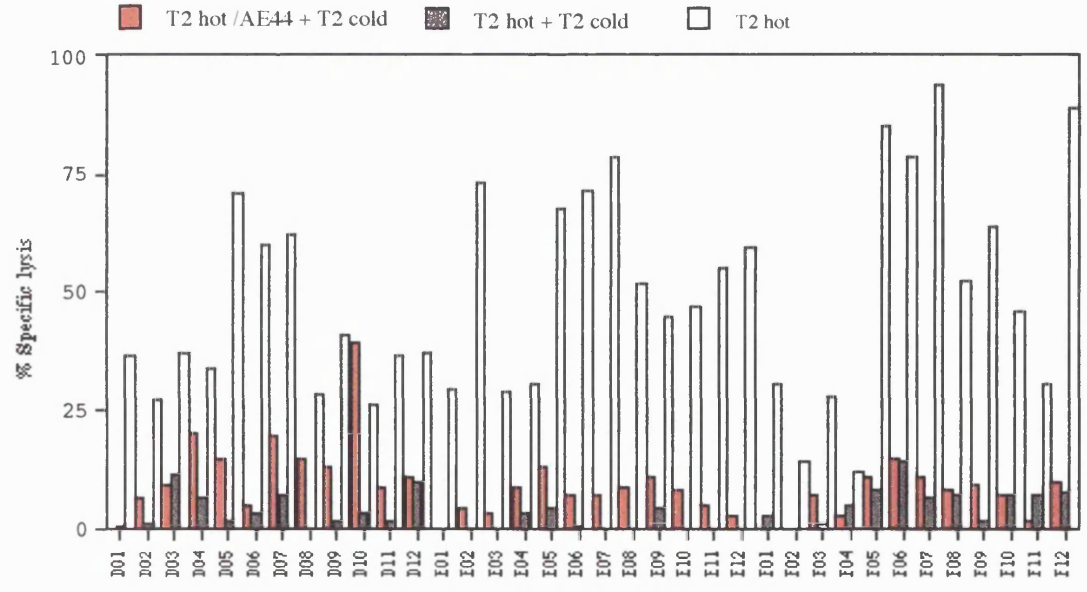
Cultures were established using 2000 PBMCs per well, which were stimulated with irradiated, peptide-pulsed T2 cells, at a stimulator to responder cell ratio of 1:2.5. IL-2 was added to the cultures on day 3 and 6, before a secondary re-stimulation on day 10 with irradiated, peptide-pulsed T2 cells, IL-2 and irradiated autologous PBMC as feeder cells. This re-stimulation protocol was repeated weekly.

The cytotoxic potential of responder cells was assessed in a 4 h ^{51}Cr release assay on day 24. Preliminary experiments had suggested that T2 cells when used as stimulator cells, induced a very high background response and that the lysis attributed to this would mask peptide-specific responses (data not shown). Thus in subsequent cytotoxicity assays, to address the problem of high T2 backgrounds, a strategy using cold target lysis inhibition was employed. A ten-fold excess of non-^{51}Cr labelled (cold) T2 target cells, was added to each well containing ^{51}Cr labelled target cells, which were either non-pulsed T2 cells or T2 cells pulsed with the relevant peptide. This strategy allowed the lysis induced by potentially low frequency peptide-specific CTLs to be elucidated. Figure 5.1 shows that CTL lines generated against peptides Flu-M (58-66), and pp65 peptides AE42 and AE44 were able to demonstrate a peptide-specific response. Although in the case of CTLs stimulated with peptide AE44, the peptide-specific response was much lower than that observed with the other two peptides. The reactivity attributable to T2 cells alone was assessed by the use of a control in which only ^{51}Cr labelled T2 cells were used as target cells.

a) Lines stimulated with T2 cells + pp65/AE42 peptide



b) Lines stimulated with T2 cells + pp65/AE44 peptide



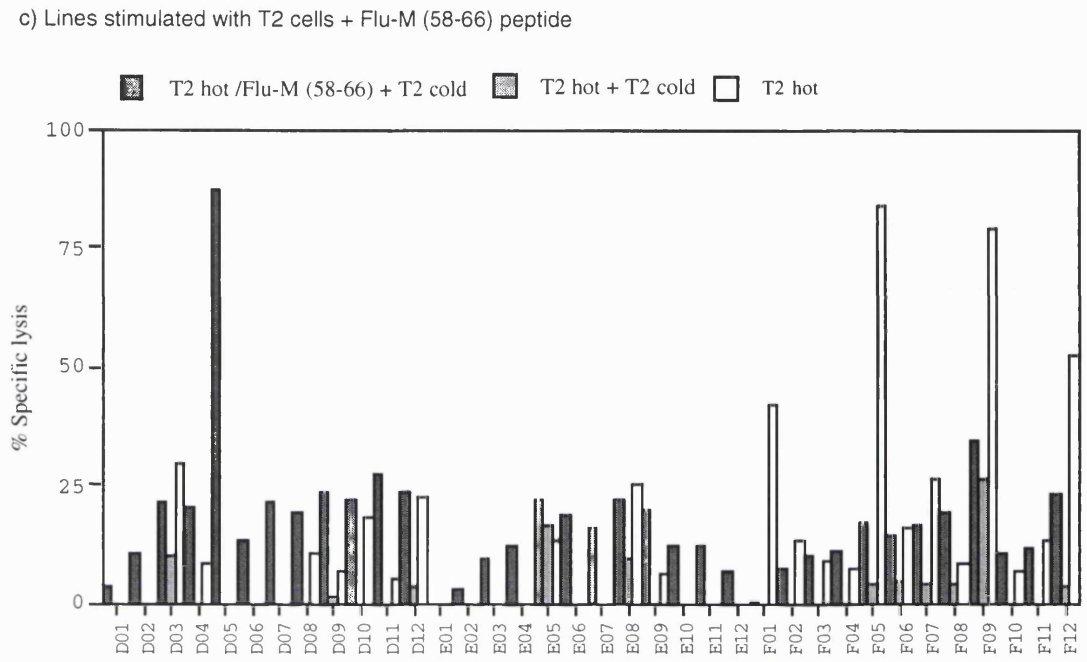


Figure 5.1 Initial reactivity of polyclonal cultures generated using T2 cells pulsed with peptide.

PBMC from the HLA-A2 and CMV seropositive donor I were primed using irradiated T2 cells pulsed with the influenza matrix peptide Flu-M 58-66 (c) or the pp65 peptides AE42 (a) or AE44 (b). CTL activity was determined in a 4 h Chromium release assay by split well analysis, in which 150 µl was taken from each test well and split to be analysed against three different target cells: labelled T2 cells pulsed overnight with 50 µg/ml of the appropriate peptide, or labelled T2 cells in the presence of a ten fold excess of non-labelled T2 cells (cold) as competitors, or labelled T2 cells alone. The figure shows the representative results from 36 out of 96 wells for each peptide.

5.2.2 The generation of cytotoxic T lymphocytes using peptide-pulsed autologous PHA blasts as antigen presenting cells

Although the results from the use of the T2 cell line as a stimulator cells for presentation of peptide showed that it was possible to induce peptide-specific CTL lines, clearly the induction of a strong non peptide-specific response by the T2 cell background was a drawback, since the response of peptides with low or intermediate affinity for HLA-A2 was masked by a stronger non-specific response.

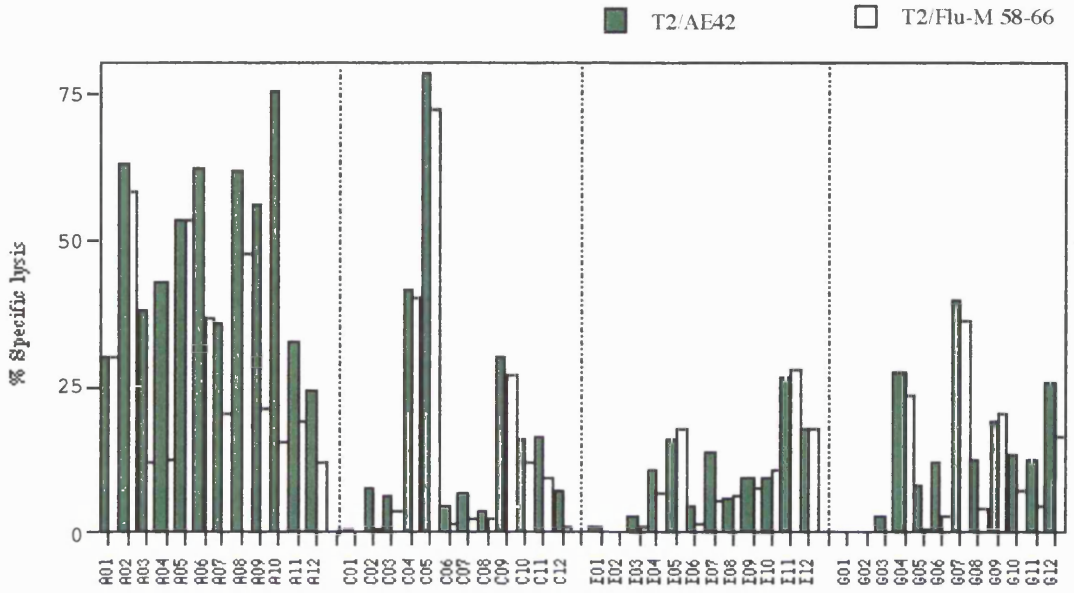
In order to circumvent this non-specific response an alternative approach was adopted for the generation of CMV-specific CTLs using synthetic peptides derived from pp65. This alternative approach utilised autologous PHA blasts, pulsed with pp65 peptides, for the first three cycles of stimulation. The benefit of using PHA blasts cells was their lack of non-specific background stimulation in the absence of peptide (Gagliardi *et al.*, 1995). Additionally, they have been shown to be effective in priming memory responses in previously published work (Azuma et al., 1993; Barnaba *et al.*, 1994).

Therefore, PHA blasts generated from the PBMCs obtained from the same normal CMV seropositive HLA-A*0201 individual, donor I, used in the experiments described in section 5.2.1, were pulsed *in vitro* with pp65 peptides AE42, AE44, and Flu-M 58-66. Four cultures were established in parallel using stimulator to responder cell ratios of 1:10, 1:5, 1:2.5 and 1:0.4. IL-2 was added as previously described on days 3 and 6, and cells were re-stimulated on day 10, using irradiated peptide-pulsed PHA blasts, with the addition of irradiated autologous PBMCs as feeder cells, also in the presence of IL-2. This re-stimulation protocol was repeated twice. For subsequent re-stimulations, peptide-pulsed T2 cells were used as stimulator cells, the assumption being that the use of autologous peptide-pulsed PHA blasts in the first two rounds of stimulation would be sufficient to focus the response to the peptide-HLA-A2 complex. This scheme was

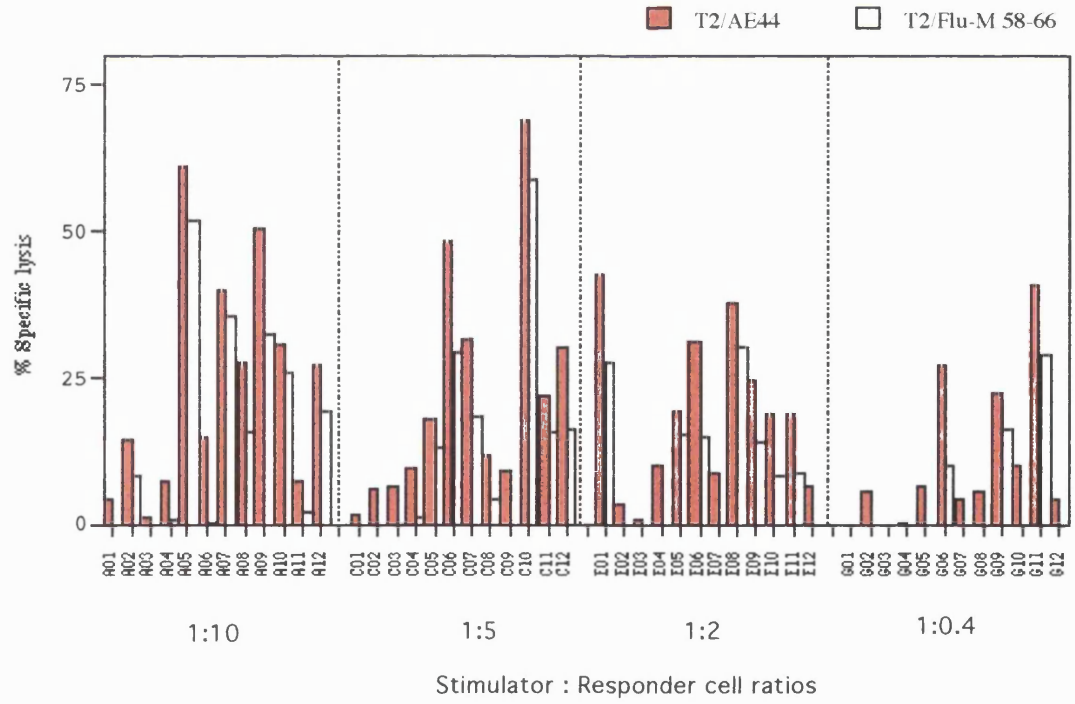
adopted as it allowed the convenience of using a defined cell line (T2), which could be maintained for long periods in culture with no change in surface phenotype.

Following three consecutive re-stimulations, using the combined protocol of stimulation by T2 cells and PHA blasts, the peptide specificity of the cultures was assessed by measuring their cytotoxic potential against T2 cells pulsed with relevant or irrelevant peptide. Although the majority of the CTL lines generated showed recognition of the irrelevant peptide, some of these lines, for example A10 and C06, showed a higher lysis of target cells pulsed with the relevant peptides, AE42 or AE44 respectively. The highest proportion of peptide-specific responder cells was observed on the cultures which had been induced using a ratio of stimulator to responder cells of 1:5 or 1:10 (Figure 5.2). The low level of specificity of these CTL lines was attributed to the polyclonal nature of the cultures.

a) Lines stimulated with PHA blasts + pp65/AE42 peptide



b) Lines stimulated with PHA blasts + pp65/AE44 peptide



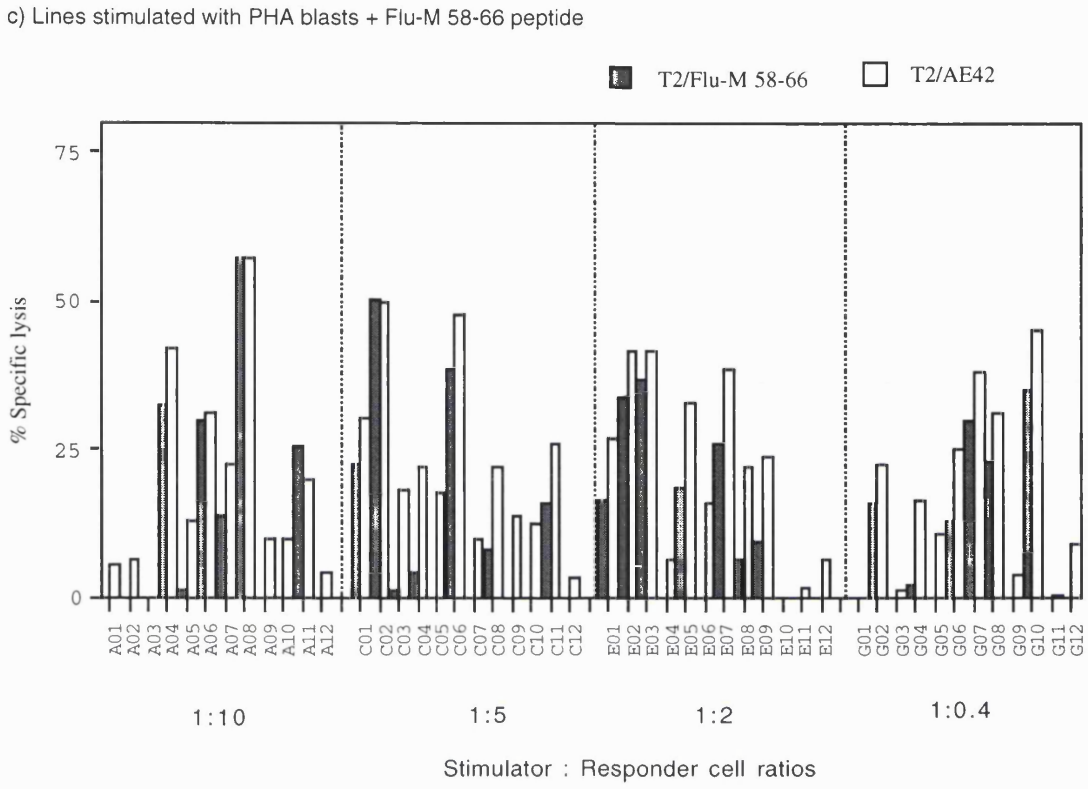


Figure 5.2 The initial reactivity of polyclonal cultures generated by a combined stimulation strategy: using peptide-pulsed autologous PHA blasts followed by T2 cells pulsed with peptide.

PBMC from the HLA-A2 and CMV seropositive donor I were primed using irradiated autologous PHA blasts cells pulsed with the pp65 peptides AE42 (a) or AE44 (b) or the influenza matrix peptide Flu-M 58-66 (c), for the first three stimulations, and peptide-pulsed T2 cells for subsequent re-stimulations. Stimulator to responder ratios at which cultures were established (1:10, 1:5, 1:2 or 1:0.4) are indicated. CTL activity was determined in a 4 h Chromium release assay by split well analysis, in which 150 µl was taken out of each well to test and analysed against three different target cells: labelled T2 cells pulsed overnight with 50 µg/ml of the appropriate peptide, or labelled T2 cells in the presence of ten fold excess of non-labelled T2 cells (cold) as competitors, or labelled T2 cells alone. The different lines were designated by the position of the wells in a 96 well plate. The figure shows representative results from 48 out of 96 wells for each peptide.

5.2.3 The expression of pp65 and HLA class I molecules in target cells

The efficient presentation of endogenously processed pp65 by a CMV-infected cell depends both upon the expression of sufficiently high levels of class I HLA molecules and the presence of pp65 antigen. However, it has been reported that CMV causes the downregulation of HLA class I molecules on infected cells. Thus, prior to the measurement of the peptide-specific CTL responses to CMV-infected cells, it was necessary to ensure that at the time post infection at which infected cells were used as targets in cytotoxicity assays, they were expressing pp65 and also that the expression of HLA class I had not yet been extensively downregulated.

To evaluate the levels of HLA class I molecules on the surface of CMV-infected fibroblasts, subconfluent fibroblast monolayers were infected with a virus inoculum which infected a high percentage of cells (5 pfu/cell, >92% infection). The level of HLA class I expression on the surface of uninfected or infected fibroblasts was evaluated at 24 and 48 and 72 h post infection by staining with the monoclonal antibody W6/32. Stained cells were then analysed by flow cytometry. As shown in Figure 5.3 the level of expression of class I molecules on infected cells at 24 h post infection was 85% of that expressed on uninfected cells. At 48 h post infection the level of class I expression was reduced to 52% of that observed on the uninfected cells and by 72 h this level was further reduced to 25%. These results indicated that at 24 h post infection, although the level of class I expression on CMV-infected cells was somewhat reduced, it was still relatively high. Thus it was assumed that cells which had been infected with CMV for 24 h would still be capable of efficient antigen presentation.

To evaluate the relative levels of pp65 expression at different time points, in particular at 24 h, fibroblasts were seeded into 8 well slide chambers and infected as above. Cells

were stained for pp65 expression at 6, 24 and 48 h post infection and analysed under an epifluorescence microscope. While pp65 expression was not evident at 6 h post infection, pp65 was clearly detected at 24 and 48 h post infection. Figure 5.4 shows the nuclear localisation of pp65 at 24 h post infection and both nuclear and cytoplasmic expression of pp65 at 48 h post infection.

These results suggested that 24 h post infection with CMV was an appropriate time at which to evaluate CTL responses, since relatively high levels of HLA class I molecules remained on the cell surface in the presence of detectable pp65 levels.

