THE EFFECT OF CYTOMEGALOVIRUS INFECTION ON MOLECULES AFFECTING LEUKOCYTE RECRUITMENT AND MIGRATION

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

Doctor of Philosophy

in the Faculty of Medicine

by

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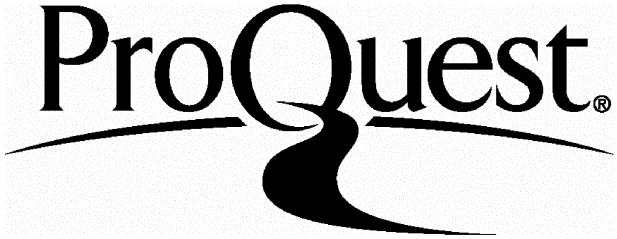
November 1996



Dedicated to my parents

ABSTRACT

The recruitment of particular subsets of leukocytes to sites of inflammation and infection is thought to be governed by specific patterns of adhesion molecule expression and chemokine production. The changes in the expression of adhesion molecules, Class I HLA, and the production of chemokines following CMV infection of fibroblasts were investigated. Class I HLA was downregulated on the surface of CMV infected fibroblasts, and upregulated on uninfected bystander cells in infected cultures, the latter by a mechanism involving Interferon β. CMV infection was found to upregulate the surface expression of ICAM-1 and LFA-3 in fibroblasts. This was found to be a direct effect of viral infection, and was independent of cytokine production by infected cells. The upregulation of ICAM-1 and LFA-3 were not prevented by treatment of the cells with the antiviral agents ganciclovir and foscarnet, showing that expression of CMV late proteins was not required for the effect. In addition, the adhesion molecule upregulation was prolonged and enhanced by antiviral treatment. The functional effects of adhesion molecule upregulation on T cell adhesion were investigated. It was found that adhesion of both resting and activated T cells took place at increased levels to CMV infected fibroblasts, but that this was not due to selective adherence of a particular subset of T cells to infected cells. Adhesion of activated T cells to CMV infected fibroblasts also occurred at increased levels compared to uninfected fibroblasts, and was mediated predominantly via the ICAM-1/LFA-1 adhesion pathway.

Expression of two chemokines, interleukin-8 (IL-8), an α chemokine, and MCP-1 (monocyte chemoattractant protein-1), a β chemokine, were investigated. IL-8 was found to be produced at increased levels by infected fibroblasts, and infected supernatants were found to enhance neutrophil migration across endothelial monolayers. This was specifically due to increased IL-8 levels in the supernatants. Steady state levels of MCP-1 mRNA were increased following CMV infection, but this was not accompanied by an increase of MCP-1 protein in the infected supernatants. In contrast, supernatants from infected cells had lower levels of MCP-1 than those from uninfected cells. Recombinant MCP-1 was lost from solution following incubation with CMV infected cells. This was probably due to the sequestering of MCP-1 by infected cells by the CMV-encoded β chemokine receptor. Migration of T cells from some donors across endothelial monolayers was increased in response to supernatants from infected fibroblasts. T cells of the memory phenotype (CD45 RO and CD45 RB_{low}) migrated at increased levels in response to supernatants from either

infected or uninfected fibroblasts, particularly those with high expression of LFA-1. There is a fine balance between a beneficial and destructive immune response. Increased adhesion and migration of leukocytes in response to changes in adhesion molecule and chemokine expression may contribute to immune mediated tissue damage, which is a characteristic of certain types of CMV disease.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Dr. Jane Grundy, for her advice and guidance throughout my years in the laboratory, and during the preparation of this thesis, and for initiating my career in CMV research. I am also grateful to my second supervisor, Dr. Arne Akbar for his introduction to the world of T cells. Special thanks are due to Dr. Nicola Borthwick, for her good advice, supplies of antibodies, collaboration in T cell migration studies, critical reading of sections of the thesis, and moral support. I am also grateful to Dr. Kwee Yong for assistance with leukocyte migration assays, and to Luci MacCormac, for her assistance with confocal microscopy and other techniques. My thanks are also due to Dr. Nicola Jordan at the University of Bath, for carrying out some of the chemokine mRNA analysis, and for helpful discussions.

Financial support from The Wellcome Trust is gratefully acknowledged, as is the financial assistance from Dr. David Webster and Dr. Geoff Dusheiko for the final stage of my studies.