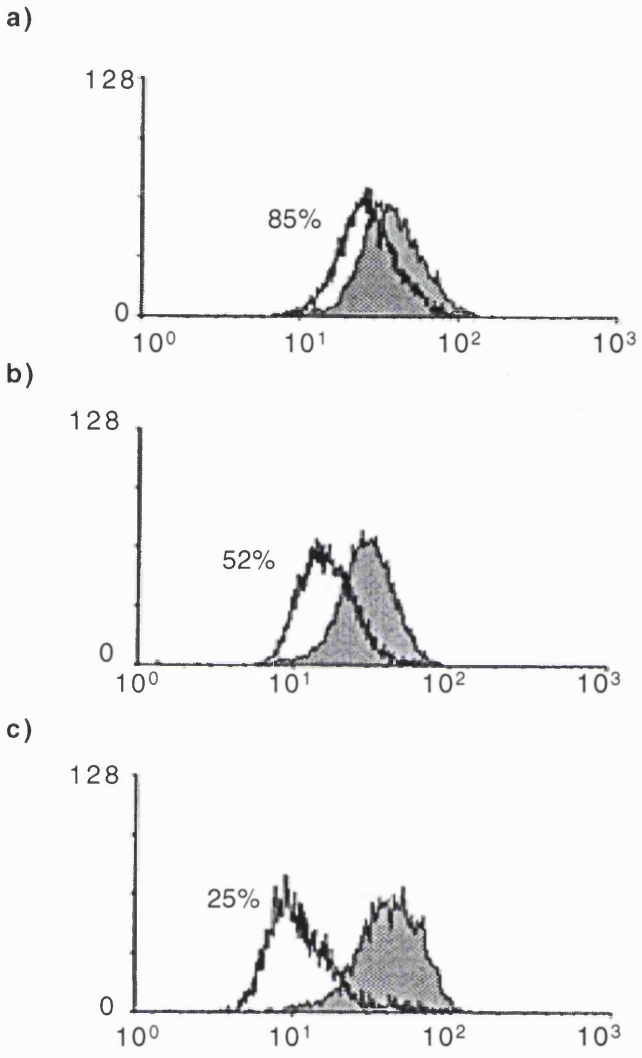
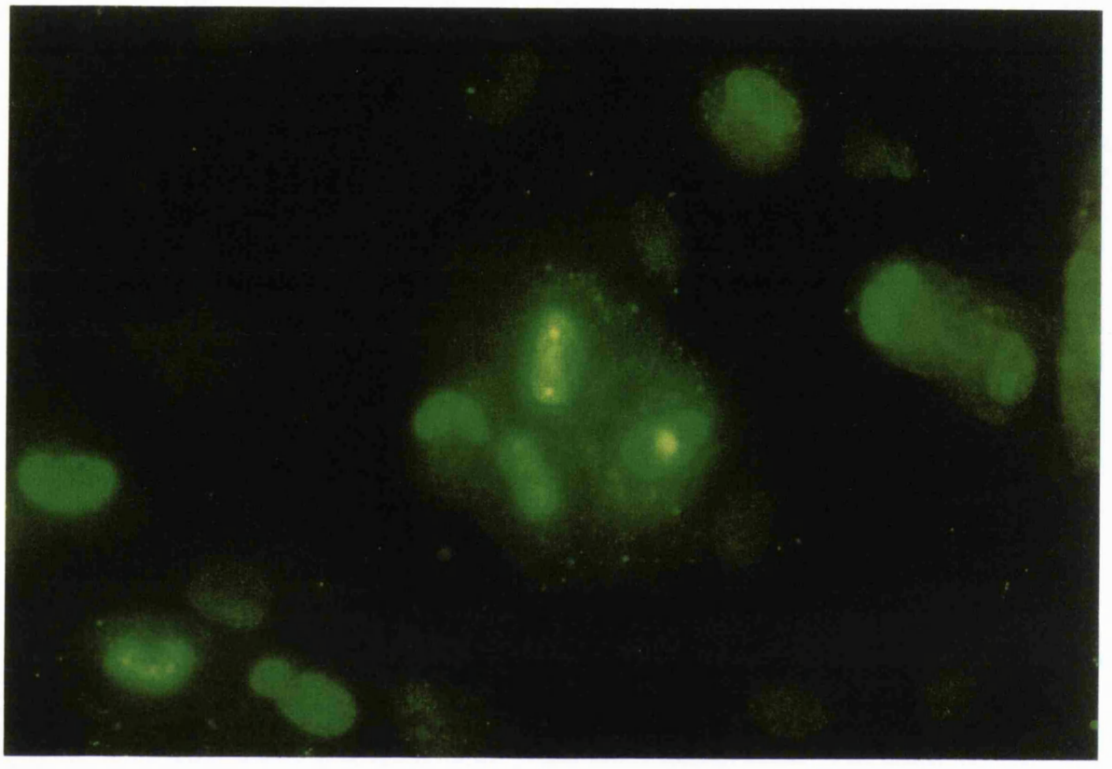


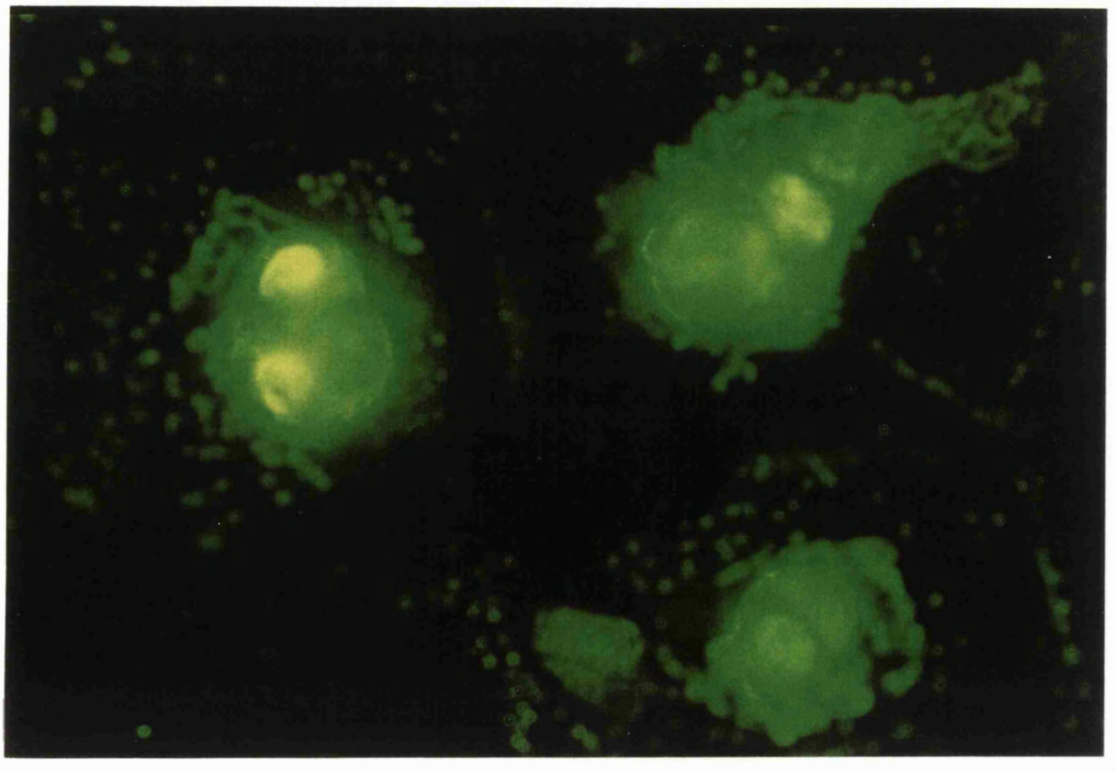
Figure 5.3 Flow cytometric analysis of HLA class I expression on CMV-infected and uninfected fibroblasts at different times post infection.

Fibroblasts were infected with the CMV strain AD169 at an MOI of 2 pfu/cell (unfilled histograms), or left uninfected (filled histograms). At a) 24, b) 48 and c) 72 h post infection the fibroblasts were harvested by trypsinisation and stained for the cell surface expression of HLA class I molecules. The x axis of the histograms show the expression of HLA class I molecules, where a shift to the left on the histogram indicates a decrease in fluorescence intensity. The number of cells with a particular fluorescence intensity is indicated on the y axis. The value shown represents the level of fluorescence intensity on the infected fibroblasts as compared to that on uninfected cells, expressed as a percentage.

a) CMV-infected fibroblasts 24 h post infection



b) CMV-infected fibroblasts 48 h post infection



c) Uninfected fibroblasts

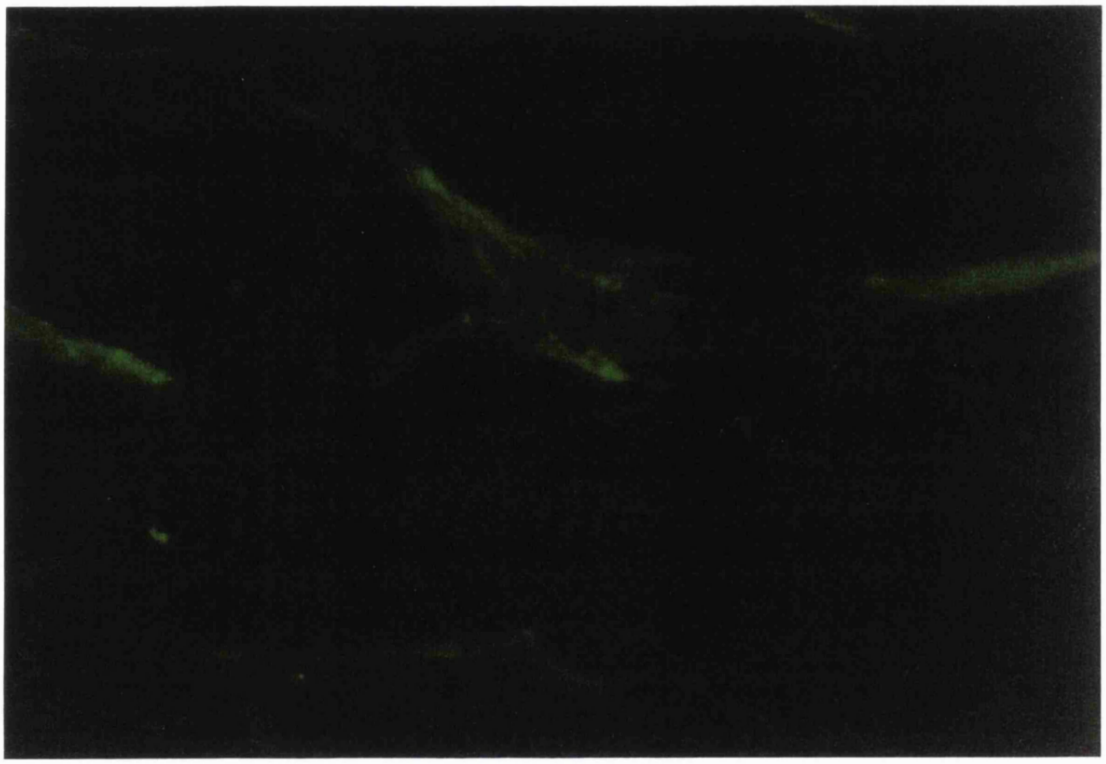


Figure 5.4 Expression of pp65 in fibroblasts at different times post-CMV infection

MRC-5 fibroblasts were seeded in 8 well slide chambers and were either left uninfected or infected with the CMV strain AD169 at an MOI of 2 pfu/cell. Cells were fixed, permeabilised and stained for pp65 expression at 24 and 48 h post infection and examined by epifluorescence microscopy. The figure shows photomicrographs of the immunostained cells. Magnification x400.

CMV-infected fibroblasts were also stained with appropriate isotype control antibody (data not shown).

5.2.4 The recognition of CMV-infected cells by peptide induced CTLs.

In order to determine whether CTLs generated using either T2 cells or PHA blasts pulsed with the pp65 synthetic peptides were able to recognise endogenously processed pp65, the various CTL lines were tested for cytotoxicity against the HLA-A2 positive fibroblast cell line MRC-5, infected with the CMV strain AD169. Uninfected MRC-5 fibroblasts were used as a negative control in these experiments. As shown in Figure 5.5, the CTL lines generated with PHA activated T cells pulsed with peptides AE42 or AE44 in combination with peptide-pulsed T2 cells, were capable of recognising endogenously processed pp65 in the CMV-infected MRC-5 fibroblasts. As can be seen from the graphs, in these cultures there was no significant lysis when uninfected fibroblasts were used as target cells. In contrast, as shown in Figure 5.6, CTL lines induced with T2 cells pulsed with the peptides AE42 or AE44 were unable to recognise the HLA-$A2^{+}$/ CMV-infected fibroblasts. Furthermore, some of these cell lines showed cross recognition of the uninfected target cells (Figure 5.6)

In the lines generated using peptide-pulsed T2 cells, increasing the effector to target cell ratios might have allowed the detection of CMV-specific responses, but may also have potentially increased the detection of non-specific responses. Therefore, the use of PHA activated T cells as stimulator cells in the initial stimulations was adopted when testing the immunogenicity of other pp65 derived peptides.

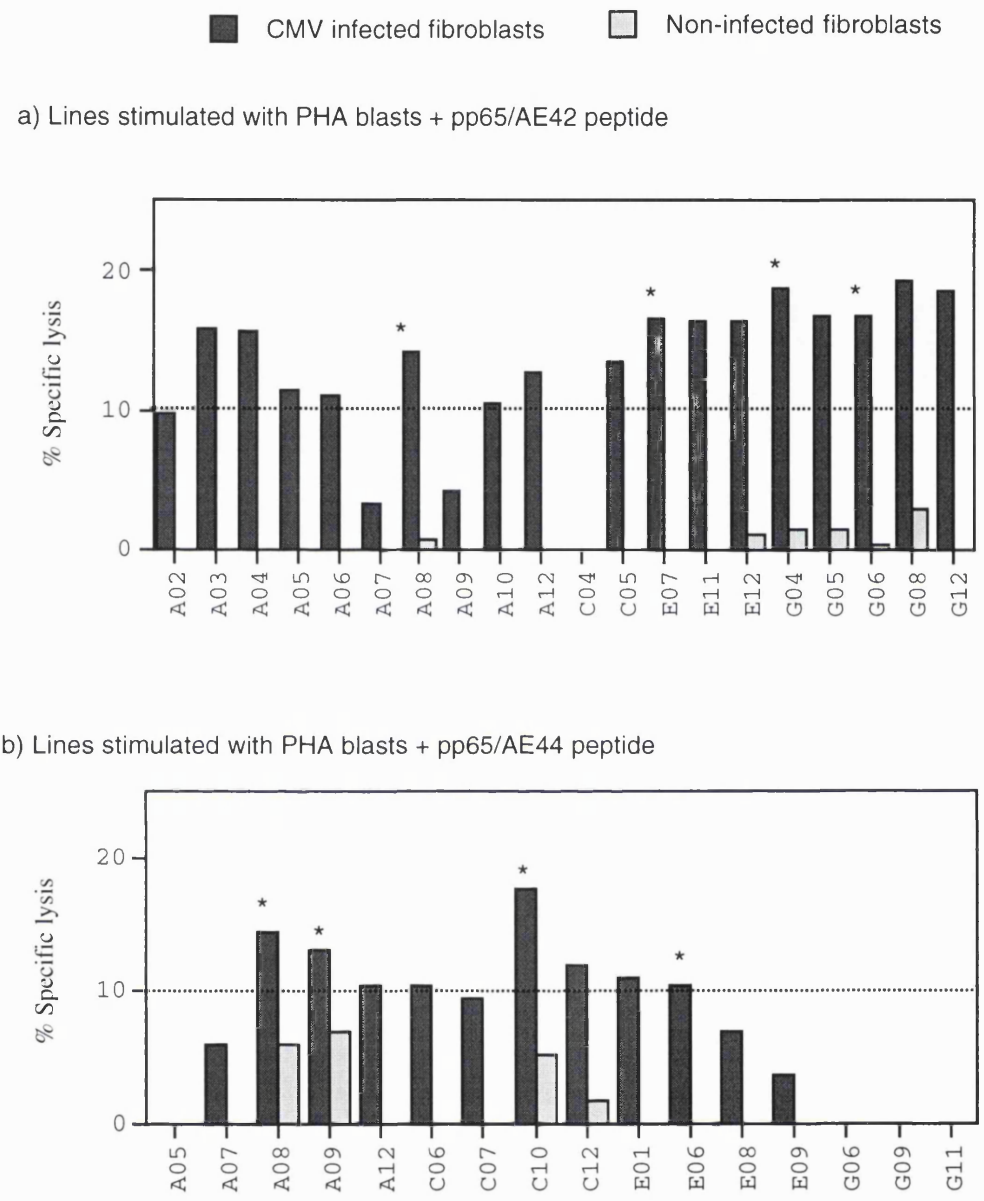


Figure 5.5 The recognition of CMV-infected fibroblasts by pp65 peptide-specific CTL lines induced using peptide-pulsed PHA activated T cells followed by re-stimulation with peptide-pulsed T2 cells .

Peptide-specific CTLs generated using PHA activated T cells pulsed with the pp65 derived peptides AE42 (a) or AE44 (b), followed by re-stimulation with peptide-pulsed T2 cells were tested in a 4 h ^{51}Cr release assay at an effector to target cell ratio of 10:1. MRC-5 fibroblasts either uninfected or infected with the CMV strain AD169 (24 h post infection) were labelled with ^{51}Cr and used as target cells. Asterisks (*) above bars represent selected lines which were subsequently depleted of $CD4^+$ cells (see section 5.2.6).

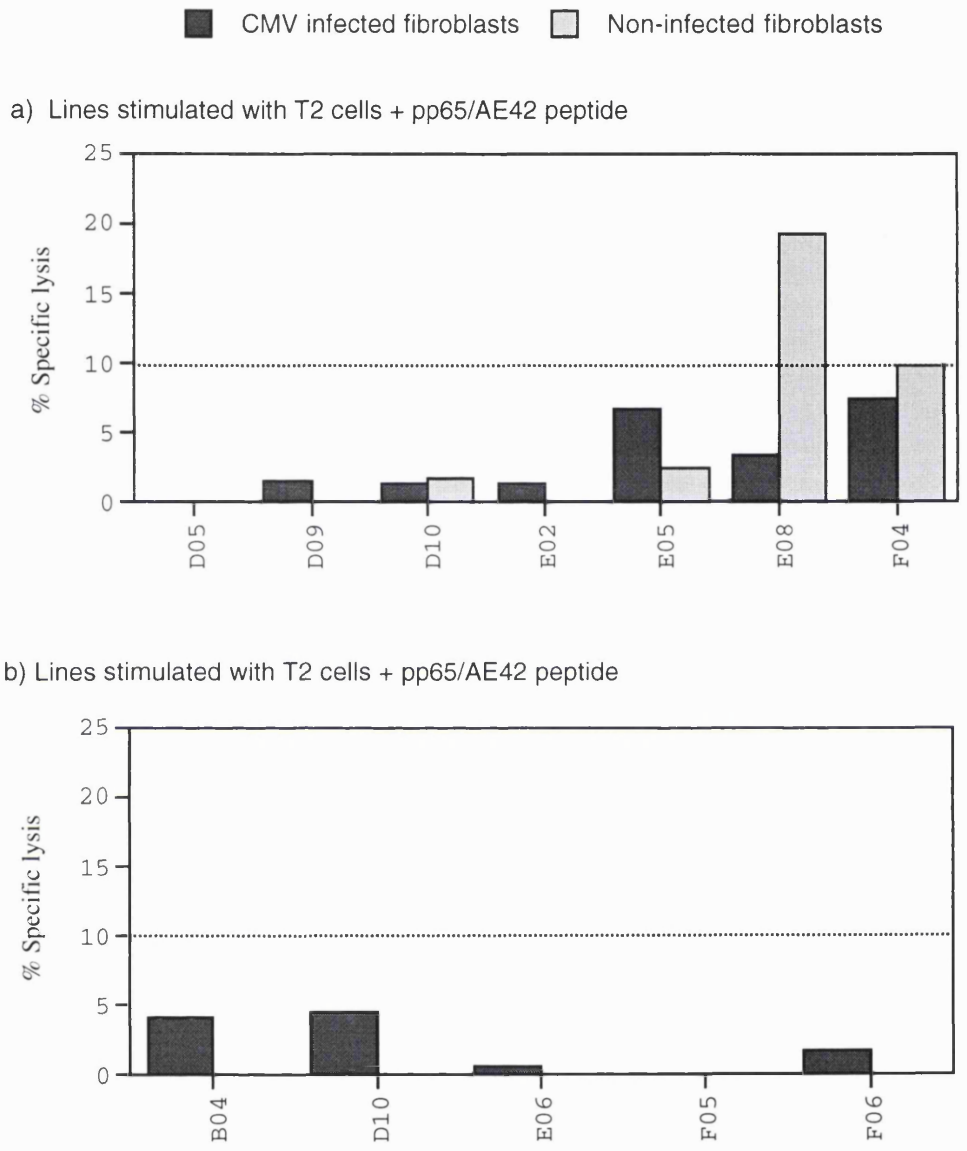


Figure 5.6. The recognition of CMV-infected fibroblasts by pp65 peptide-specific CTL lines induced using peptide-pulsed T2 cells.

Peptide-specific CTLs generated using T2 cells pulsed with the pp65 derived peptides AE42 (a) or AE44 (b) were tested in a 4h ^{51}Cr release assay at an effector to target cell ratio of 10:1. For these assays MRC-5 fibroblasts were infected with the CMV strain AD169, and at 24 h post infection these cells were labelled with ^{51}Cr and used as target cells. Uninfected MRC-5 fibroblasts were processed in parallel. The dotted line represents the minimum level of lysis considered significant.

5.2.5 The generation of AE45 peptide-specific CTLs

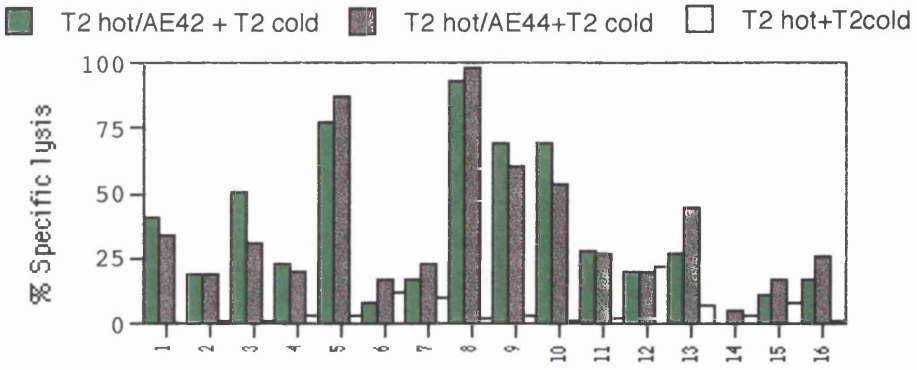
The results described in section 5.2.2 suggest that the strategy employed was able to promote the induction of peptide-specific CTLs which were also able to recognise the native antigen. In order to test whether such a strategy would induce similar CTL responses in a different CMV seropositive and HLA-A2 positive individual, new cultures were established using PBMCs from a second donor. Peptides AE42 and AE44, plus 2 further peptides, AE45 and AE47, were tested for their capacity to induce CMV-specific CTL responses from donor II (HLA A*0201, 19, B7, 27). Peptides AE45 and AE47, had been previously shown (Chapter 3) to have affinity for HLA-A2 in the competition binding assay and the T2 stabilisation assay. Since the strategy employed in the CTL induction involved the use of T2 cells pulsed with peptide for the re-stimulations of responder cells, peptides which gave negative results in the T2 stabilisation assay, such as peptides AE48 and AE49, were excluded from these experiments, despite the fact that they showed affinity for HLA-A2 in the peptide-binding competition assay (Table 3.2).

After 4 weeks in culture, responder cells stimulated with peptides AE42, AE44, AE45 and AE47, were tested for cytotoxicity against T2 cells pulsed with relevant or irrelevant peptides. The results from these experiments demonstrated that peptides AE42 and AE44 were again capable of inducing peptide-specific CTLs using cells from a different donor, which recognised target cells infected with CMV (Figure 5.7). From the new peptides tested, peptide AE45 was also able to generate a peptide and CMV-specific CTL response similar to the response observed when peptide AE44 was used (Figure 5.7). The lysis of T2 cells pulsed with the irrelevant peptide observed when peptides AE42 and AE44 were used as stimulator cells was also observed in cultures generated with peptide AE45 (Figure 5.7). Peptide AE47 was unable to stimulate any specific responses (data not shown) .

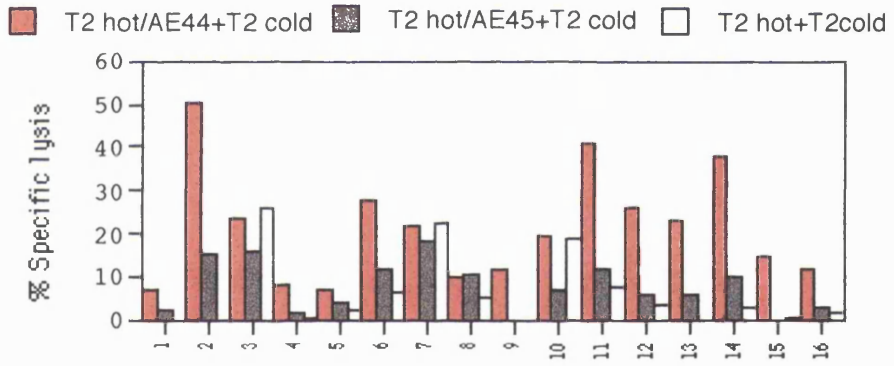
Figure 5.7. The initial reactivity of polyclonal cultures generated from donor II, using a combined stimulation strategy: autologous PHA blasts and followed by re-stimulation with T2 cells pulsed with peptide.

PBMC from the HLA-A2 and CMV seropositive donor II were primed using irradiated autologous PHA blasts cells pulsed with the pp65 peptides AE42 (a) or AE44 (b) or AE45 (c). Cultures were used at a stimulator to responder ratio of 1:10. CTL activity shown here as the percentage of specific lysis, was determined in a 4 h ^{51}Cr release assay by split well analysis, in which 150 µl was taken out of each well and analysed against three different target cells: labelled T2 cells pulsed overnight with 50 µg/ml of the relevant and irrelevant peptide or non-pulsed T2 cells, all in the presence of 10 fold excess of non-labelled T2 cells (cold) as competitors.

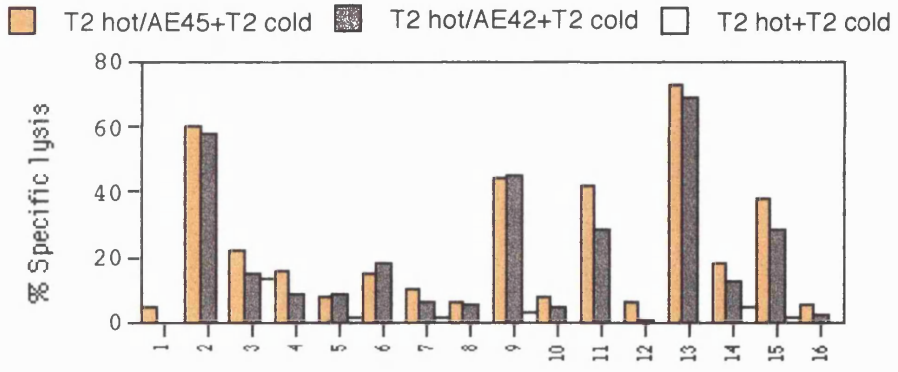
a) Lines stimulated with peptide pp65/AE42



b) Lines stimulated with peptide pp65/AE44



c) Lines stimulated with peptide pp65/AE45



5.2.6 The phenotype of peptide-specific CTLs

Dual-colour flow cytometric analysis was performed to determine the phenotype of the responding population from the cultures stimulated with PHA blasts pulsed with CMV pp65 peptides AE44, AE42 and AE45. Several CTL lines stimulated with the three different peptides were stained with a panel of monoclonal antibodies, and the results are shown in Table 5.1 The results demonstrate that the responder cells stimulated with the respective peptides, consisted of polyclonal populations, composed mainly of $CD4^+$ and $CD8^+$ T cells, while B cells ($CD20^+$) or NK cells ($CD56^+$) were present at a low percentage ranging from 0.5 to 1.5% of the responding population.

Table 5.1 The phenotype of CTL lines stimulated using PHA blasted cells pulsed with the CMV pp65 peptides AE42, AE44 or AE45.*

peptide-specificity of CTL lines	$CD3^+$, $CD4^+$	$CD3^+$, $CD8^+$	$CD3^-$, $CD56^+$	$CD3^+$, $CD56^-$	CD20+
AE44	45.7%	49.4%	0.6%	98.5%	0.5%
AE42	30.6%	56.7%	1.9%	97.7%	1.1%
AE45	33.8%	47.6%	0.5%	97.2%	0.7%

* Two colour flow cytometric analysis of 3 representative CTL lines stimulated using a combined approach of T2 cells and PHA blasts pulsed with the pp65 peptides AE42, AE44, or AE45. The results are expressed as the percentage of positive cells.

5.2.7 The effect of CD4$^+$ T cell depletion and semi-cloning on the responding cell population of pp65 peptide-specific CTL lines

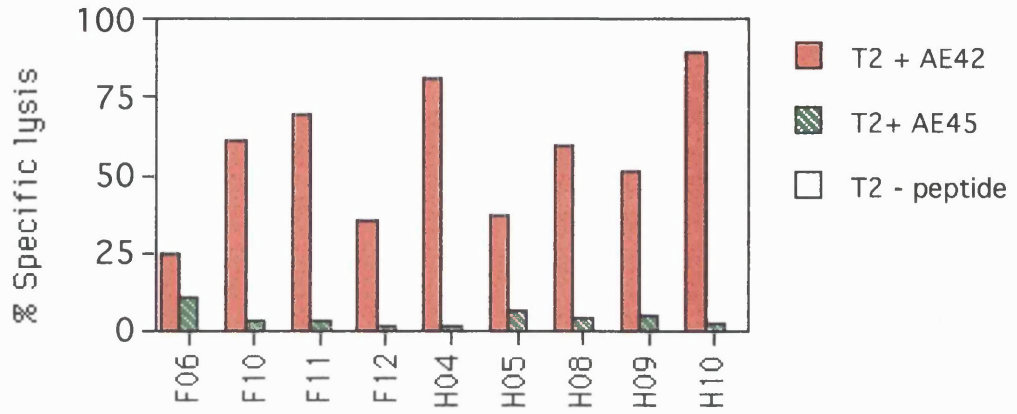
In an attempt to reduce the non-specific reactivity found in the pp65 peptide induced CTLs, and to increase the levels of CD8$^+$ cells in the cultures, selected lines marked with an asterisk (*) in Figure 5.6, were depleted of CD4$^+$ cells. The remaining CD8$^+$ enriched population was plated at 1000 cells/well. Following two rounds of stimulation and cell expansion, these CD4$^+$ depleted CTL lines were again tested for peptide specificity in a cytotoxicity assay. Approximately 20% of the sub-lines then displayed more clearly defined specificity for the relevant peptides than before the CD4$^+$ depletion. These lines no longer crossreacted with T2 cells alone, and displayed minimal recognition of irrelevant peptides (Figure 5.8).

It is therefore clear from these results that the CD4$^+$ depletion strategy followed by the generation of sub-lines, was successful in selecting only those lines which were specific for the pp65 peptides AE42, AE44 and AE45, excluding cells responsible for the non-specific lysis previously observed in cytotoxicity assays.

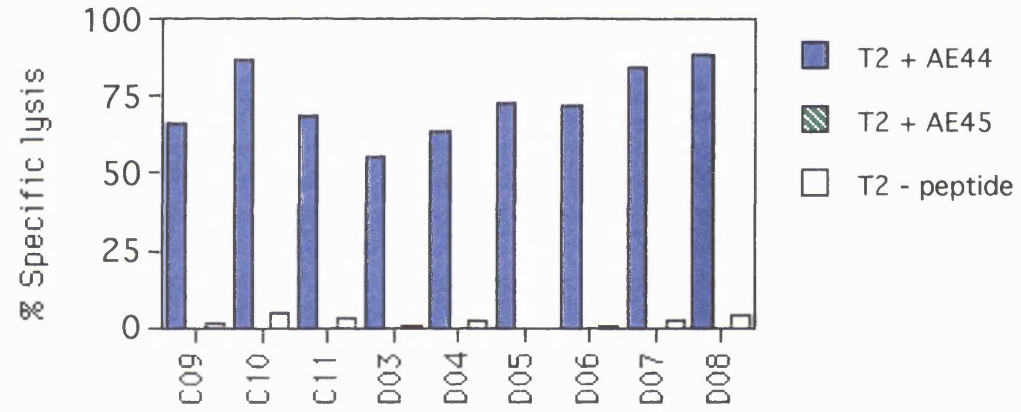
Figure 5.8 Peptide-specific cytotoxicity of CD4⁺ depleted CTL lines stimulated with pp65 derived peptides.

The figure shows representative CTL lines stimulated with pp65 peptides AE42, AE44 or AE45, tested for cytotoxicity three weeks after $CD4^+$ depletion and generation of sub-lines. Target cells in these experiments were ^{51}Cr-labelled T2 cells pulsed with 50 µg/ml of relevant peptides AE42, AE44 , AE45 respectively. T2 cells pulsed with an irrelevant peptide or T2 cells alone were used as negative controls. The effector to target cell ratio used in these experiments was 40:1.

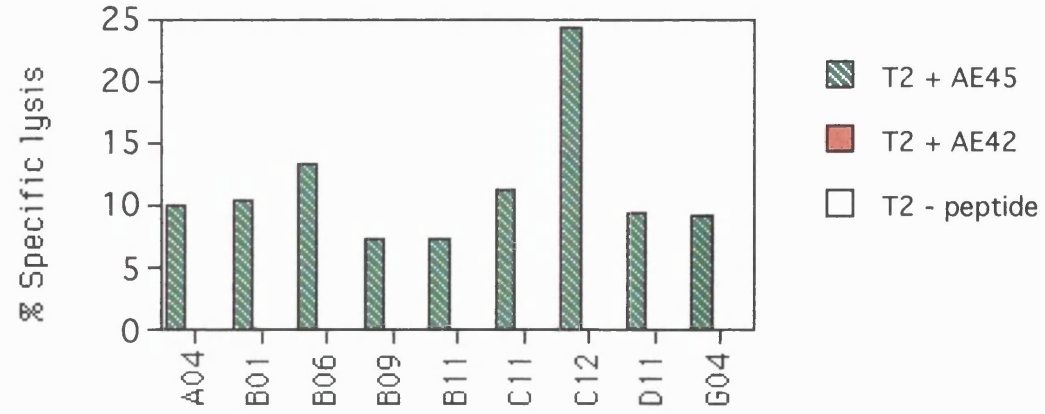
a) Lines stimulated with peptide pp65/AE42



b) Lines stimulated with peptide pp65/AE44



c) Lines stimulated with peptide pp65/AE45



5.2.8 Use of pp65 expressing cells as targets for the recognition of peptide-specific CTLs.

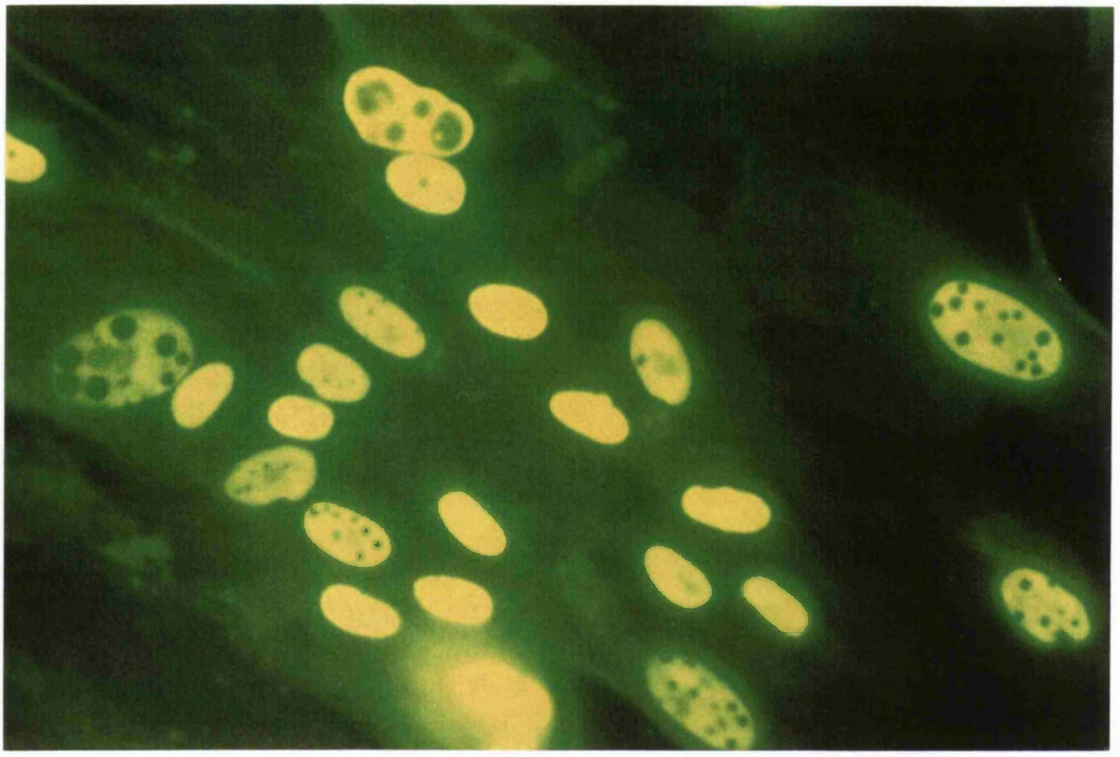
Although in section 5.2.4 it was shown that peptide-specific CTL were able to recognise CMV-infected fibroblasts, it was necessary to show that they were recognising peptides actually derived from the processing of pp65. Therefore, fibroblasts infected with the recombinant adenovirus construct carrying pp65 (RAd/pp65) described in Chapter 4 (section 4.2.11), which expressed pp65 in isolation from any other CMV proteins, were employed as target cells in the cytotoxicity assays described below.

Prior to performing cytotoxicity assays, the relative levels of expression of pp65 in target cells infected either with RAd-pp65 or CMV were compared. Experiments described in Chapter 4 showed that RAd-pp65-infected cells expressed high levels of pp65 either at 24 or 48 h, after exposure to the recombinant adenovirus. However, at 48 h post infection these cells became oversaturated and began to lose their morphology, thus a 24 h infection period was selected for target cells infected with RAd-pp65. In order to obtain comparable levels of pp65 expression in fibroblasts infected with RAd-pp65 and CMV, several different concentrations of RAd-pp65 were tested. Fibroblasts were seeded in 8 well slide chambers and infected with CMV at an MOI of 2 pfu/cell or with RAd-pp65 or RAd-35 at doses ranging from 1 to 500 $TCID_{50}$ units/cell. Figure 5.9 showed that fibroblasts infected with RAd-pp65 at a dose of 100 $TCID_{50}$ units/cell expressed comparable levels of pp65 after 24 h to those observed at 24 h post CMV infection. Infection with RAd-pp65 at higher doses produced very high levels of pp65 expression, however lysis of the cells was also observed even at 24 h.

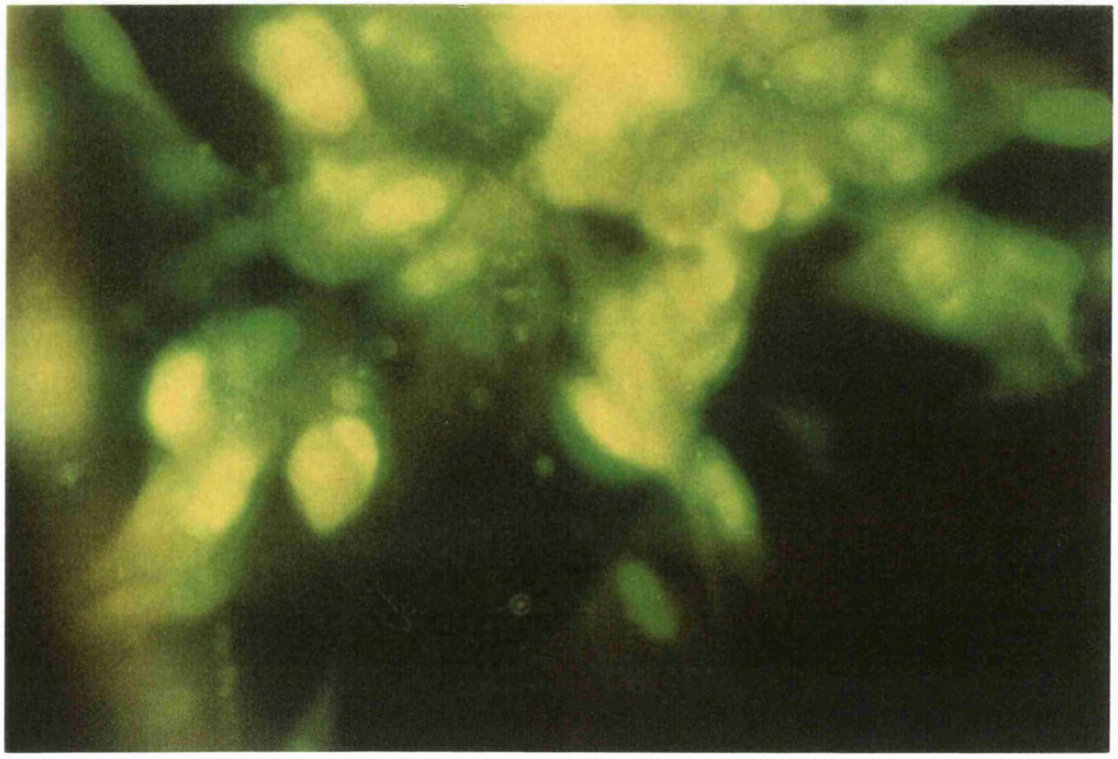
In the case of defective recombinant adenovirus, class I downregulation on infected cells has not been previously demonstrated, however, it was important to ensure that the expression of HLA class I molecules had not been altered when these cells were used as

targets in cytotoxicity assays. Therefore, RAd-pp65 or RAd-35 infected fibroblasts were analysed for the expression of HLA class I molecules at 24, 48 and 72 h post infection, however, no change in the level of cell surface expression of HLA class I molecules was observed at any of these times (data not shown).

a) RAd-pp65-infected fibroblasts



b) CMV-infected fibroblasts



c) Uninfected fibroblasts

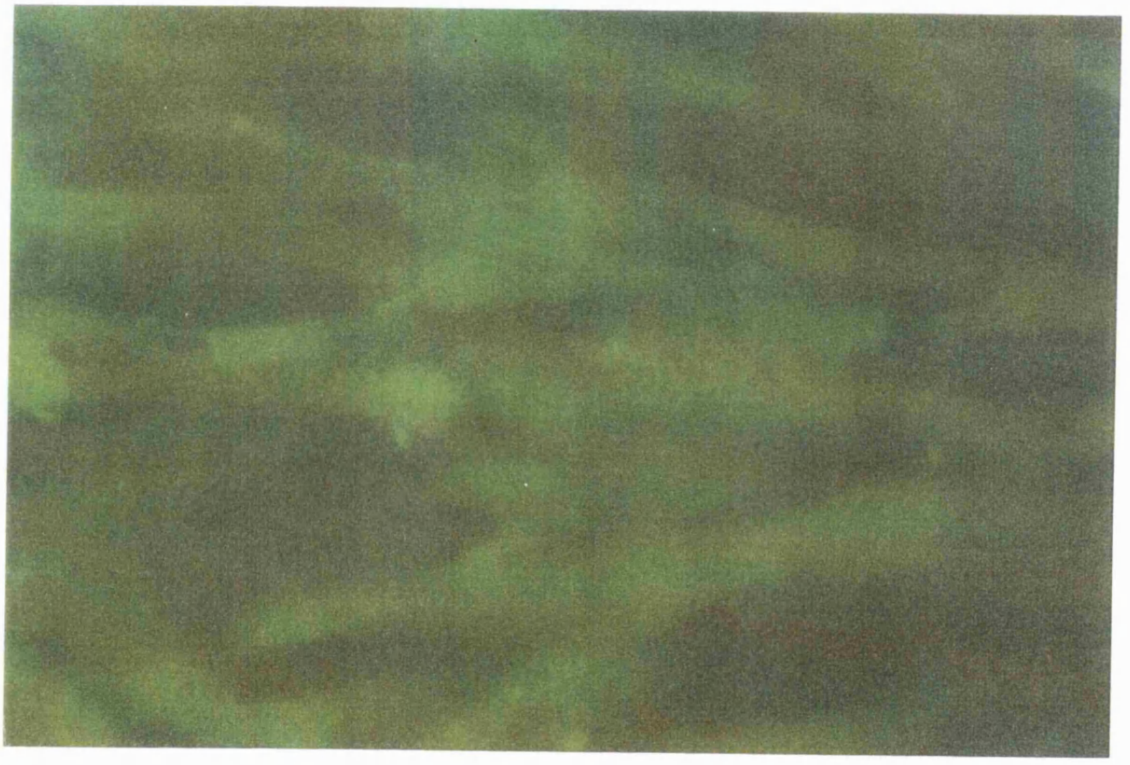


Figure 5.9 Comparison of the relative levels of expression of pp65 after infection with RAd-pp65 and CMV.