I would like to thank my friends at the Royal Free Hospital for their continual support, and for keeping me sane during the writing-up, and in particular to Jean Fletcher for the thankless task of proof-reading this thesis, and to my flatmates for putting up with me during the last few months! Finally, I would like to thank my family for their love and support throughout my studies, and especially my parents who couldn't have imagined what they were getting into when I left for London 10 years ago.

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LIST OF ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
BSA	bovine serum albumin
CMV	cytomegalovirus
CPM	counts per minute
CTL	cytotoxic T lymphocyte
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunoabsorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothyocyanate
FIU	fluorescence intensity units
G-CSF	granulocyte macrophage colong stimulating factor
GVHD	graft versus host disease
HBSS	Hank's balanced salt solution
HBV	hepatitis B virus
HEL	human embryonic lung
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMWP	high molecular weight protein
HRP	horse radish peroxidase
HTLV-1	human T cell leukemia virus type 1
HUVEC	human umbilical vein endothelial cells
ICAM-1,2,3	intercellular adhesion molecule-1,2,3
IE	immediate early
IFN	interferon
IVIG	intravenous immunoglobulin
kD	kilodaltons
LAK	lymphokine activated killer cells
LCMV	lymphochoriomeningitis virus
LFA-1,3	leukocyte function associated antigen-1,3
MAP	mitogen activated protein kinase
MCMV	murine cytomegalovirus

MCP-1	monocyte chemoattractant factor-1
MEM	minimal essential medium
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIEP	major immediate early promoter
MOI	multiplicity of infection
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSA	PBS containing 1% BSA and 0.02% sodium azide
PdBu	phorbol 12,13 dibutyrate
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule
PFU	plaque forming units
PHA	phytohaemagglutanin
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMNC	polymorphonuclear cells
RFHSM	Royal Free Hosptial School of Medicine
RNA	ribonucleic acid
RSV	respiratory syncitial virus
SD	standard deviation
SDF-1	stromal cell derived factor-1
SEM	standard error of the mean
TAP	transporter associated with processing
TCR	T cell receptor
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule 1
VLA	very late antigen
WNV	West nile virus

CHAPTER 1

MAIN INTRODUCTION

Chapter 1. Main introduction.

CMV is a widespread virus, infecting at least 50% of the population in the USA and Western Europe. Although the immune response effectively controls CMV infection in preventing the development of disease in most normal immunocompetent individuals, the virus establishes a persistent, lifelong infection. 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. Under conditions of immunosuppression, reactivation of latent virus, reinfection, or primary infection can lead to the development of CMV disease. The populations at risk for CMV disease include neonates, patients who are immunosuppressed due to preconditioning prior to solid organ or bone marrow transplantation, and human immunodeficiency virus (HIV) infected patients. Congenital CMV can be manifested as cytomegalic inclusion disease, with varying degrees of involvement of the central nervous system, resulting in mental retardation, hearing loss or visual impairment (Boppana *et al.* 1992). The dramatic and progressive cellular immunodeficiency induced by HIV infection creates optimal conditions for the reactivation of latent CMV. The development of acquired immune deficiency syndrome (AIDS), and a CD4+ve lymphocyte count below 50/μl are important risk factors for invasive CMV disease, which is associated with early death in AIDS patients. Clinical syndromes associated with CMV in AIDS patients have included disease in almost every organ system, but most frequently is manifested by diseases of the central nervous system, eyes, gastrointestinal system, and lungs (Pertel *et al.* 1992; Gallant *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 (Peterson *et al.* 1980; Paya *et al.* 1989; Smyth *et al.* 1991), increased immunosuppression (Rook, 1988), and increased risk of allograft rejection (Glenn, 1981), leading to decreased patient survival. 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 (Rubin, 1990; Bailey, 1993). A greater understanding of the biology of CMV and its interactions with the host immune system may help to generate new therapeutic strategies to help combat the effects of this disease in susceptible patient groups.