MRC-5 fibroblasts were seeded into 8 well slide chambers and exposed to either CMV AD169 viral inoculum corresponding to an MOI of 2 pfu/cell, or RAd-pp65 at 100 $TCID_{50}$ units/cell. The inoculum was allowed to adsorb for 2 h, after which time culture medium was added. Cells were fixed, permeabilised and stained for expression of pp65 at 24 h post infection. pp65 expression was analysed under an epifluorescence microscope. Magnification x400.

5.2.9 Peptide-specific CTLs recognised endogenously processed pp65

The peptide-specific CTL lines H4, C11 and D11 specific for the CMV pp65 peptides AE42, AE44 and AE45 respectively, were established as long term cultures following $CD4^+$ depletion, and were maintained in culture for 6 months. The ability of these CTLs to recognise endogenously processed pp65 was tested in a 4 h ^{51}Cr release assay against HLA-A2 positive fibroblasts infected either with CMV strain AD169 or RAd/pp65.

The results from the ^{51}Cr release assay shown in Figure 5.10 showed that CTLs generated against the three peptides used for the stimulation namely AE42, AE44 and AE45 were able to recognise endogenously processed pp65 presented by HLA-A2 fibroblasts infected with the CMV strain AD169, with minimal recognition of the uninfected HLA-A2 positive fibroblasts. Lines generated against the peptide AE45 showed a comparable percentage of lysis to lines generated against peptides AE42 or AE44, despite the fact that a low level of lysis of peptide-pulsed target cells had been observed in previous experiments shown in Figure 5.8. One possible explanation for this might be that the number of effector cells in the earlier assays was suboptimal and that the extent of the response had been underestimated.

In order to demonstrate that the pp65 peptide-specific CTL lines reacting against CMV-infected fibroblasts were recognising a peptide derived from the endogenously processed pp65, and not from any other CMV proteins, they were tested further for their ability to lyse fibroblasts infected with RAd-pp65. Fibroblasts infected with a control adenovirus construct, RAd-35, expressing the enzyme β-galactosidase were used as a control in these experiments. The data in Figure 5.10 shows that these CTL lines were able to lyse fibroblasts infected with the RAd-pp65 construct, with minimal non-specific lysis against the control RAd-35 infected fibroblasts.

To confirm that the reactivity of pp65 peptide-specific CTLs was restricted by the HLA-A2 allele, they were tested against HLA-A2 negative fibroblasts following natural infection with CMV. The target cell type used in this assay was the fibroblast cell line Hs68, infected with the CMV strain AD169. As shown in Figure 5.10, CTL lines H4, C11 and D11 were unable to recognise endogenously processed pp65 in CMV-infected target cells which did not express HLA-A2 molecules. These results confirmed that CTLs generated against peptides AE42, AE44 and AE45 clearly recognised the CMV-infected target cells in a HLA-A2 restricted manner.

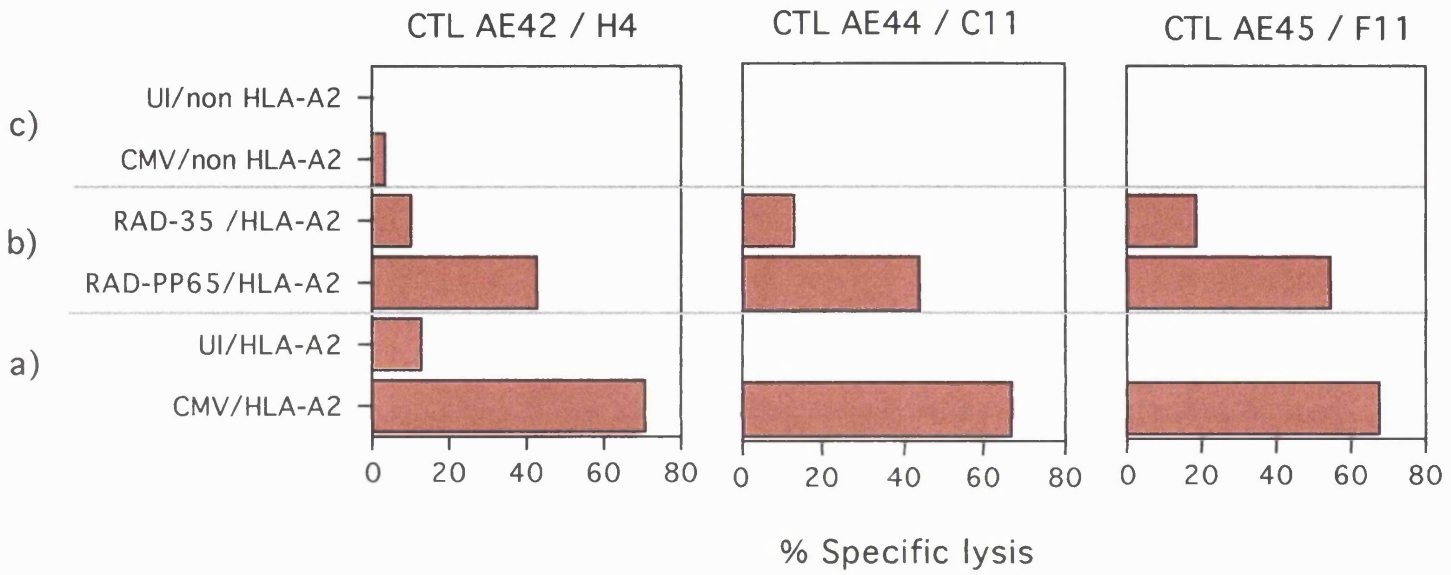


Figure 5.10. The recognition of endogenously processed pp65 protein by peptide-specific CTLs.

$CD4^+$ depleted CTL lines H4, C11, D11, generated against peptides AE42, AE44 or AE45 respectively were tested in a ^{51}Cr release assay. Fibroblasts were infected with the CMV strain AD169 or with the adenovirus construct RAd-pp65 and at 24 h post infection these cells were labelled with ^{51}Cr and used as target cells. CTLs were analysed against either HLA-A2 positive fibroblasts: a) uninfected (UI) or infected with CMV or b) infected with the adenovirus constructs RAd-pp65 or RAd-35 or c)HLA-A2 negative fibroblasts infected with CMV or uninfected (UI). These experiments were carried out at an effector to target cell ratio of 40:1, and the results are representative of two separate experiments.

5.2.10 Analysis of the HLA-A2 restricted CTL epitopes in a range of CMV isolates

The work described above was entirely based upon CTLs activated using pp65 peptides derived from the amino acid sequence of the CMV laboratory strain AD169. To be able to extrapolate these results for the possible use of these peptides in therapeutic settings, it was necessary to determine the extent to which the pp65 peptides AE42, AE44 and AE45 were conserved among other CMV strains. The significance of this being that polymorphisms present in the CMV genome corresponding to pp65 might alter these CTL epitope sequences, and thereby may abrogate the CTL recognition of other CMV strains. This phenomenon has been previously demonstrated for Epstein Barr virus (Apolloni *et al.*, 1992; de Campos-Lima et al., 1993).

DNA was extracted from cell free virus preparations of the CMV laboratory strains AD169, Towne and Davis, and four low passage clinical isolates, Toledo, CRV, R7, CIF and CIFE, and the gene encoding pp65 was amplified by PCR and sequenced by direct sequencing. When the DNA sequences were compared to the published sequence of the CMV strain AD169, several nucleotide differences were found in the other strains, however, most of these differences were conservative, that is they did not change the amino acid sequence. Only four of the differences found at the nucleotide level would result in amino acid coding substitutions which differed from the amino acid sequence of the strain AD169 (Table 5.2). The first substitution present only in the CMV strain Toledo, encoded a Ser → Asn mutation at position 371. The second substitution found in Toledo and in the Towne strain was a Ser → Ala mutation in position 447, while the third substitution which encoded an Asp → Glu mutation in position 545, was found in three strains, Davis, Toledo and CRV (Table 5.2). The fourth substitution, Ile → Ala in position 12 which was present only in the Towne strain, had been previously detected by others (Pande *et al.*, 1984).

Importantly, as Figure 5.11 shows, none of the coding substitutions identified affected the amino acid sequence of any of the pp65 derived peptides used in the present study, either the peptides which showed to be HLA-A2 restricted CTL epitopes or even those peptides which showed affinity for the HLA-A2 molecule in binding assays.

The amino acid substitutions observed in some of the CMV strains may, however affect CTL epitopes restricted to other HLA class I antigens. Therefore peptide binding motifs for a range of HLA molecules were analysed to investigate whether these substitutions affected potential binder peptides for these antigens. Twenty five HLA class I antigens found in the Caucasoid population were analysed by the peptide binding motif computer algorithm described in Chapter 3. The most interesting results were found with the antigen HLA-B*4403, in which a peptide at position 364-372 of pp65 (SEHPTFTSQY) was found to bear the appropriate binding motif and had a high score in the prediction of the possible binding peptides. This peptide spans the mutation found in the CMV strain Toledo in position 371 (Ser → Asn), corresponding to position 8 on this peptide. Two more peptides, found to be potential binders for HLA-B*2705 position 539-547 (RRRHRQDAL) and HLA-B*3701 position 544-552 (QDALPGPCI) respectively, were found to span the substitution in position 545 (Asp → Glu) present in the Davis, CRV and Toledo strains of CMV, and these peptides were predicted to bind the antigens with scores of 6000 and 40 respectively. Surprisingly, when the three mutations were considered and the hypothetical sequences submitted for the algorithm prediction, no effect was found with the scores previously assigned for wild type peptides. One explanation is that, particularly in the case of a substitution in position 545, the change is relatively conservative.

Table 5.2. Amino acid sequences of pp65 peptides with coding substitutions in different CMV strains*

CMV strain	Encoding substitutions							
	25							48
AD169	CCC	GAA	ATG	ATA	TCC	GTA	CTG	GGT
	P	E	M	I	S	V	L	G
				12				
R7								
C1F								
C1FE								
TOWNE	P	E	M	A	S	V	L	G
DAVIS								
CRV								
TOLEDO								
	1102							1125
AD169	ACC	TTC	ACC	AGC	CAG	TAT	CGC	ATC
	T	F	T	S	Q	Y	R	I
				371				
R7								
C1F								
C1FE								
TOWNE								
DAVIS								
CRV								
TOLEDO	T	F	T	N	Q	Y	R	I
	1335							1359
AD169	TGC	ACG	TCG	GGC	GTT	ATG	ACA	CGC
	C	T	S	G	V	M	T	R
			448					
R7								
C1F								
C1FE								
DAVIS								
TOWNE	C	T	A	G	V	M	T	R
CRV	C	T	A	G	V	M	T	R
TOLEDO	C	T	A	G	V	M	T	R
	1627							1650
AD169	CGG	CAA	GAC	GCC	TTG	CCC	GGG	CCA
	R	Q	D	A	L	P	G	P
			545					
R7								
C1F								
C1FE								
TOWNE								
DAVIS	R	Q	E	A	L	P	G	P
CRV	R	Q	E	A	L	P	G	P
TOLEDO	R	Q	E	A	L	P	G	P

* The DNA obtained from the different CMV strains was amplified by PCR using pp65-specific primers, and the DNA sequences obtained translated into their coding amino acids. Predicted amino acid changes with respect to the AD169 CMV sequence in 4 different regions of the pp65 protein are shown in bold. The nucleotide sequence is shown only for the AD169 strain.

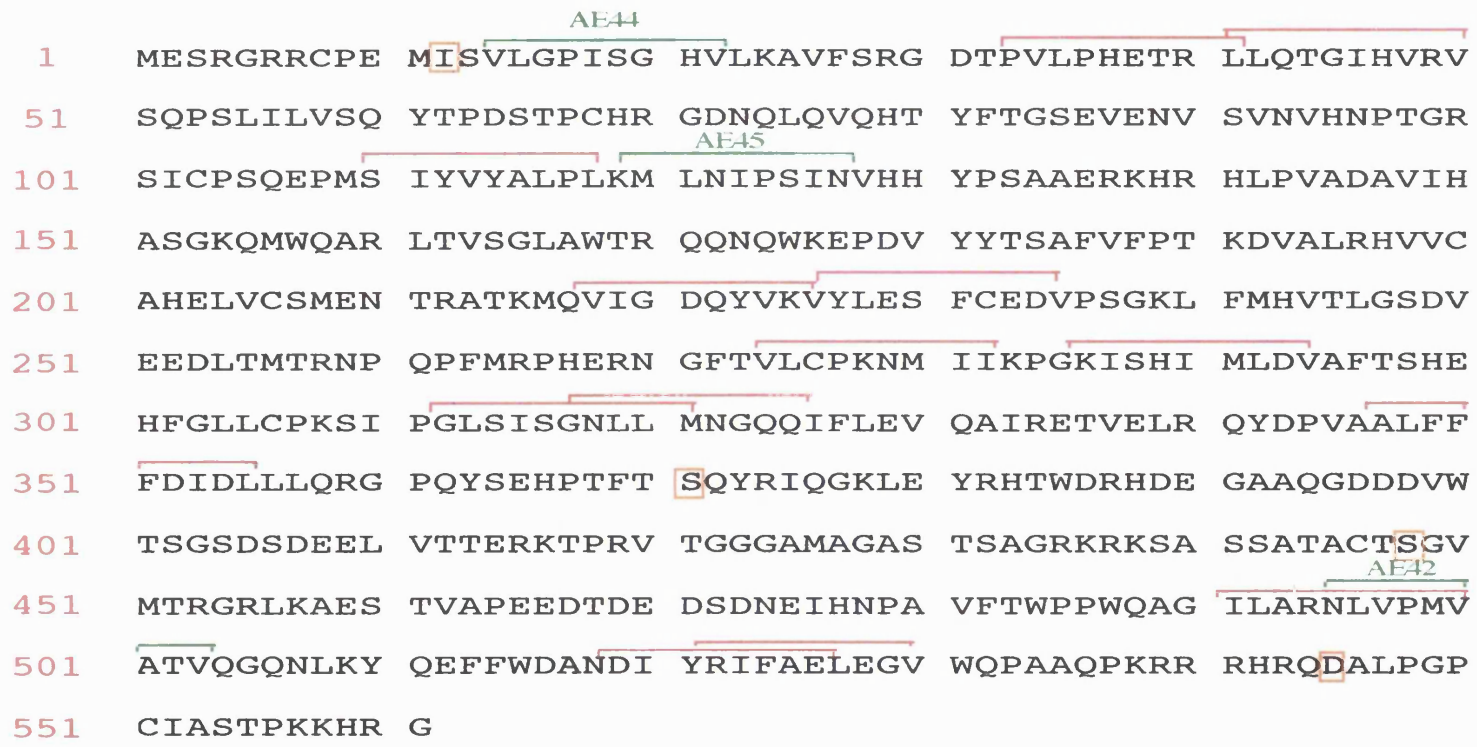


Figure 5.11. Location of the amino acid variations found in the pp65 sequence of different CMV isolates shown in Table 5.2.

Peptides used in this study are shown with a line above. Peptides used in this thesis have been named. The CTL peptide epitopes AE42, AE44 and AE45 found in pp65 are highlighted in blue. The variations Ile → Ala in position 12, Ser → Asn in position 371, Ser → Ala in position 447, and Asp → Glu in position 545 are highlighted by the orange boxes.

5.3 Discussion

In the present study, three of the pp65 derived peptides which displayed high or intermediate affinity binding to HLA-A2 were shown to be able to induce peptide-specific CTLs. Such peptides, namely AE42, (495-503, NLVPMVATV) from the carboxyl terminal, and AE44 (14-22 VLGPISGHV) and AE45 (120-128, MLNIPSINV) from the amino terminal end of the pp65 amino acid sequence, were confirmed to be the actual CTL peptide epitopes presented in a natural infection in the context of HLA-A2.

A strategy was developed in order to induce a CMV-specific CTL response restricted to HLA-A2, by stimulation of effector cells with the HLA-A2 binding peptides identified previously in Chapter 3. Although the use of T2 cells pulsed with peptide as stimulator cells had previously been successfully employed in the generation of EBV-specific CTLs with peptides derived from the EBV protein EBNA-4 (Reali et al., 1996), when peptide-pulsed T2 cells were used as stimulator cells in the present study, they consistently induced the generation of non-specific CTL responses. Furthermore, this stimulation strategy led to the induction of CTLs that did not recognise the endogenously processed pp65, which might be due to the stimulation of low affinity CTLs. Similar observations were reported by Gagliardi et al, who found that the use of T2 stimulator cells was associated with a very strong non-peptide-specific response which obscured any peptide-specific responses (Gagliardi et al., 1995). The background lysis observed when T2 cells were used as stimulator cells might be due to the presentation of endogenous peptides possibly derived from endoplasmic reticulum (ER)-resident proteins, which do not require peptide transporters to access surface HLA-A2 molecules on T2 cells (Henderson et al., 1992). It has been shown that peptides derived from the 30-Kd intracellular polypeptide, IP-30, and calreticulin, can be found in association with HLA-A2 molecules on T2 cells (Hunt et al., 1992; Hunt *et al.*, 1986; Luster *et al.*, 1988). Furthermore, the proteolysis of signal peptide domains in the ER has been shown to be a possible second mechanism for the

processing and presentation of peptides by HLA class I molecules (Henderson et al., 1992). However, peptides could also be imported into the ER by other TAP independent mechanisms, since this has been demonstrated for the RMA-S cell line (the mouse counterpart of the T2 cell line), which has been shown to present certain viral and minor histocompatibility antigens on MHC class I molecules, albeit at a low level (Hosken and Bevan, 1992; Zhou *et al.*, 1993). Moreover, the generation of primary CTL specific for TAP deficient cell lines has also been reported (Wolpert *et al.*, 1997). Therefore, it was not surprising to find a high level of background lysis when these cells were used as stimulator cells to present peptides which do not have a high affinity for the HLA-A2 molecule. Possibly these peptides do not have the capacity to displace the low level of ER peptides already bound to this molecule on T2 cells.

Another disadvantage of using of peptide-pulsed T2 cells to induce peptide-specific CTLs was that such CTLs were unable to recognise endogenously processed pp65. This finding might be due to the fact that the stimulation with these cells promoted the generation of low affinity CTLs that lysed target cells in the presence of relatively high concentrations of exogenous peptide, as in the case of peptide presented by T2 cells. However, these potentially low affinity CTLs were apparently unable to recognise epitopes from endogenously processed pp65 which, in the naturally infected cells, might be presented at levels insufficient for recognition by the CTLs, as previously observed for other antigens (Christinck *et al.*, 1991; Ohno, 1992; Schild *et al.*, 1991).

A second stimulation strategy which involved the use of autologous PHA blasts pulsed with the pp65 derived peptides for the first two rounds of stimulation, followed by stimulations using peptide-pulsed T2 cells, was able to overcome the problems of low specificity and also the lack of recognition of endogenously processed pp65 found when peptide-pulsed T2 cells were used exclusively as stimulators. This alternative strategy was conducive to the production of effector cells that were not only able to recognise peptide-

pulsed T2 cells as targets, but also CMV-infected cells, and cells infected with an adenovirus construct expressing pp65, in an HLA-A*0201 restricted manner. These results imply that the three pp65 derived peptides identified expressed in association with HLA-A2 on the surface of infected cells are a result of endogenous pp65 processing.

Therefore, the latter protocol of CTL stimulation was adopted, because it afforded the convenience of using a defined cell line that could be maintained for long periods in culture. Following this protocol, two of the peptides which displayed "good" binding to HLA-A2, namely AE42, (495-503, NLVPMVATV) from the carboxyl terminal, and AE44 (14-22 VLGPISGHV) from the amino terminal of the pp65 protein, were able to induce significant CTL responses, whereas peptide AE47 failed to do so. The failure of AE47 to induce CTLs correlated with its poor ability to stabilise HLA-A2 molecules on T2 cells. Peptide AE45 (120-128, MLNIPSINV) only induced a weak CTL response, although it consistently bound strongly to HLA-A2 in the two peptide binding assays. In spite of this initially poor CTL response, continuous stimulation with this peptide allowed the establishment of long term CTL lines. Responses to peptides AE42 and AE44 were found in two CMV seropositive and HLA-A2 individuals, while responses to peptide AE45 were detected in the only individual tested.

An interesting aspect of the present study was the demonstration of responses to subdominant CTL epitopes which were not detected in polyclonal cultures obtained by conventional stimulation protocols (Diamond et al., 1997; McLauglin-Taylor et al., 1994; Wills et al., 1996). Following a different strategy, two independent groups identified a peptide sequence which lies in the same amino acid region of one of the peptides found in this study, AE42 (495-503, NLVPMVATV). Wills *et al.* designed overlapping peptide sequences from pp65 and tested them for their recognition by CMV-specific CTLs, and reported that such CTLs recognised a 15-mer (493-507) and a 10-mer (495-504) presented in the context of HLA-A*0201(Wills et al., 1996). Diamond and colleagues (Diamond et

al., 1997) mapped the recognition of a pp65-specific T cell clone restricted by HLA-A*0201 to an 84-amino acid region in the carboxyl terminal end of pp65. From this region they designed synthetic peptides based on MHC binding motifs, and found the peptide pp65 (495-503, NLVPMVATV; known in the present study as AE42) to be the only epitope recognised by their pp65-specific T cell clone. They also reported that this peptide was able to induce CTLs, both *in vivo* and *in vitro*, which lysed HLA-A*0201 positive CMV-infected fibroblasts.

Thus the results presented in this chapter have not only identified the peptide AE42 (NLVPMVATV) as a CTL epitope, but have also identified three peptides derived from pp65, peptides AE44 (14-22 VLGPISGHV) and AE45 (120-128, MLNIPSINV), which were able to elicit a CMV-specific CTL response in the context of HLA-A2. Conflicting observations for peptide AE44 have been reported by Diamond *et al.* In their study preliminary immunisations of HLA-A2 transgenic mice with pp65 peptides starting at amino acids 14, 40 and 227, which corresponded to peptides AE44, AE48 and AF88 respectively, were capable of eliciting peptide-specific responses in the HLA-A2 mice. However, unlike the peptide pp65 495-503, the splenic CTLs raised against such peptides were unable to recognise endogenously processed pp65 (Diamond et al., 1997). The discrepancies between the results reported here and the results obtained in the Diamond study, might be related to the system used and the antigen presenting cells used by the latter study in raising peptide-specific CTLs, since it is not known if the immune system in concert with the peptide degradation pathway would select the same epitopes to be recognised by CTL when it is presented by different antigen presenting cells. These observations, together with the results obtain in the present study, suggest that stimulation with synthetic peptides in association with an appropriate stimulator cell can be an efficient tool for the selective expansion of CTL precursors which are present in low numbers in the memory T cell pool. It is noteworthy that subdominant epitopes are likely to be a good target for immune intervention, since they are less prone to mutate as a consequence of *in*

vivo immunoselection. The protocol of stimulation described in this chapter may therefore be the strategy of choice for use in large scale production of CTL for immunotherapy protocols.

The results described in this chapter also demonstrated that the amino acid sequences of the three peptides identified as HLA-A2 restricted CTL epitopes were conserved amongst different wild type strains of CMV from renal transplant patients (R7 and CRV), an AIDS patient (C1F and C1FE) and a congenitally infected infant (Toledo), as well as three laboratory strains of the virus (AD169, Towne and Davis). Although the analysis of a larger number of wild type CMV samples would be desirable, these observations suggest that these CTL peptide epitopes could potentially be used as synthetic peptide vaccines, or for other therapeutic strategies aimed at HLA-A2 positive individuals. Furthermore, these observations must be taken into consideration when the search for CTL peptide epitopes is extended to other HLA class I alleles, as these mutations may span potential CTL epitopes for other HLA molecules, such as HLA-B*4403, HLA-B*2705 and HLA-B*3701.

6. Chapter 6. General discussion

Given the importance of CTL in controlling CMV infection, there has been a great deal of interest in designing methods for the induction and augmentation of the CMV specific CTL response in immunocompromised individuals. Several therapeutic strategies have been attempted in order to achieve this aim, including vaccination and adoptive T cell therapy.

A number of studies have demonstrated that the immunodominant target antigen in an anti-CMV CTL response was the structural phosphoprotein pp65 (Boppana and Britt, 1996; Diamond et al., 1997; Li et al., 1994; McLauglin-Taylor et al., 1994; Riddell and Greenberg, 1994; Wills et al., 1996). Given the relevance of the pp65 protein in the overall CTL response to CMV, a detailed analysis of its recognition in the context of HLA class I antigens is crucial in the design of immunological methods to augment CMV-specific immunity in immunocompromised individuals. Thus the identification of immunogenic pp65-derived peptides in the context of high frequency HLA-A and -B molecules is required.

In the present study a systematic approach was adopted in order to rapidly identify CTL peptide epitopes from the CMV protein pp65. Potential antigenic peptides were first selected from the known sequence of pp65 (Ruger et al., 1987), for the presence of HLA-A2 anchor residues (Falk et al., 1991), and then selected by two different methods for their capacity to bind to HLA-A2 molecules. Peptides which showed high or intermediate affinity for the HLA-A2 molecule were then tested for their ability to stimulate a CMV specific memory CTL response. Three HLA-A2 restricted cytotoxic T cell peptide epitopes were identified in the CMV pp65 protein. These nonamer peptides, referred to as

AE44 (14-22 VLGPISGHV), AE45 (120-128, MLNIPSINV) and AE42, (495-503, NLVPMVATV), were all able to induce peptide-specific CTLs. Crucially the peptide-specific CTL's were shown to be capable of recognising the naturally processed pp65 in an HLA-A*0201 restricted manner as demonstrated by the lysis of CMV infected target cells and target cells infected with an adenovirus construct containing the gene encoding the pp65 protein. The results obtained in the present study with peptide AE42, have recently been confirmed by two other groups (Diamond et al., 1997; Wills et al., 1996). In these other studies however, peptides AE44 and AE45 were not identified as CTL epitopes. Thus, the approach taken in this thesis had the advantage that it not only identified the peptides that are immunodominant as AE42 seems to be, but was also able to identify two more pp65 peptides which might be subdominant CTL epitopes. Thus the strategy detailed here for the prediction of CTL epitopes has been demonstrated to be both practical and successful in the identification of CTL epitopes derived from the pp65 protein, and could be used to identify CTL epitopes not only derived from other CMV, proteins but many other immunogenic proteins.

Similar approaches have been successfully employed in the prediction of CTL epitopes for other microorganisms. The most notable instance of this has been the identification of a peptide binding motif for H-2K^d which conferred protective CTL immunity to *Listeria* monocytogenesis in mice (Pamer et al., 1991). Similarly, a CTL epitope from *Plasmodium falciparum* was identified which might protect individuals who express HLA-B*5301 from severe malaria (Hill et al., 1992).

The induction of protective immunity has been achieved classically by immunising individuals at risk with attenuated or inactivated pathogens. In the case of CMV, several trials with a live but attenuated strain of the virus, Towne vaccine have been performed (Plotkin et al., 1994; Plotkin et al., 1991; Plotkin *et al.*, 1990; Plotkin *et al.*, 1989). This vaccine was shown to induce a level of response in seronegative renal transplant recipients

similar to that given by previous natural infection with CMV (Plotkin et al., 1991). The relative protective effect of this vaccine was associated with a proliferative immune response, although the induction of a CMV specific CTL response was not described (Plotkin, 1991). More recently a new batch of the live attenuated Towne vaccine was tested in three open label trials involving 140 individuals, including women of childbearing age and children (Adler *et al.*, 1998). This vaccine was found to induce a broad range of immune responses including the production of neutralising antibodies, lymphopoliferative responses and CTL responses (Adler et al., 1998). Although this type of approach would appear to be relatively successful, certain concerns persist about the safety and efficacy of such vaccines, in part because of the complexity of the virus life cycle, which includes latency and reactivation. Thus, it is not known if this CMV vaccine strain could reactivate with time, or under certain conditions, and cause pathology. Furthermore this approach might not work in the severely immunocompromised, such as BMT patients. It may even be dangerous in the context of AIDS patients where a CMV protein has been demonstrated to operate as a superantigen, creating a T cell pool preferentially infected by HIV (Dobrescu et al., 1995)

Other approaches to producing viral vaccines have included the use of either live canary pox, baculovirus or vaccinia virus vectors to encode immunogenic proteins (Borysiewicz et al., 1996; Cadoz *et al.*, 1992; Gonczol *et al.*, 1995; Hruby, 1993; Khanna *et al.*, 1992; Kulkarni *et al.*, 1993; Plotkin *et al.*, 1995; Xiang *et al.*, 1996). In the case of CMV this type of approach has been used mainly for glycoproteins (Gonczol et al., 1995; Marshall and Plotkin, 1990; Marshall *et al.*, 1990). However these type of strategies must also be used with caution in immunocomppromissed patients, since live viral vectors could cause disease, for example disseminated vaccinia infection, in this group of people. In the mean time, efforts to develop CMV immunotherapies that are based on different technologies, such as subunit or peptide vaccines and adoptive T cell immunotherapy, are also developing (Diamond et al., 1997; Riddell and Greenberg, 1994; Riddell et al., 1992b).

Synthetic viral peptides have recently been shown to be effective in priming naive animals to generate virus-specific CTL (Deres *et al.*, 1989; Vitello et al., 1995). Using such an approach would have the advantage of eliciting CTL responses to CMV in immunocompromised patients, without the need for exposure to infectious virus. However there are several important factors to consider when identifying immunogenic epitopes for use in a peptide vaccination strategy. These include (1) the survival and immunogenicity of the peptide in vivo, (2) the breadth of epitopes necessary to generate an effective immune response in the majority of individuals, (3) the efficient identification of these epitopes, and (4) the population genetics of the HLA system.

Peptide vaccines may have several advantages over traditional vaccines (1) they can be synthesised in large quantities with high reproducibility and excellent purity, (2) they are safe, and (3) they can be designed to induce well-defined monofunctional immune responses. The major disadvantages of peptide vaccines are their low immunogenicity and the monospecificity of the response induced, especially in outbred populations (Ertl and Xiang, 1996). Furthermore, peptides injected into an individual will probably be subjected to proteolytic degradation, alternatively they might be absorbed rapidly onto irrelevant structures and become inaccessible to the immune system. Some of these problems, such as the low immunogenicity and the rapid degradation of peptides can be corrected by modification of the peptides (Otvos *et al.*, 1995; Otvos *et al.*, 1994), or by their incorporation into controlled release formulations (Ertl *et al.*, 1996). Although, the modification of peptides may adversely affect the binding of peptides to HLA molecules, this strategy can result in a efficient CTL response against the modified peptide (Diamond et al., 1997). As mentioned above, the pp65 derived peptide AE42 (495-503) identified in this study as a CTL epitope was recently described by others as the only HLA-A2 restricted CTL epitope (Diamond et al., 1997). In their study, Diamond and colleagues tested the immunogenicity of this peptide in vivo in a HLA-$A2^+$ transgenic mouse model. In order to stimulate an immune response in the transgenic mouse without the use of adjuvants, they

modified the amino terminus of the pp65 peptide (495-503) by the addition of a lipid molecule. This 'lipidated' peptide was covalently attached to a helper T cell epitope, and used to immunise the HLA-$A2^+$ transgenic mouse. Thus, the chimeric and lipidated pp65 (495-503) peptide was shown to be able to induce an in vivo immune response, capable of recognising the endogenously processed form of pp65 in CMV infected cells (Diamond et al., 1997). These results show that the lipidated pp65 (495-503) peptide can be used to elicit CTL effector cells without adjuvant, making this epitope a prime candidate as a vaccine for clinical studies.

In some model systems, single epitope immunisation has provided complete protection (Kast *et al.*, 1991; Schulz *et al.*, 1991), but it remains unclear whether responses to more than a single epitope are necessary for optimum, biologically significant immunity in humans. However for some pathogens, especially those with high mutation rates, immune selective pressures may drive the expansion of escape mutants, where the immunodominant peptide epitope is altered. In the present study, the existence of two more HLA-A2 restricted pp65 derived CTL epitopes AE44 and AE45 was demonstrated, a finding which may be useful in the design of multi-epitope peptide vaccines.

A practical approach to the induction of CTL against CMV in a wide variety of individuals, would be to use CTL peptide epitopes restricted to different HLA alleles as part of a multi-CTL epitope vaccine. One of the strategies for linking multiple epitopes to achieve optimal CTL priming is the use of genetic or polynucleotide vaccines, which are also called naked DNA vaccines. Several studies have validated the usfulness of this approach in animals (Ciernik *et al.*, 1996; Davis, 1997; Xiang *et al.*, 1995). It has been shown that the inoculation of vectors encoding a foreign protein under the control of a suitable promoter, and injected into the muscle or skin of an animal, causes uptake of the DNA into cells close to the injection site (Ertl and Xiang, 1996; Williams *et al.*, 1991). Using this strategy, DNA encoding two CTL epitopes for a pair of distinct MHC antigens were complexed in a

single DNA vaccine which provided protection for two different strains of mice (Oldstone *et al.*, 1993). Multi-epitope vaccines have shown in several instances to provide effective protection for individuals of different MHC backgrounds (Bergmann *et al.*, 1996; Ciernik et al., 1996; Ertl and Xiang, 1996; Thomson *et al.*, 1998). These observations validate the feasibility of inserting multiple sets of sequences from pp65 and other viral proteins that are recognised in the context of a wide variety of HLA antigens and hence offering the potential to generate protection against CMV in a given population.

The strategy described in the present study for pp65 is simple and reproducible, and could be used to identify peptides derived from the same protein that are recognised in the context of several different HLA alleles, and which could potentially be used as a multi-epitope vaccine. Although the T2 stabilisation assay used in this study is only useful for assessing the binding of peptides to HLA-A2 molecules, the second method used, namely the peptide-binding competition assay, could be adapted for the identification of peptides which compete for the binding to any HLA class I molecule, provided that an EBV-transformed cell line expressing the desired HLA allele is available.

Peptide binding motifs are now known for a wide range of HLA class I molecules, making it possible to predict potential immunogenic residues from the protein sequence of a pathogen (Rammensee et al., 1995). This strategy may be imprecise, as evidenced by the observation that only about one-third of the peptides tested in this study containing appropriate HLA-A2 binding motifs were shown to actually bind. To further complicate prediction of immunogenic peptides, CTL epitopes which do not contain known MHC-binding motifs have been described (Calin-Laurens *et al.*, 1993; Nijman et al., 1993). This anomaly may be due to the lack of information concerning both the requirements for peptide-MHC interactions and the interactions of peptide-MHC complex with the TCR. Currently it appears that peptide elution and sequencing techniques are far less sensitive than the T cell (Ignatowicz *et al.*, 1996; Sykulev *et al.*, 1996).

In the case of common HLA alleles such as HLA-A2, the prediction of CTL epitopes by their binding motifs and peptide binding affinity might be more accurate, since these HLA class I molecules have been extensively studied by different groups (Bjorkman et al., 1987a; Hogan and W., 1988; Matsui, 1994; Matsui *et al.*, 1993; Saper et al., 1991), and the presence of the binding motif and secondary anchor residues has been confirmed by several different methods (Bednarek et al., 1991; Falk et al., 1991; Hunt et al., 1992; Parker et al., 1994; Ruppert et al., 1993). Furthermore, although most functionally defined CTL peptide epitopes possess the appropriate HLA class I binding motifs, only a minority of peptides containing binding motifs are actually immunogenic (Moore *et al.*, 1988; Rotzschke *et al.*, 1991). Clearly there are other factors which can alter the recognition of peptides by CTLs. These factors include proteolytic mechanisms which might be altered by the presence of enzymatic cleavage sites in flanking sequences of the peptide (Ossendorp *et al.*, 1996), peptide stability, and peptide transport. All of these factors may strongly influence the repertoire of the peptides presented to the CTLs (Neisig *et al.*, 1995; Schumacher *et al.*, 1991). Likewise, T cell avidity and the repertoire of the T cell receptor, all regulate CTL induction, and must be kept in mind when designing peptide vaccines.

The design of a useful peptide-based vaccine against CMV must consider the identity of the immunogenic proteins of CMV and the HLA class I antigen frequencies within the population. As mentioned previously, pp65 has been shown to be the focus of the CTL immune response. Additionally, a small component of the $CD8^+$ T cell mediated immune response is targeted to pp150, with very little measurable response detected to the other CMV proteins tested (Boppana and Britt, 1996; Gilbert et al., 1996; Wills et al., 1996). Immunogenic peptide identification should therefore concentrate on pp65 and perhaps pp150. The second consideration, HLA class I antigen frequencies, is complicated by the extreme polymorphism of the HLA system (Bodmer et al., 1997; Schipper *et al.*, 1995). However in the context of vaccine design this complication can be overcome. Several antigens are present at a relatively high frequency within the Caucasoid population, for

example HLA-A2, which is present in close to 50% of the British population. Therefore the identification of CMV-derived CTL epitopes for a small number of high frequency antigens could be useful to design a multi-epitope vaccine for a large proportion of the population. For example, over 70% of the British population would be expected to express either one or two of the antigens HLA-A1, -A2 and -A3. The, selection of the HLA antigens for which peptides could be included may be influenced by the HLA usage of the memory T cell response. In the case of EBV the CTL response appears to favour certain HLA antigens (Khanna *et al.*, 1997), and this should be investigated in the CMV setting.

If selecting antigens from HLA-A and -B for immunogenic peptide identification, linkage disequilibrium between the loci should be considered. Due to the close proximity of the loci and low recombination rates, many HLA alleles from several loci are commonly inherited together in what is termed a haplotype. Perhaps the best example of this is that of the HLA-A1, -B8 haplotype, which has a frequency close to that of the individual HLA-B8 allele (9.7% vs 13.7%) (Tsuji *et al.*, 1991). Clearly in order to be effective in a large proportion of the population, a more efficient strategy would be to identify immunogenic CMV-derived peptides from antigens which are not in positive linkeage disequilibrium, perhaps concentrating on those on a single locus.

Since the emergence of BMT as a therapy for haematological malignancies, one of the most difficult infectious complications is pneumonia caused by CMV infection (Quinnan and Burns, 1982; Reusser et al., 1991). Various attempts over the last decade have been made to control this disease. Pharmacological agents that limit virus replication such as aciclovir, and more recently ganciclovir and foscarnet, have become methods of choice for the prevention of CMV infection over the past 5 years (Prentice *et al.*, 1994; Reusser, 1996; Reusser *et al.*, 1992), since it has been demonstrated that treatment of the established disease in most cases is unsuccessful (Prentice and Kho, 1997). However, as mentioned

in the introduction there are significant side effects of these drugs, especially in the setting of immunosuppression after BMT (Appelbaum *et al.*, 1988; Conti *et al.*, 1995; Goodrich et al., 1991; Hibberd *et al.*, 1995).

An alternative strategy to prevent CMV infection in the case of transplant recipients is the use of adoptive T cell therapy. This approach, currently being explored in clinical studies by the research team led by Riddell and Greenberg, attempts to reconstitute immune responses to CMV in allogeneic bone marrow transplant recipients (Li et al., 1994; Riddell and Greenberg, 1995b). It involves the isolation and expansion in vitro, of $CD8^+$ CMV-specific T cell lines from the peripheral blood of the respective bone marrow donor using CMV infected autologous fibroblasts as stimulator cells (Goodrich et al., 1991; Li et al., 1994). The findings presented in this thesis suggest that the use of synthetic peptides in association with an appropriate antigen presenting cell could be an efficient tool for the selective expansion of CMV specific CTL precursors. The advantage of using this alternative approach over the current strategy currently used by the group of Riddell and Greenberg, would be the use of peptide-pulsed autologous PHA blasts as stimulator cells, in contrast to the use of autologous skin fibroblasts infected with CMV which require at least a month to expand. The stimulation protocol described in the present study may therefore be the strategy of choice for the large scale production of CTL for T cell immunotherapy. However certain modifications would have to be made to the protocol for ethical reasons, for example avoiding the use of T2 cells at any stage of the CTL induction, as it is an immortalised EBV cell line which has been shown to be able to elicit alloreactive responses (Wolpert et al., 1997).