1.1 BIOLOGY OF CMV

1.1.1 Virion characteristics

CMV is classified as a β herpesvirus, on the basis of its restricted host range and relatively slow replication cycle in comparison to other herpes viruses (Roizman, 1993). The virion consists of a core containing linear double stranded DNA, an icosahedral capsid, an amorphous layer of proteins called the tegument, and an envelope containing protruding viral glycoprotein spikes. The genome of the laboratory strain, AD169, has been sequenced, and contains 208 predicted open reading frames, encoding more than 100 known proteins (Chee *et al.* 1990a). 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. The function of a large proportion of CMV-encoded proteins is not known at present. Many of the proteins encoded by CMV are non-essential for growth of the virus in culture, but may have other effects on the host cells or be important for the success of the virus *in vivo* (Jones and Muzithras, 1992).

1.1.2 Structural proteins of CMV

Prior to sequencing of the CMV genome, nomenclature of viral proteins was based on various characteristics of the protein, such as molecular weight, post translational modification, location in the virion or temporal expression, leading to proteins with several different names, and confusion in cross-referencing CMV proteins in the literature. It has now been decided to name CMV proteins according to the alphanumeric open reading frame designation of the protein (Chee *et al.* 1990a), prefixed by pp (phosphoprotein), gp (glycoprotein), or p (a protein which is neither of the above, or whose post-translational modification is as yet undefined) (Landini and Spaete, 1993).

The mature CMV capsid consists of at least 3 proteins: the 153kD major capsid protein (pUL86), the 34kD minor capsid protein (pUL46), and a small 12kD capsid protein, encoded by a previously unidentified reading frame between UL48 and UL49 (Gibson *et al.* 1996). The CMV virion has 3 main tegument proteins, the basic phosphoprotein (pp150, ppUL32), and the upper (pp71, ppUL82) and lower (pp65, ppUL83) matrix proteins. Together these proteins account for approximately 40% of the virion mass. Three other tegument proteins have been characterised, namely pUL48, ppUL99, ppUL65. All the

tegument proteins are phosphorylated with the exception of pUL48, the 212kD high molecular weight protein (Spaete *et al.* 1994). The capsid and tegument are enclosed by a lipid bilayer envelope, derived from either the inner nuclear or cytoplasmic membranes (McGavran and Smith, 1965; Parry, 1988). At least 8 viral envelope glycoproteins have been identified, although there are likely to be many more minor envelope glycoproteins present, as sequence analysis of the CMV strain AD169 genome has identified approximately 55 open reading frames potentially encoding envelope glycoproteins (Chee *et al.* 1990a). The three envelope glycoprotein complexes that are best characterised are designated gCI, gCII, and gCIII (Gretch *et al.* 1988c). The gCI complex, or gB (gpUL55), is the most abundant CMV envelope glycoprotein. This is a disulphide linked protein consisting of a 116kD surface component, and a 55kD transmembrane portion, which are proteolytically cleaved from a precursor protein (Gretch *et al.* 1988a). This protein is thought to be involved in virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells (Navarro *et al.* 1993). The gCII complex consists of at least two distinct proteins which are disulphide linked to each other, designated gp47-52 (Kari *et al.* 1990). These have been identified as the UL100 (Kari *et al.* 1994) and US6 (Gretch *et al.* 1988b) gene products. The latter is dispensable for growth in culture (Villareal *et al.* 1992). The gCIII complex includes glycoprotein H (gH, UL75) which is proposed to be in a stable complex with gL (UL115), as is the case for the herpes simplex homologues of these proteins (Spaete *et al.* 1994). gH appears to be involved in cell to cell spread and fusion of the virion with the host cell plasma membrane (Keay and Baldwin, 1991; Germain, 1994).

1.1.3 Receptor/virus entry.

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). The initial interaction between cytomegalovirus virions and the cell surface involves the binding of viral envelope proteins to extracellular heparin sulphate. CMV binding was sensitive to heparin competition and heparinase treatment, and did not occur on cells defective in synthesis of heparin sulphate (Compton *et al.* 1993). Multiple envelope glycoproteins are capable of binding to heparin sulphate, but glycoproteins gB and gC-II are strong candidates (Kari and Gehrz, 1993; Kari and Gehrz, 1992; Compton *et al.* 1993). The binding of the virus to heparin sulphate is converted rapidly to high affinity, heparin resistant attachment. The high affinity receptor responsible for the heparin resistant interaction has not

been identified, but such viral attachment correlates with the expression of a 34kD membrane protein, which has been shown to specifically bind to CMV virions (Taylor and Cooper, 1990; Nowlin *et al.* 1991), although the viral protein involved at this stage has not been identified. Expression of this protein correlates with viral attachment only, not with fusion and penetration of the virion. The envelope glycoprotein, gH is thought to be involved in the fusion step, by binding to a 92.5kD cellular receptor (Keay *et al.* 1989; Keay and Baldwin, 1991). Viral penetration appears to take place by a pH-independent fusion event between the viral envelope and the host cell plasma membrane (Compton *et al.* 1992), leading to the release of the nucleocapsid into the cytoplasm and initiation of a replicative cycle.