The expansion of donor-derived antigen-specific T cell clones in vitro, and their subsequent adoptive transfer to BMT recipients, has been effective in limiting disease in the case of EBV infection (Heslop *et al.*, 1996; Kuzushima *et al.*, 1996; Sing *et al.*, 1997). Similar approaches have also been applied to the treatment of tumours (Evans *et al.*, 1997; Melief, 1992; Rickinson, 1995), HIV infection (Riddell *et al.*, 1992a), and again in the

BMT setting, for CMV. While this strategy has proven relatively successful at controlling CMV infection in the case of allogeneic HLA-matched BMT recipients, it may be less appropriate in other settings where CMV infection represents a problem.

In the case of AIDS patients for example, it is likely that treatment by the transfer of CMV-specific T cells would need to be prolonged, and this could present both logistical and financial obstacles. When considering an adoptive T cell therapy to be applied in the context of the different transplant settings, several issues must be kept in mind. Since this approach takes advantage effector cells derived from the transplant donor, who must be CMV seropositive in order to stimulate a CMV specific memory T cell response. Therefore this approach would exclude a large proportion of CMV seropositive patients for who have a CMV seronegative donor. Even more problematic could be the situation in solid organ transplantation such as liver, heart, and heart-lung transplants where the transplanted organ is often the site of CMV infection. Clearly in these situations a cadaver donor is normally used, and so obtaining viable T cells for expansion is likely to be difficult. Further the expansion of effector cells ex vivo is time-consuming, and CMV infection may occur before these cells are available for use. Immunologically, the use of adoptive T cell therapy in such transplant situations has several drawbacks. HLA matching in organ transplantation is limited at best in most cases, raising the problems of alloreactivity. HLA-incompatible donor-derived T cells are likely to be recognised rapidly and killed even in an immunosuppressed patient. This may even serve to increase alloreactivity towards the donor organ, raising the chance of rejection. A second option is to use the patient as a source of CMV-specific CTL. However as patient and donor are generally HLA incompatible to a greater or lesser extent, the donor organ may not express the correct HLA restriction elements for successful recognition of CMV-derived peptide antigens. A final consideration when using adoptive T cell therapy is the potential for effector cells which recognise both the viral antigen in the context of self-HLA and also cross-react with foreign MHC (Burrows *et al.*, 1994). While there is some attempt to HLA match patient and

donor in renal transplantation, generally incompatibilities remain, and so the problems outlined above remain.

The requirements for a CMV vaccine might be different for each at risk group. To prevent congenital CMV infection, a vaccination program would have to include the general population, such as with the measles, mumps or rubella vaccine, so that pregnant women would not experience primary infection, which has been shown to be associated with the highest risk of infection to the new-born (Stagno et al., 1986). When evaluating CMV infection in bone marrow recipients a good vaccine would have to provide protection early after transplant and cover the period of maximal immuno-incompetence after BMT, which is approximately 6 months (Atkinson, 1990; Lum, 1987; Lum, 1990). Since, many BMT recipients are unable to mount an effective immune response immediately after transplant, when the risk of CMV infection is the greatest, vaccinating the bone marrow donor could possibly overcome this problem. However, although humoral immunity has been shown to be transferred from the vaccinated donor to the patient (Atkinson, 1990; Wimperis *et al.*, 1986) the transfer of cellular immunity would still have to be investigated. Vaccination of BMT donors with live attenuated measles, mumps and rubella vaccines showed almost no transfer of immunity to the recipient (Ljungman *et al.*, 1989), and donor reactivity to varicella zoster virus was rarely transmitted to recipients. On the other hand, it has been shown that it is possible to transfer cellular immune responses against CMV, hepatitis B, EBV and myeloma idiotype, from the donor to the recipients of BMT (Boland *et al.*, 1992; Kuzushima et al., 1996; Kwak *et al.*, 1995; Shouval and Ilan, 1995).

Whether a live vector, DNA, peptide or subunit vaccine incorporating sequences from pp65 is used, or even when using an adoptive T cell therapy approach, it is important to know whether the pp65 amino acid sequence is conserved amongst wild type strains of CMV. Interestingly, the three pp65 CTL epitopes identified in the present study were found to be widely conserved among several virus isolates, and had the capacity to

stimulate in vitro CTL response using lymphocytes from CMV seropositive individuals. Two of the peptides identified in this study, AE42 and AE44 were effective CTL epitopes in two CMV seropositive individuals, and peptide AE45 was identified to be a CTL epitope in the one CMV seropositive individual tested. This suggests that these pp65 immunogenic epitopes may provide a useful basis for the development of potential peptide vaccines or as part of multi-epitope vaccine, which could serve to generate a primary CMV specific CTL response, or to amplify a memory CTL response to CMV. However, the present study demonstrated that amongst the seven strains tested there was strain variation in positions that could be potential CTL epitopes for other HLA class I molecules. Further studies would thus be needed to determine if such variations interfered with the recognition of any of those potential CTL epitopes and to extend the pp65 sequencing studies presented here to a larger number of clinical isolactes of CMV.

7. Chapter 7 Appendices

7.1 Preparation of solutions and reagents

7.1.1 Virus detection and titration

5 % paraformaldehyde - 2% sucrose in PBS: 25 g of paraformaldehyde was added to 100 ml of distilled water and 200 µl of 1 M NaOH, the mixture was heated gently without boiling until dissolved and made up to 500 ml by the addition of 350 ml of distilled water, 50 ml of 10X PBS and 10 g of sucrose.

0.5 % NP-40, 10% sucrose, 1% FCS in PBS: 50 g Sucrose plus 2.5 ml of NP-40 and 5 ml FCS were dissolved in 500 ml and stored at 4 °C

10 X Phosphate Buffered Saline (PBS): 100 PBS tablets were added to 1 litre bottle of sterile water. Stored at 4 °C.

Methyl Cellulose overlay: 2% Methyl Cellulose solution was prepared by autoclaving 10 g Methyl Cellulose (Sigma) and 500 ml distilled water separately. These were then combined while still hot, and left stirring overnight to allow the powder to dissolve. The Methyl Cellulose overlay medium was prepared by combining 10 ml 10x MEM, 30 ml L-15 medium, 5 ml FCS, 3 ml 7.5% sodium bicarbonate, 1 ml antibiotic/antimycotic solution, and 0.5 ml L-glutamine with 50 ml of 2% methyl cellulose and mixed well by inversion several times.

10 % Paraformaldehyde: 10 g paraformaldehyde was added to 100 ml PBS and heated to 70 °C in a fume hood for 2 h. The stock solution was filtered through 0.22 µm filters and stored at 4 °C. This stock solution was diluted 1/100 in RPMI-1640 prior to use

7.1.2 Sequencing reagents

Annealing Buffer: 1M Tris-HCl (pH 7.6), 100 mM $MgCl_2$ and 160 mM DTT.

Stop Solution: 0.3% each Bromophenol Blue and Xylene Cyanol FF; 10 mM EDTA (pH 7.5) and 97.5% deionized formamide.

10X Cloned Pfu Buffer:
200 mM Tris-HCl (pH 8.75)
100 mM KCl
100 mM $(NH4)_2SO_4$
20 mM $MgSO_4$
1% Triton" X-100
1 mg/ml bovine serum albumin (BSA)

7.1.3 Bacterial culture media

SOB Medium:

Reagent	Final concentration (w/v)	Amount per litter
Bacto Tryptone	20%	20 g
Bacto Yeast extract	0.5%	5 g
NaCl	0.5%	5 g
Distilled H_2O		1000 ml

10 ml of 250 mM KCl was added when the components had fully dissolved. Prior to use, 5 ml of a filter sterilised solution of 2M Magnesium (1M $MgCl_2$ + 1M $MgSO_4$) was added.

SOC Medium: This medium was prepared as SOB medium with the addition of only 0.5 ml 2M Mg Solution plus 1ml 2M glucose stock (final 20 mM). The medium was filtered thorough a 0.2 mM filter and pH adjusted to 7.0 + 0.1

YT Agar plate

Reagent	Final concentration (w/v)	Amount per litter
Bacto Tryptone	0.8%	8 g
Bacto Yeast extract	0.5%	5.0 g
NaCl	0.5%	8 g
Agar	1.5%	15 g
Distilled H_2O		1000 ml

The powders were dissolved into 1000 ml of distilled H_2O. The agar was then added to the flask with a magnetic stirring bar. The mixture was autoclaved 15-20 min at 121 °C, then cooled to 45 °C, mixed and poured into plates (approximately 15 plates). Plates were stored at 4 °C until use.

YT Top Agar preparation: It was prepared following the formula above substituting 8.0 g/l (0.8%) for the 15 g/l agar in the protocol. After autoclaving the medium was stored at room temperature and melted as needed in a microwave oven.

BB Broth Plate preparation

Reagent	Final concentration (w/v)	Amount per litter
Bacto Tryptone	1.0%	10 g
NaCl	0.8%	8 g
Agar	1.5%	15 g*
Distilled H_2O		1000 ml

The agar was prepared and plated as described previously.
*In the case of BB Top agar, 7g (0.7%) of agar were substituted for the 15 g/l agar in the plating suspension.

Table 7.1 Oligonucleotides used to PCR amplify pp65 DNA

Primer	Restriction Site	Sequence
5' end	pp65 KpnI	5' CGGCGGTACCATGATATCCGTACTGGGTCCCATT 3'
3' end	pp65 BamHI	5' CCGCGGATCCTCAACCTCGGTCCTTTTTGGGCGT 3'

A KpnI site has been engineered into the 5' primer and a BamHI site has been incorporated into the 3' primer. A GC tail has been also incorporated in each primer

Table 7.2. Sequences of sequencing oligonucleotides and their position in the pp65 DNA

Primer	Sequence	pp65 region
5' end	Same as PCR pp65 KpnI	1- 24
INT 1	5' ACACGTACTTTACGGGC 3'	206 -222
INT 2	5' GTTCGTGTTTCCCACCAAG 5'	525 - 543
INT 2.5	5' TTACGGTGTTGTGTCC 3'	785 - 800
INT 3	5' GTACAGCGAGCACCCCACC T 3'	1056 -1075
INT 4	5' ACCGTCGCGCCCGAAG 3'	1351 - 1366

Table 7.3 Composition of Sequencing mixes

	T7Sequencing' mixes	Deaza GA T7Sequencing' mixes
"A" mix1	840 mM each dCTP, dGTP and dTTP: 9.3 mM dATP; 14 mM ddATP	95 mM each c7dATP and c7dGTP; 840 mM dCTP and dTTP; 2mM ddATP;
"C" mix	840 mM each dATP, dGTP and dTTP: 9.3 mM dATP; 14 mM ddCTP	95 mM each c7dATP, c7dGTP and dCTP; 840 mM dTTP; 10 mM ddCTP
"G" mix	840 mM each dCTP, dATP and dTTP: 9.3 mM dATP; 14 mM ddGTP	95 mM each c7dATP and c7dGTP; 840 mM each dCTP and dTTP; 4m ddGTP
"T" mix	840 mM each dCTP, dGTP and dATP: 9.3 mM dATP; 14 mM ddTTP;	95 mM each c7dATP, c7dGTP and dTTp; 840 mM each dCTP; 6mM ddTTP
Labelling mix-dATP	1.375 mM each dCTP, dGTP and dTTP; 333.5 mM NaCl	1.37 mM each dCTP, c7dGTP and dTTP; 333.3 mM NaCl

1. All mixtures where prepared in 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl

Table 7.4 Symbols for amino acids

Amino acid	One-letter symbol	Three-letter symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

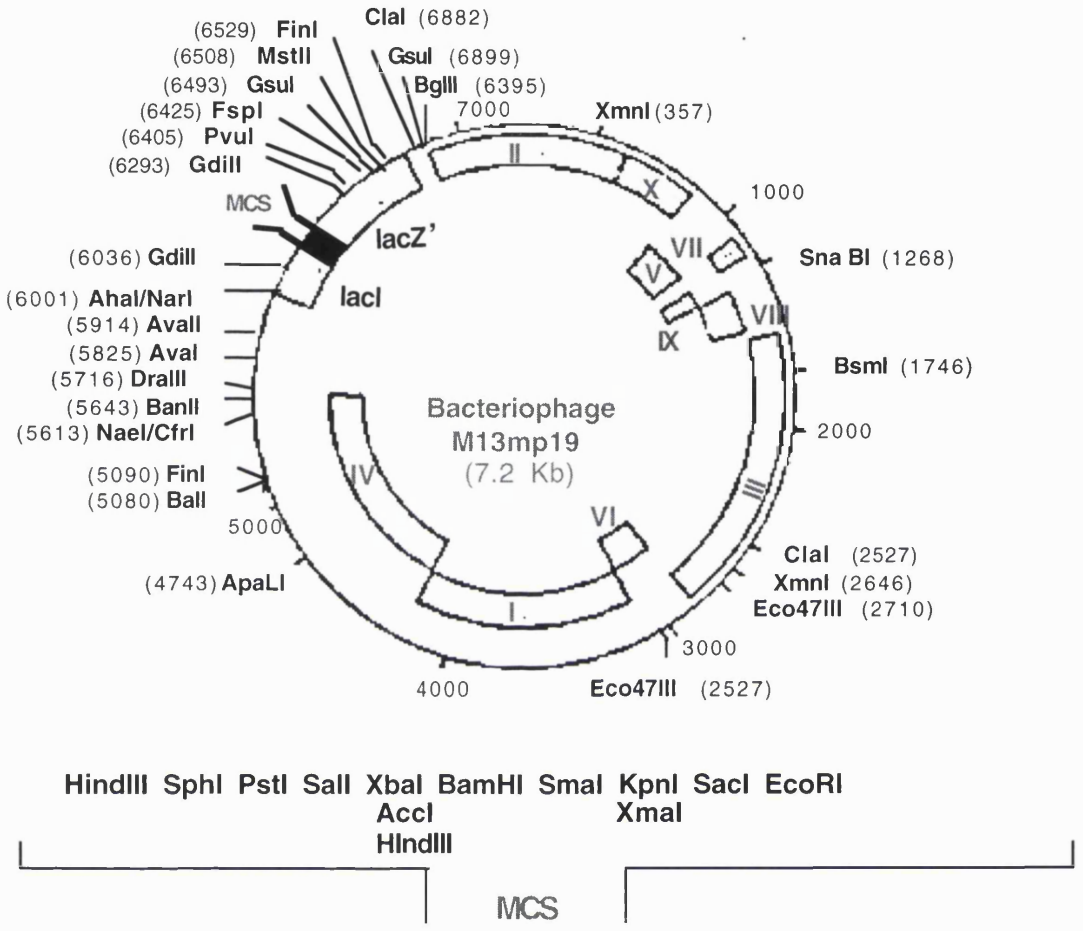


Figure 7.1 Restriction map of the bacteriophage M13 vector M13mp19.
The multiple cloning site (MCS) at which restriction enzymes can be used to cleave the double-stranded replicative form of M13mp19 is shown at the bottom of the figure.

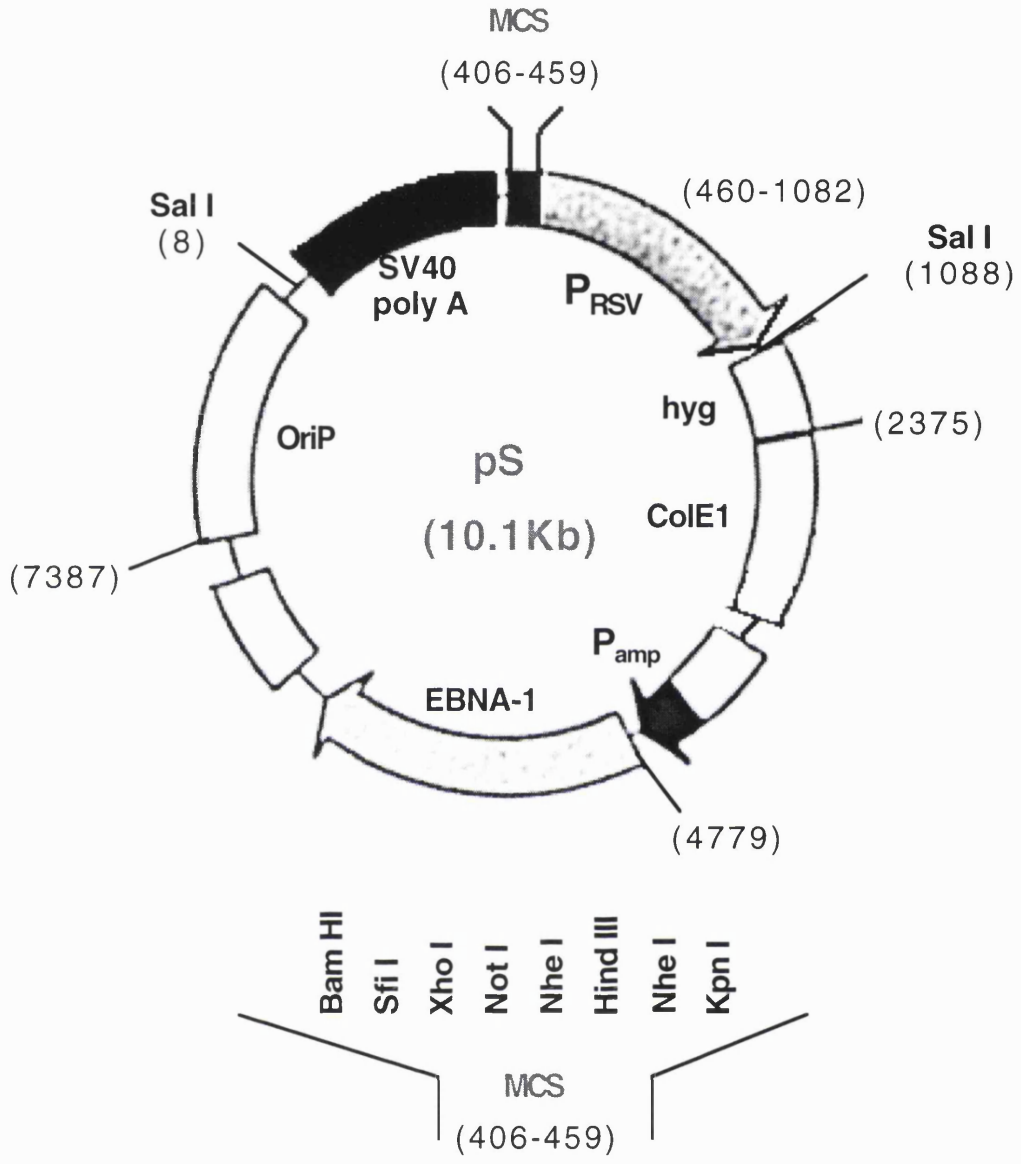


Figure 7.2 Restriction map and multiple cloning site (MCS) of vector pS. This vector contains a Rous sarcoma virus long terminal repeat promoter (PRSV), a hygromicin resistance cassette (Hygr) and polyadenylation signals from the simian virus, SV40 (SV40 polyA) . It also contains a Epstein Barr virus origin of replication (Ori P) and nuclear antigen (EBNA-1) coding sequences for episomal replication in mammalian cells. The ColE1 origin (Col E1) and the ampicillin resistance cassette (Pamp) allow replication and selection in E.coli.

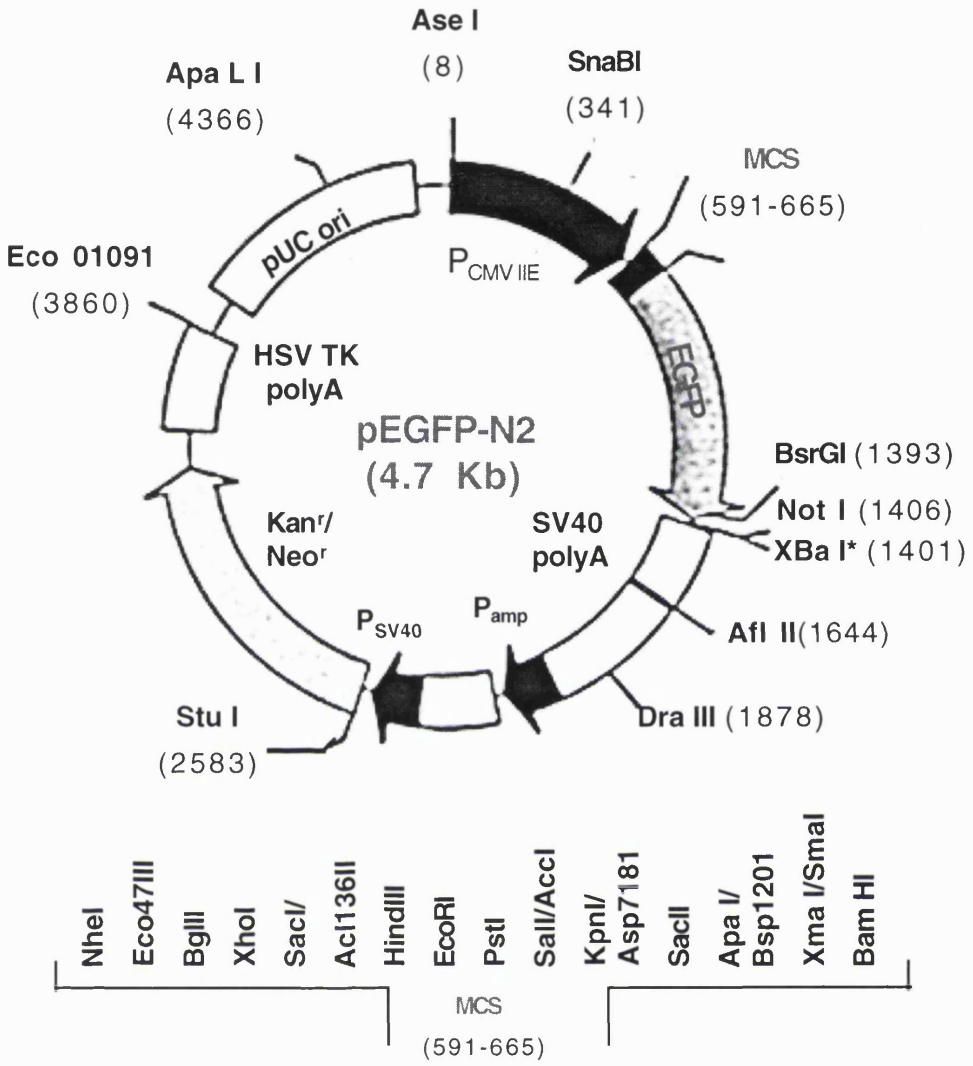


Figure 7.3 Restriction map and multiple cloning site (MCS) of pEGFP-N2. Unique restriction sites are in bold. The MCS in pEGFP-N2 is between the immediate early promoter of CMV (PCMV IE) and the EGFP coding sequences. The vector backbone also contains, SV40 polyadenylation signals (SV40 poly A) and SV40 origin of replication in mammalian cells (SV40 ori P). A neomycin-resistance cassette (kanr/neor), consisting of the SV40 early promoter (PSV40e), the neomycin / kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene (HSV TK poly A). A bacterial promoter upstream of this cassette (Pamp) expresses kanamycin resistance in E.coli. This vector backbone also provides a pUC19 origin of replication for propagation in E.coli and an f1 origin for single-stranded DNA production

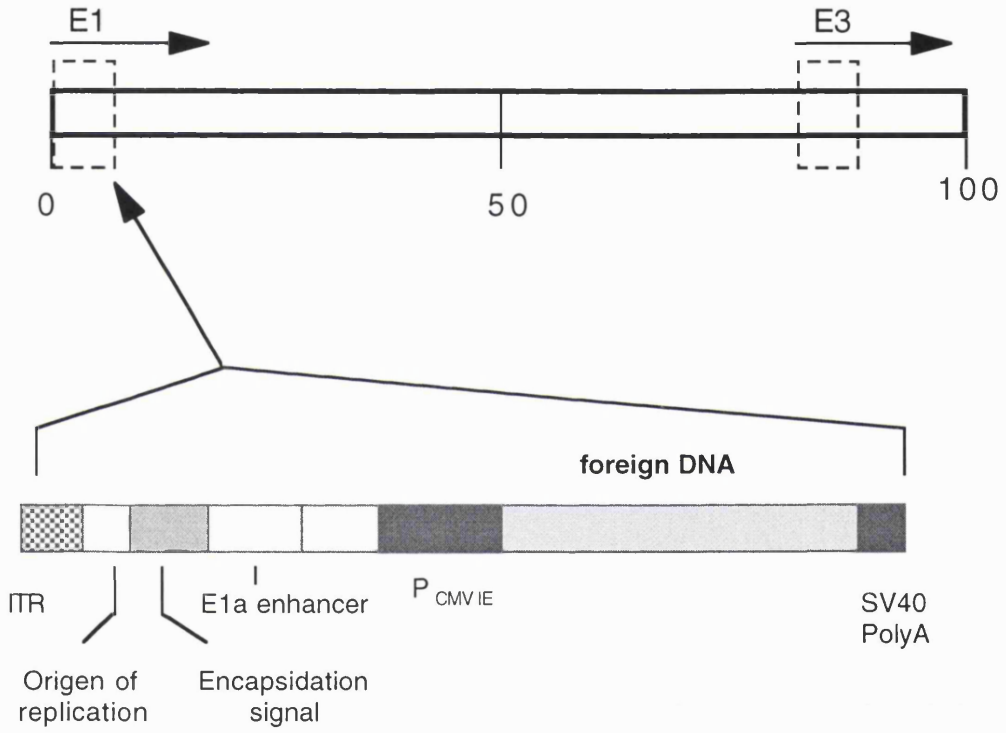


Figure 7.4. Representation of the cassette for the defective adenovirus vector

The cassette shows the 5' inverted terminal repeat (ITR), the adenovirus origin of replication, the encapsidation signal, and the E1a enhancer upstream from the CMV immediate early promoter (PCMV IE), which drives the inserted gene. This is followed by the pp65 DNA and a polyadenylation signal from the SV40 virus.

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class I molecules. This review will summarise those recent findings that were not previously included in section 1.2.11

8.1.1 The breakdown of cytosolic proteins within the cytosol

The degradation of endogenous proteins is modulated in various ways. Some proteins with sequences rich in proline, glutamine, serine and threonine seem to be rapidly degraded (Rogers et al., 1986). Other proteins contain sequences referred to as destruction boxes that confer cell cycle-specific degradation (Glotzer et al., 1991). Another factor that influences the stability of cytosolic proteins is the identity of their N-terminal amino acids (Varshavsky, 1992), with some amino acids inducing a more rapid degradation than others. The most clearly understood and apparently most general entrance to the cytosolic protein degradation pathway involves the conjugation of ubiquitin to lysine residues of targeted proteins (Ciechanover, 1994; Hochstrasser, 1997). The mechanisms involved in ubiquitination are complex and involve three enzymatic activities mediated by proteins called E1, E2 and E3 (Ciechanover, 1994; Hochstrasser, 1997). These enzymes work in series, first activating ubiquitin and then covalently linking it to specific lysine residues in the target protein. Polyubiquitin chains are then generated by conjugating additional ubiquitin moieties to lysine residues of already conjugated molecules (Hochstrasser, 1997). Polyubiquitin chains may serve two major purposes: to unfold the protein and to function as recognition elements, which target this protein for cytosolic proteasome degradation.

As mentioned previously in section 1.2.11, proteasomes mediate the majority of endogenous cytoplasmic protein degradation (Rock et al., 1994). Rock and colleagues demonstrated that proteasomes degrade both short-lived and long-lived proteins, and that in the absence of proteasome-mediated protein degradation, MHC class I molecules remain in the endoplasmic reticulum, deprived of peptides. The proteasome is a 20S-26S high

8. Chapter 8. Post-viva addendum

The purpose of this addendum is to address questions raised by the examiners about this thesis during the viva examination.

The two main topics covered in the first section are: 1) the antigen processing and assembly of MHC class I molecules in the ER and 2) the recognition of the peptide-MHC class I complex by T lymphocytes, and the resulting signalling cascade which leads to T cell activation and eventually lysis of the antigen presenting cell.

The second section of this appendix consists of a more detailed discussion of results obtained with the peptide binding assays used in this thesis (described in sections 3.2.4 and 3.2.6). This includes a statistical comparison of the results obtained by these methods with the predicted values obtained by the computer algorithm described in section 3.2.7.

Finally the HPLC profiles of peptides included in this study will be shown in order to demonstrate their purity.

8.1 Antigen processing and presentation by MHC class I molecules

The degradation of cytosolic proteins is an essential step in the generation of most peptide epitopes presented by MHC class I molecules. Although how those peptides are funnelled into protein degradative pathways remains an important question, several recent studies have facilitated the understanding the process of degradation of such proteins into antigenic peptides, their transport to the endoplasmic reticulum (ER) and their assembly onto MHC

(Levitskaya et al., 1995). However proteases other than those in the proteasomes may be involved in the generation of MHC class I-associated peptides, as shown recently by Glass et al (Glas et al., 1998). For example, peptides of 17 amino acids expressed endogenously can be processed for presentation by MHC class I molecules in the presence of proteasome inhibitors, implying that other proteases are involved in the generation of the CTL epitopes (Yang et al., 1996). Since TAP can transport peptides that are longer than most MHC class I-associated peptides, it is possible that additional "trimming" proteases are present in the ER (Snyder et al., 1994). Evidence for such proteases comes from experiments using TAP-deficient cells in which antigenic peptides are transported into the ER behind a conventional signal sequence (Anderson et al., 1991). Interestingly, epitopes with N-terminal extensions are processed poorly, suggesting that the proteasome has some aminopeptidase activity (Snyder et al., 1994). The existence of a mechanism for processing in the secretory pathway has been suggested by several groups (Elliott et al., 1995; Hammond et al., 1993; Lee et al., 1996; Snyder et al., 1997; Snyder et al., 1994), and recently confirmed by the group of Del Val et al (Gil-Torregosa et al., 1998). The latter group demonstrated that the antigen processing of the Hepatitis B (HB) virus secretory core protein occurs in the Golgi or post Golgi compartment, and that this processing was attributed to furin, a protease member of the subtilisin family (Gil-Torregosa et al., 1998).

molecular weight multicatalytic cytosolic protease complex consists of up to 14 subunits in mammalian cells. Each subunit has a barrel-shaped structure in which the outer rings are composed of seven α-subunits, which are thought to have primarily structural and regulatory functions. T he inner rings are composed of seven distinct β-subunits that form the catalytic sites and are responsible for the different peptidase activities of the proteasome (Fenteany, 1995; Lowe et al., 1995). Exposure of cells to γ-interferon (which can happen after a viral infection), results in the expression of LMP2, LMP7 and MECL-1 (LMP10). The two β-subunits, LMP2 and LMP7 are encoded by genes within the MHC class II region (Belich et al., 1994; Goldberg and Rock, 1992; Martinez and Monaco, 1991; Monaco, 1992). Upon expression the LMP2, LMP7 and LMP10 subunits replace the constitutively expressed Y (δ) and X (MB1) and Z subunits, respectively, modifying the catalytic activities of the proteasome and enhancing the presentation of at least some antigens (Nandi et al., 1996; Ortiz-Navarrete et al., 1991).

8.1.2 The generation of MHC class I binding peptides by the proteasome

The eukaryotic proteasome expresses five distinct proteolytic activities (Harding et al., 1995) which can be distinguished by the nature of the amino acid residue preferred for cleavage. These include a trypsin-like activity cleaving after basic residues, a peptidyl-glutamyl peptide-hydrolysing (glutamyl) activity cleaving after hydrophobic residues, and a chymotrypsin-like activity, cleaving after hydrophobic residues including aromatic residues. An enzymatic activity cleaving preferentially after leucine, isoleucine or valine was recently described, and an activity cleaving preferentially between two small neutral amino acids has also been identified (Cardozo and Kohanski, 1998). Single amino acid substitutions that prevent proteasome-mediated peptide cleavage have been shown to prevent antigen presentation (Ossendorp et al., 1996). Importantly, some proteins derived from pathogens may contain sequences that reduce antigenicity by this mechanism

8.1.3 The assembly of MHC class I molecules

The efficient assembly of MHC class I molecules in the ER requires the formation of a complex between the class I molecule and the TAP peptide transporter molecules (Ortmann et al., 1994; Suh et al., 1994). Recent work by several laboratories has identified a group of ER resident proteins that appear to associate with the TAP-class I complex and are involved in the class I assembly process (Hughes and Cresswell, 1998; Morrice and Powis, 1998; Sadasivan et al., 1996; Suh et al., 1996). Such proteins identified as calnexin, calreticulin, tapasin and ERp57 seem to contribute to the efficiency of peptide loading onto MHC class I molecules.

Although the exact sequence of events in MHC class I molecule-peptide assembly is not quite known, the current view of this process suggested by Lehner and Trowsdale (Lehner et al., 1998) is summarised in figure 8.2. Nascent heavy chains of MHC class I molecules, translocated into the ER membrane are rapidly glycosylated (Figure 8.2, stage i). At this stage class I molecules associate with the transmembrane chaperone calnexin (Figure 8.2, stage ii), a non-glycosylated 65 kDa protein expressed predominantly in the ER (Williams and Watts, 1995). Calnexin seems not to be essential for MHC class I assembly, as demonstrated by the normal MHC class I assembly and peptide loading in a calnexin-negative cell line (Sadasivan et al., 1995; Scott and Dawson, 1995). In human cells the association of MHC class I heavy chain with β2m causes dissociation of calnexin, which is replaced by its soluble homologue calreticulin (Figure 8.2, stage iv) (Sadasivan et al., 1996).

The next stage in the assembly is the interaction of the MHC class I heavy chain-β2m-calreticulin complex with a second transmembrane protein, known as tapasin (Figure 8.2, stage iv). Tapasin is a 48 kDa , MHC encoded protein which, like the class I heavy chains, is a member of the immunoglobulin superfamily (Li et al., 1997). Analysis of the

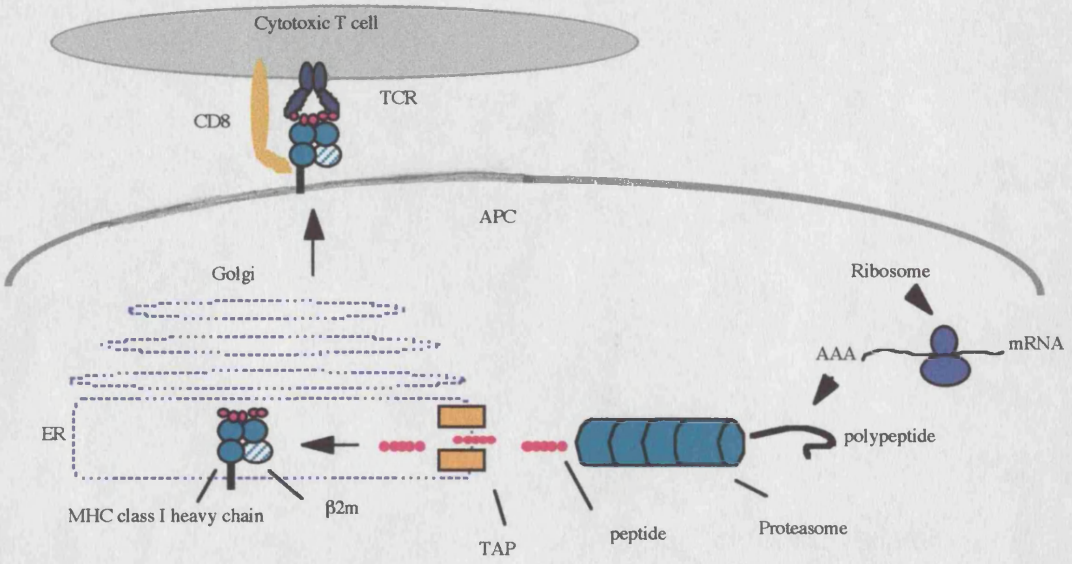


Figure 8.1 Processing and presentation by MHC class I molecules of endogenously processed proteins. See text for details.

The translocation and subsequent binding of a peptide to an appropriate MHC class I heavy chain causes release of the folded MHC class I molecule, and transport of the MHC class I complex to the cell surface. However, as it becomes apparent that several molecules are important in the assembly of this complex, it is also clear that these molecules may be the targets of activity by viral proteins. The subsequent disruption of MHC class I assembly may result in the evasion of immune recognition by an infected cell.

tapasin negative mutant cell line, 220, provided evidence that tapasin bridges class I and TAP proteins and plays a critical role in class I assembly, as in its absence cell-surface MHC class I levels are decreased (Grandea et al., 1995; Ortmann et al., 1997; Sadasivan et al., 1996). Tapasin expression also allows more peptide to be translocated into the endoplasmic reticulum. Up to four MHC class I-tapasin complexes have been found to bind to each TAP molecule (Lehner and Cresswell, 1996; Ortmann et al., 1997).

Recently a fourth protein, ERp57, has been found to take part in the MHC class I assembly process. Like calnexin and calreticulin, ERp57 binds newly synthesised ER glycoproteins after their N-linked glycans have been trimmed by glycosidase I and II, and it has therefore been suggested to act as an ER molecular chaperone (Hirano et al., 1995; Oliver et al., 1997). Studies from Hughes and Cresswell suggest that ERp57 associates with assembled dimers of MHC class I heavy chain-β2m at the same time as both calreticulin and tapasin (Figure 8.2, stage iv) (Hughes and Cresswell, 1998). While Lindquist et al, observed ERp57 in association with class I heavy chain before binding to β2m (Figure 8.2, stage iii) (Lindquist et al., 1998). The amino acid sequence of ERp57 shows similarities to protein disulphide isomerases, and it has been shown to make and break disulphide bonds in vitro (Hughes and Cresswell, 1998; Lindquist et al., 1998). This activity may be used in the oxidation of MHC class I or tapasin molecules. Since MHC class I molecules complexed with β2m have completely oxidised disulphide bonds, while calnexin-associated MHC class I heavy chains do not, ERp57 has been postulated as responsible for the formation of this complex (Sadasivan et al., 1996; Tector et al., 1997). ERp57 has also been suggested to have a cysteine protease activity. Thus as suggested by Hughes and Cresswell, ERp57 associated with MHC class I heavy chains could be involved in the trimming of long peptides translocated to the ER by TAP (Hughes and Cresswell, 1998).

8.1.4 The recognition of antigen by CD8⁺ T cells

Antigen recognition by the TCR is mediated by the clonotypic (TCRαβ or TCRγδ) chains that are derived from the products of site-specific DNA recombination events during development (Fowlkes and Pardoll, 1989). The remaining chains of the TCR, which include the CD3γ, δ and ε chains and a ζ family dimer, are responsible for signal transduction (Weiss, 1993). The subunits of the TCR are first assembled in the ER and subsequently transported to the cell surface (Klausner et al., 1990). In mature T cells a complete TCR complex (i.e. TCRαβ, CD3γεδε, ζζ) must first be assembled intracellularly in order for efficient cell surface expression (Shores and Love, 1997).

The ability of the ζ and CD3 components to transduce signals derives from a shared functional sequence, the immunoreceptor tyrosine-based activation motif (ITAM), within their cytoplasmic domains. ITAMs consist of two tyrosine-containing paired sequences ($YxxL/Ix_{6-8}$ YxxL/I; where x denotes non-conserved residues). Phosphorylation of ITAM tyrosine residues represents one of the earliest events in the TCR signalling cascade and results in recruitment of specific Src homology (SH_2) domain containing proteins to the TCR complex. As the CD3 and ζ chain do not posses intrinsic catalytic activity, their ability to recruit other molecules to the TCR complex is essential for TCR signal transduction (Cantrell, 1996).

The current view of early events in T cell activation suggests that phosphorylated TCR ITAMs initiate the signalling cascade by serving as docking structures for signal transduction molecules. Following TCR engagement, the TCR ITAMs are rapidly tyrosine phosphorylated by the Scr family protein tyrosine kinases, lck and fyn. The protein tyrosine kinase ZAP-70 is then recruited to the TCR complex through high affinity interaction between its tandem SH2 domains and the two phosphotyrosine ITAM residues.

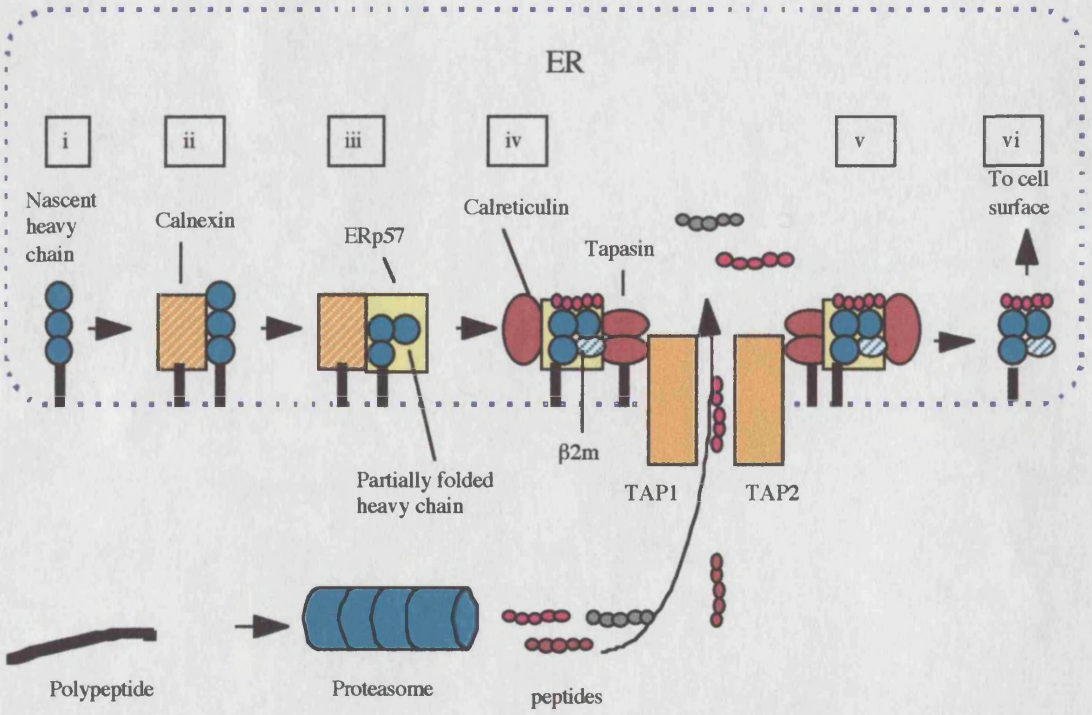


Figure 8.2. Assembly of MHC class I molecules in the endoplasmic reticulum. See text for details. Adapted from Lehner and Trowsdale (Lehner et al., 1998)

binding of exogenous synthetic peptides. In contrast, in the T2 stabilisation assay, although T2 cells are deficient in the transport of cytoplasmic peptides to the ER, it has been demonstrated that they can present peptides derived from ER resident proteins or signal proteins (Henderson et al., 1992). Therefore, exogenously added peptides possibly would have to compete for the binding to potentially occupied HLA class I molecules, rather than binding to empty molecules; these processes may not be directly comparable

A potential alternative explanation to the lack of stabilisation of class I molecules on T2 cells by certain peptides which showed either high or intermediate affinities in the peptide binding competition assay (peptides AE48, AE47, AE49), is that those exogenously added peptides are internalised and trimmed further possibly into the ER, to peptides which might not bind to HLA-A2 molecules. This hypothesis could be tested experimentally by the use of inhibitors of proteases resident in the secretory pathway.