1.1.4 Replication cycle

In common with other members of the herpesvirus family, the CMV genome is expressed in three temporally regulated phases during a productive infection. Immediate early gene expression occurs immediately following infection, and is defined as not requiring prior synthesis of other viral proteins. During or after attachment of the virion to the cell, signal transduction pathways are triggered by the CMV upper matrix protein, pp71 (ppUL82), leading to activation of protein kinase pathways and cellular transcription factors, which subsequently act on the enhancer/promoter region of the CMV major immediate early protein (MIEP) (Liu and Stinski, 1992). Immediate early (IE) proteins are synthesised throughout the replication cycle of the virus. They are non-structural viral proteins, which accumulate in the nucleus, and are important in the regulation of the expression of other viral genes, controlling the switch to early gene expression. The most abundantly expressed immediate early region has two genes, called IE1 (UL123, exon 1) and IE2 (UL122, exons 2-5). Alternative splicing of the 5 exons of UL122 and UL123 results in the expression of a series of proteins. The most abundant immediate early protein is the 72 kD major immediate early protein ($IE1_{491aa}$), which accumulates in the nucleus of infected cells following infection. Other abundant IE proteins are the 86kD protein, $IE2_{579aa}$, and a further IE2 gene product, $IE2_{425aa}$. $IE1_{491aa}$, and $IE2_{425aa}$ autoregulate the MIEP, but to a lesser extent than pp71 (Malone *et al.* 1990; Baracchini *et al.* 1992), while $IE2_{579aa}$ is a repressor of this promoter. The latter is thus believed to play the central regulatory role, alongside $IE1_{491aa}$, in the switch from immediate early to early gene expression, as well as the shut off of IE1 and IE2 gene expression which takes place during DNA replication. A final IE2 gene product, $IE2_{338aa}$, also has a negative effect on immediate early gene

expression, and a positive effect on early and late gene expression. Other immediate early genes include UL36, UL37, UL69, IRS1, TRS1, and US3, and are required for the activation of certain viral promoters, and expression of early and late genes (Colberg-Poley *et al.* 1992; Pari and Anders, 1993; Pari *et al.* 1995).

Early gene expression can be divided into two classes, β_1 (early, transcription begins at 4-8 hours post infection, and is not affected by inhibitors of DNA replication) and β_2 (delayed early, transcription is first detectable from 8 to 24 hours post infection). Early genes encode many proteins required for viral DNA replication, such as the major DNA binding protein, p52 (UL44), the viral DNA polymerase (UL54), which interact to form a stable complex (Ertl and Powell, 1992), and the major early DNA binding protein UL57.

Late CMV transcripts are first detected at 12-36 hours post infection, but there is a temporal lag before proteins are detected. Late proteins accumulate at high levels after DNA replication (48-72 hours), and are predominantly structural proteins, such as the viral envelope, capsid and tegument proteins.

1.1.5 Mechanism of action of antiviral agents

The current antiviral agents licensed specifically for the treatment of CMV infection are ganciclovir and foscarnet. These agents act by inhibition of the viral DNA polymerase and thus prevent viral DNA replication, the formation of CMV late proteins and the generation of new virions (Matthews and Boehme, 1988; Crumpacker, 1992). The two compounds, although exerting similar effects on the viral replication cycle, do so by very different mechanisms. Intravenous immunoglobulin (IVIG) is also licensed for prophylaxis of CMV disease, and appears to ameliorate the symptoms of CMV disease, but the mechanism for this is not known (Snydman, 1990).

1.1.6 Ganciclovir

Ganciclovir (9-[1,3-dihydroxy-2-propoxy)methyl]guanine; DHPG) is an analogue of the purine nucleotide, guanosine. The structure of ganciclovir differs from that of guanosine by the absence of the 2' carbon atom of the deoxyribose moiety, making the sugar acyclic. However, the equivalent of the 3' and 5' hydroxyl moieties required for chain elongation are still present, distinguishing

ganciclovir from the related compound, acyclovir, which has no 5' hydroxyl group and is thus an obligate chain terminator. Ganciclovir is a prodrug which must be phosphorylated to its active triphosphate form (ganciclovir-triphosphate) in order to be incorporated into the DNA (Biron *et al.* 1985). Ganciclovir-triphosphate has a half life of 10 hours, begins to accumulate at 48 hours post infection, and is stable for several days in infected cells *in vitro* (Vere-hodge and Perkins, 1989). As with all effective antiviral agents, a major requirement is that the drug should be active in infected cells only, so that toxicity in uninfected cells is limited as much as possible. In the case of ganciclovir, this selectivity is due to the action of the CMV UL97 gene product in the infected cell which converts ganciclovir to its monophosphate form (Littler *et al.* 1992; Sullivan *et al.* 1992). Cellular kinases complete the conversion from the monophosphate to the triphosphate (Matthews and Boehme, 1988). Ganciclovir is a more potent inhibitor of the CMV DNA polymerase than of the cellular DNA polymerases (Mar *et al.* 1985; Freitas *et al.* 1985). However, despite these selective mechanisms, ganciclovir has significant toxicity, particularly for rapidly dividing cells such as bone marrow progenitor cells and lymphocytes, and is associated with neutropenia and immunosuppressive effects (Bowden *et al.* 1987; Fletcher and Balfour, 1989).

Ganciclovir inhibits CMV DNA synthesis in two ways. Firstly, it competitively inhibits binding of the normal substrate dGTP to the DNA polymerase and hence its incorporation into the DNA. Secondly, although ganciclovir is not an obligate chain terminator, ganciclovir-monophosphate terminated DNA is a poor substrate for chain elongation. Such chains elongate more slowly and terminate prematurely compared to those containing only unmodified nucleotides (Reid *et al.* 1988; Matthews and Boehme, 1988). One explanation for this is that although both 3' and 5' hydroxyl groups are present on the sugar part of the molecule, the 3' hydroxyl is preferentially phosphorylated, resulting in the incorporation of unphosphorylated 5' hydroxyl groups which are not capable of chain elongation (Marshalko *et al.* 1995). It has also been demonstrated that the presence of a deoxyribose sugar in the backbone contributes significantly to the overall stability of a DNA duplex molecule, and that the presence of an acyclic sugar such as that in ganciclovir results in decreased stability of the DNA (Marshalko *et al.* 1995).

1.1.7 Foscarnet

Foscarnet (trisodium phosphonoformate) is a pyrophosphate analogue which inhibits the action of the viral DNA polymerase by non-competitive inhibition at the pyrophosphate binding site of the enzyme. This inhibits the formation of the 3'-5'-phosphodiester bond between the primer and the substrate thus preventing chain elongation. It selectively inhibits viral DNA polymerases (and HIV reverse trancriptase) at concentrations which do not affect cellular DNA polymerases (Oberg, 1989; Chrisp and Clissold, 1991; Crumpacker, 1992).

1.1.8 Cell tropism.

Permissive replication of CMV takes place only in primary, differentiated human cells, with the exception of an immortalised fibroblast line which was recently created (Compton, 1993). Most of the knowledge gained so far about the molecular biology and replication of CMV has been derived from infection of fibroblasts, due to the ease with which high titre virus replication can be achieved in this cell type. *In vivo,* lung tissue, epithelial cells and fibroblasts are the predominantly infected cell types, lending importance to the *in vitro* findings using these cells. Endothelial cells, smooth muscle cells, and tissue macrophages are also target cells for CMV *in vivo*, expressing CMV immediate early and late proteins, suggesting that these cells support a full viral replication cycle (Sinzger *et al.* 1995; Sinzger *et al.* 1996). Endothelial cells (Somoza *et al.* 1993), smooth muscle cells (Tumilowicz *et al.* 1985), and macrophages (Ibanez *et al.* 1991; Lathey and Spector, 1991) are permissive for CMV replication *in vitro*, either with clinical isolates adapted for growth in endothelial cells, or following stimulation of the cells with either sodium butyrate or hydrocortisone.