8.2.1 The validation of the computer algorithm designed to predict MHC binding peptides.

As described in section 3.2.7, a computer algorithm designed by Kenneth Parker (Parker et al., 1994) was employed to search for all the possible HLA-A2 binding peptides in the amino acid sequence of the CMV phosphoprotein pp65. This method was employed in order to determine whether this computer program would predict the same potential HLA-A2-binding pp65-derived peptides identified by an earlier analysis in this study (Table 3.1)

The theoretical half life of β_2m dissociation (t1/2) for each peptide shown in Table 3.2, suggested that all 17 peptides identified previously, with the exception of AF90, which had a t1/2 greater than 5 minutes, were predicted to form complexes with HLA-A2. Seven of these peptides were tested by the peptide binding competition assay and six found to bind

lck and fyn also phosphorylate ZAP-70, resulting in an increase in Zap-70 catalytic activity (Qian and Weiss, 1997). Other proteins, including additional tyrosine kinases, tyrosine phosphatases and adapter molecules, may also associate directly or indirectly with the TCR, forming a multimolecular activation complex that links the TCR to downstream signalling pathways which regulate calcium mobilisation and the activation of Ras (Cantrell, 1996; Chan and Shaw, 1996; Wange and Samelson, 1996).

8.2 Peptide binding assays

As described in chapter 3, 17 peptides derived from the pp65 protein were synthesised and tested for their binding to the HLA-A2 molecule. Two assays were employed to test the binding of seven of these peptides: the peptide binding competition assay and the T2 stabilisation assay (described in material and methods, section 2.3).

Although peptides AE42, AE44 and AE45, consistently resulted in high affinity binding to HLA-A2 by the T2 stabilisation assay (Figure 3.4) and the peptide binding competition assay (Figure 3.6), the results from the other 4 peptides tested were more variable. Consequently, when these two assays were analysed statistically by the Spearman rank order correlation, no correlation was found between the two assays ($r=-0.234$, $p>0.5$; Figure 8.3a). This lack of correlation is likely to reflect the different parameters of peptide binding that the two assays measure. While the T2 stabilisation assay measures peptide off rates and thus the stability of binding, the peptide binding competition assay is likely to be more influenced by on rates, which, given that the off-rates of bound peptides are usually slow, means that those peptides which bind faster compete better.

Another possibility for the lack of correlation might be related to the nature of the assays. In the case of the peptide binding competition assay, JY cells have been acid-treated to remove the peptides from the folded MHC class I molecules. This could facilitate the

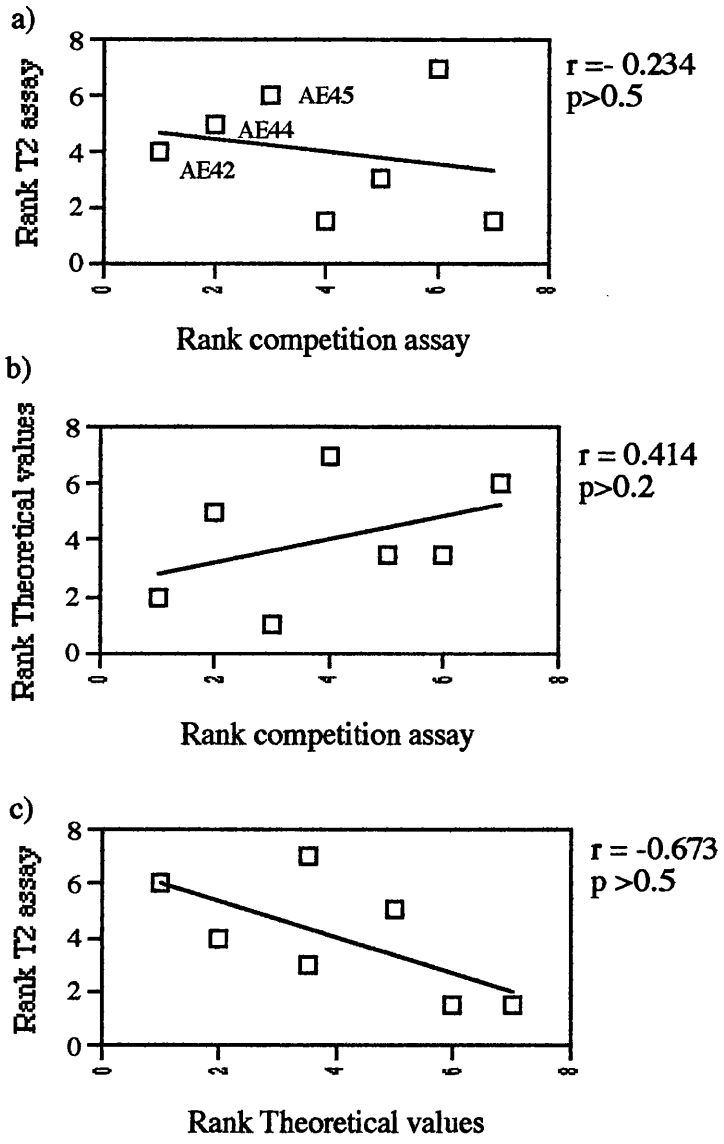


Figure 8.3 Observed and predicted binding of pp65 derived peptides to HLA-A2 molecules. IC_{50} values measured in competition assays were transformed by the formula 1 divided by the IC_{50} value, and ranked from 0 to 7. IC_{50} rank values were plotted against ranked values of peptides tested in the T2 stabilisation assay (a) and against ranked values obtained from the theoretical half life of β_2m dissociation (t1/2) predicted for individual peptides (b). T2 stabilisation rank values were also plotted against ranked predicted values of t1/2 (c). Spearman rank order correlation was performed, and the values of correlation coefficients (r) and probability values (p) are given next to each plot.

to HLA-A2 molecules with either a high or an intermediate affinity (Figure 3.6). These results suggested that the algorithm developed by Parker was able to predict the binding of these peptides to HLA-A2 molecules. However, in order to obtain a statistical correlation of the theoretical versus the experimental values, these results were analysed by the Spearman rank order correlation. The statistical analysis showed that there was no significant correlation between the prediction analysis and the results obtained by the peptide binding competition assay ($r=0.414$ $p>0.20$; Figure 8.3b). No correlation was also found when the predicted values were compared to the results obtained by the T2 stabilisation assay ($r=-0.673$, $p>0.5$; Figure 8.3c).

the estimates of binding stability based on theoretical coefficients (Table 8.1) (Parker et al., 1995).

In order to analyse the statistical significance of the correlation between the Parker prediction method and the Kast experimental data, the same statistical analysis carried out for the pp65 peptide was employed for these 14 HPV E6-E7 derived peptides. Spearman rank order correlation showed that these two methods did not have a significant correlation with a p value similar to the one obtained for pp65 peptides ($r=0.235$, $p>0.20$, Figure 8.4).

In summary no correlation of the results from either technique could be found. However it is likely that each could be used independently to identify potentially immunogenic peptides in the context of HLA-A2. Furthermore, although the T2 peptide stabilisation assay is limited to the HLA-A2 molecule the competition assay and algorithm could be used to screen peptides in the context of a range of common HLA class I antigens. Perhaps the Parker algorithm would be the most convenient for the initial screening, with binding affinities then measured by the peptide-binding competition assay for those peptides scoring above the predetermined threshold for predicted binding. It is however important to stress a) that these peptides may not be generated by the antigen processing machinery of the cell and b) that they may not be immunogenic. Since immunogenicity depends not only on the presence of particular binding motifs, but also on several other factors, a better understanding of the processing and presentation of peptides on HLA molecules and their recognition by T cells will certainly lead to a better prediction of the rules of peptide antigenicity.

The results of this statistical analysis might reflect the limitations of the use of a prediction algorithm in the search for potential CTL epitopes. One possibility for the problem in the predictions might be the presence of cysteines in the amino acid sequence of the peptides whose binding was predicted less well. This could be due to complications resulting from peptide dimerization and oxidation, which would affect peptide binding (Parker et al., 1995).

The comparison of both in vitro and algorithm methods involved a small number of samples (7 tested by all methods). These peptides were selected on the basis of their possession of amino acid residues compatible with known HLA-A2 peptide binding motifs. This selection bias means that all are likely to bind to HLA-A2 to some extent, a hypothesis largely supported by functional assays (Figure 3.4 and 3.6). However, it is not surprising that a strict correlation between the methods used does not exist in such a small and selected sample. A more fair test of the correlation of the methods used to identify HLA-binding peptides might be to screen a large panel of peptides without prior selection, namely by MHC binding motifs or predicted coefficients. In this situation it is likely that those peptides which appear to bind HLA-A2 molecules with high or intermediate affinity would generally do so for each technique.

In order to evaluate the efficiency of prediction by his algorithm, Parker et al compared the predicted t1/2 of dissociation obtained by their theoretical coefficients (Parker et al., 1994) with the experimental result obtained by several groups employing different binding assays (Kast et al., 1994; Nijman et al., 1993; Sette et al., 1994). In the analysis reported by Parker, they calculated all the potential HLA-A2-binder peptides derived from the human papilloma virus E6 and E7 proteins (Parker et al., 1995). Their results were compared to the data obtained by Kast et al, whom had previously tested the binding of this group of peptides to HLA-A2, employing a peptide binding competition assay (Kast et al., 1994). Parker reported concordance of the experimental measurements of binding affinities with

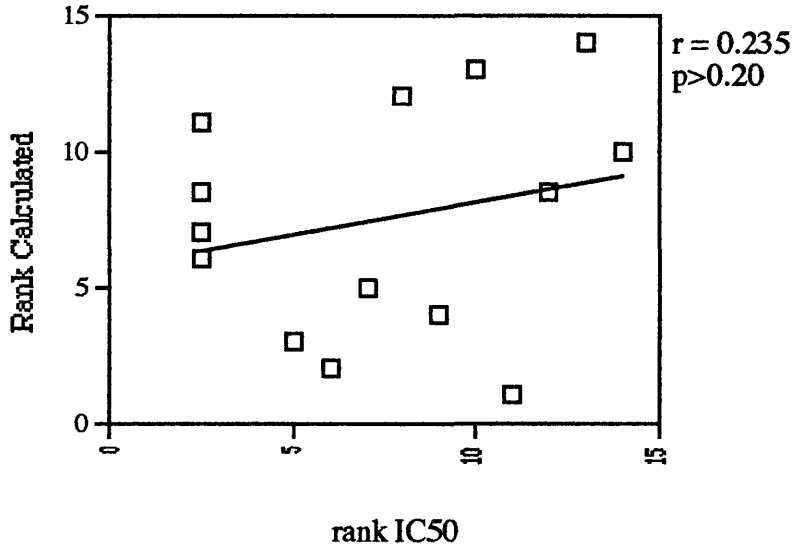


Figure 8.4 Observed and predicted binding of HPV E6 and E7 proteins derived peptides. IC_{50} values measured in competition assays were transformed by the formula 1 divided by the IC_{50} value, and ranked from 0 to 14. IC_{50} rank values were plotted against ranked values obtained from the theoretical half life of β_2m dissociation (t1/2) predicted for each peptide. Spearman rank order correlation was performed and the values of correlation coefficients (r) and probability values (p) are given next to the plot.

Table 8.1 Comparison of experimental and predicted values of binding to HLA-A2 of peptides derived from human papilloma virus type 16 E6 and E7 proteins

Peptide	Calculated[a]	IC_{50} [b]
Peptides that behaved as predicted		
YMLDLQPET	370	49
TLHEYMLDL	190	188
KLPQLCTEL	74	328
LLMGTLGIV	56	8
FAFRDLCIV	16	130
Peptides that bound ,but were not predicted to bind		
MLDLQPETT	2.4	462
GTLGIVCPI	1.2	193
LQTTIHDII	0.39	3,157
AMFQDPQER	0.20	1,818
TLGIVCPIC	0.016	153
Peptides which were predicted to bind, but which did not bind		
RLCVQSTHV	72	>5,000
QLCTELQTT	16	>5,000
CVYCKQQLL	5.8	>5,000
KCLKFYSKI	5.3	>5,000

[a] Table taken from (Parker et al., 1995) Comparison of the data of Kast et al (Kast et al., 1994) to t1/2 calculated using coefficient predictions from Parker (Parker et al., 1994).

[b] t1/2 of dissociation, calculated using the coefficients described in section 2.3.4.

[c] IC_{50}, defined as the concentration in nM that results in 50% inhibition of binding of ^{125}I-labelled FLPSDYFPSV to HLA-A2 molecules purified from the human lymphoblastoid cell line JY.

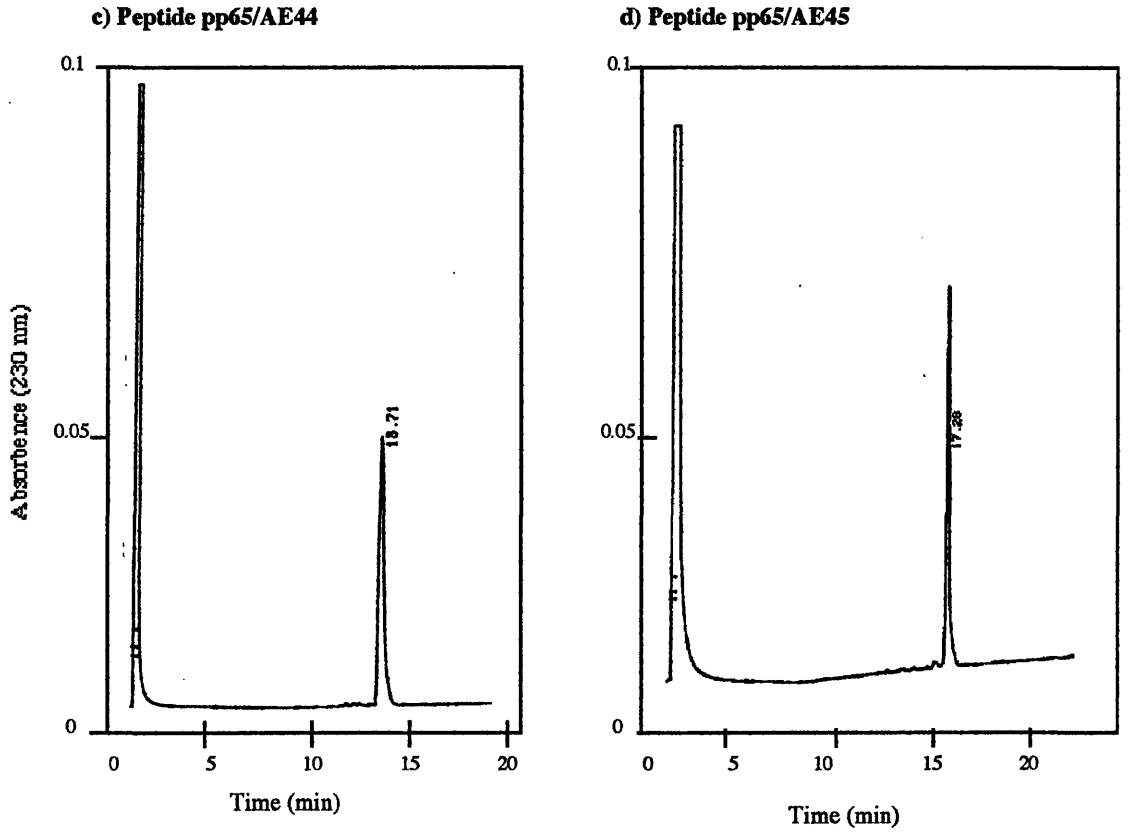
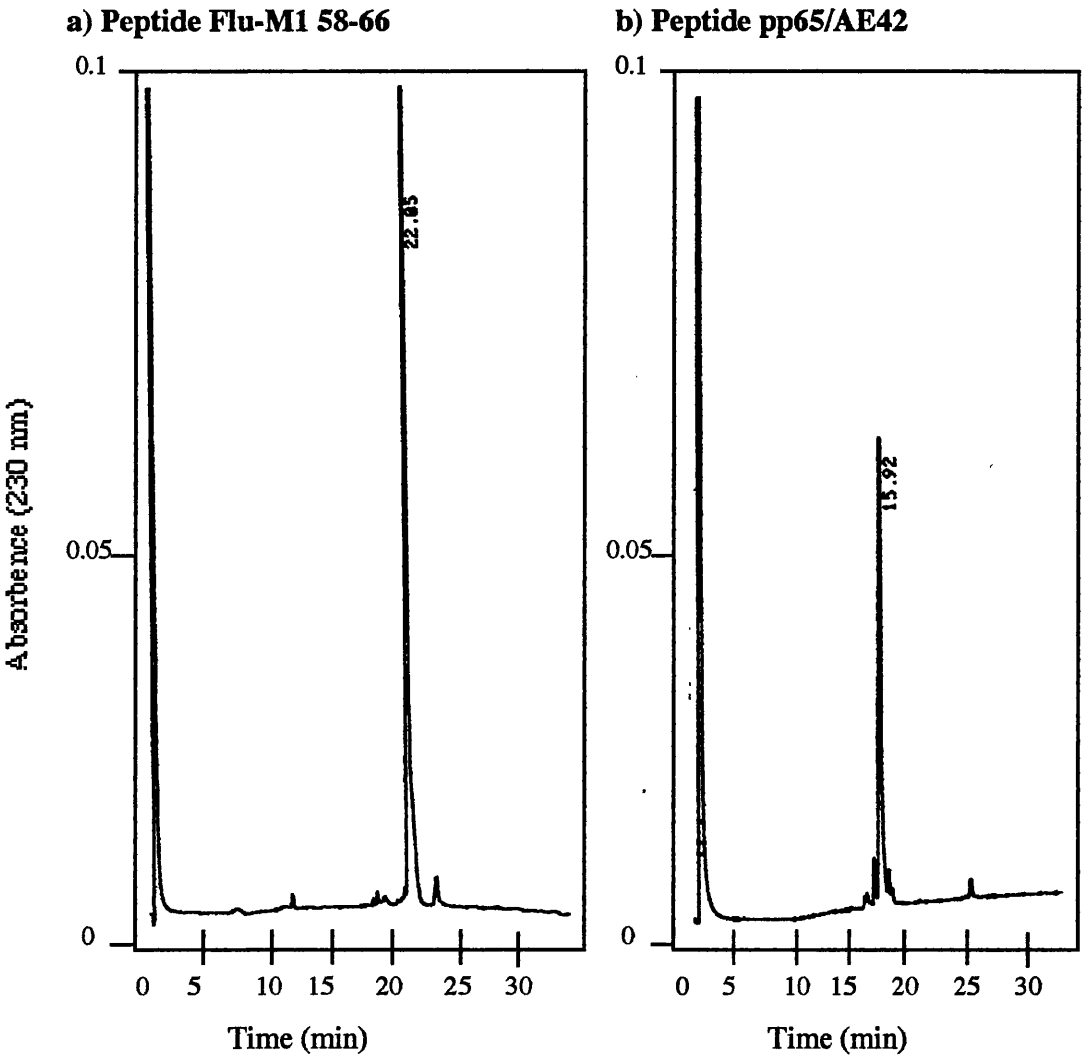


Figure 8.5 HPLC profiles of 4 representative HLA-A2 binder-peptides. All peptides employed in this study were synthesised as described in section 2.3.1. Peptides were dissolved in DMSO at 10 mg/ml and purified by HPLC using a C18 column and eluted in a gradient of 0 to 60% acetonitrile.


a) Peptide Flu-M1 58-66
b) Peptide pp65/AE42
Absorbence (230 nm)
0.1
0.05
0
22.45
15.92
0 5 10 15 20 25 30
Time (min)

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