Whether a permissive or abortive infection takes place in a particular cell type depends on the differentiation state of the cell. Human cell lines that can be induced to differentiate have been used to demonstrate the dependence of CMV replication on the differentiation state of the cell. These are the monocytic cell line, THP-1 (Weinshenker *et al.* 1988), and a teratocarcinoma cell line which differentiates in response to retinoic acid (Gonczol *et al.* 1985). Undifferentiated cells contain a DNA binding protein (designated MBF1) which negatively regulates the expression of the immediate early genes by binding to the 'modulator' region upstream of the major immediate early promoter (MIEP). Following differentiation, this protein is either altered or absent, and

transcription factors are activated, leading to transcription from the MIEP (Shelbourn *et al.* 1989; Lubon *et al.* 1989; Sinclair *et al.* 1992).

It is well established that CMV can be transmitted by blood transfusion (Adler, 1983), and that the incidence of transmission by this means can be dramatically reduced by depletion of leukocytes prior to transfusion (Yeager *et al.* 1981). However, the ability of each population of leukocytes to support CMV replication is still not clear. Most groups have found it difficult to productively infect monocytes and polymorphonuclear cells (PMNCs), although expression of CMV immediate early genes has been documented in *in vitro* infected monocytes, PMNCs and also T and B lymphocytes (Rice *et al.* 1984; Einhorn and Ost, 1984). One group has reported release of infectious virus by *in vitro* infected monocytes and CD3+/CD8+ large granular lymphocytes, although this awaits confirmation by others. The cellular expression of CD13 correlated with the subset of cells which could be infected *in vitro* (Soderberg *et al.* 1993).

Numerous studies have been carried out in an attempt to determine the blood cells infected with CMV in both viraemic patients and healthy seropositive individuals, and the extent of viral replication taking place. In healthy individuals it is accepted that the major site of persistence in the blood is in monocytes (Taylor-Wiedeman *et al.* 1991), as CMV DNA is not detected in polymorphs in healthy CMV positive individuals (Taylor-Wiedeman *et al.* 1993). In contrast, both monocytes and polymorphs contain CMV IE antigens and pp65 protein (UL83, CMV lower matrix protein) during an active infection. However, mRNA encoding pp65 is absent, demonstrating that the abundance of the structural protein pp65 in polymorphs is likely to be a result of uptake of this protein by phagocytes, rather than *de novo* synthesis (Grefte *et al.* 1994). This is in agreement with the findings of earlier studies on viraemic patients, in which viral DNA and infectious virus was isolated predominantly from polymorphs (Saltzman *et al.* 1988), although the viral DNA was localised to the cytoplasm, suggesting that it was harboured in phagosomes (Turtinen *et al.* 1987). Some discrepancies exist between different groups regarding the expression of mRNA for CMV late proteins in leukocytes, many of which may stem from differences in the accuracy of the purification methods used (some of which may not exclude circulating endothelial cells), combined with increased sensitivity of the assays used to detect viral nucleic acid (Gozlan *et al.* 1993; Bitsch *et al.* 1993).

1.1.9 Dissemination of CMV.

Following infection, CMV is present in many bodily fluids, including blood, saliva, urine, breast milk and semen, indicating the involvement of the cervix, salivary glands, and urinary tract as sites of productive infection. Primary CMV infection may thus take place by a number of routes, including infection via an epithelial route (respiratory or sexual transmission), transfusion with seropositive blood, or transplantation of an infected organ. In the blood, the leukocytes are the major carriers of the virus; almost no virus can be isolated from red cells or plasma. CMV is a strongly cell associated virus, and the spread of CMV infection through tissues is believed to take place via cell to cell spread from infected epithelial cells and fibroblasts, leading to eventual infection of endothelial cells (Sinzger *et al.* 1995). The latter is thought to be the central event for conversion from local subclinical infection to systemic infection, providing a possible mechanism for the acquisition of virus by circulating leukocytes and its transport to other organs via the bloodstream. Viral gene expression has been found in both the monocyte/macrophage and granulocyte populations in the lung, gastrointestinal tract and placenta. In tissue macrophages, viral genes from all three phases of the replication cycle have been detected, which strongly suggests that CMV can replicate and produce progeny virions in this cell type (Ibanez *et al.* 1991; Lathey and Spector, 1991; Sinzger *et al.* 1996). While monocytes display only restricted expression of viral genes when isolated from the blood (Grefte *et al.* 1994), differentiation of monocytes into macrophages results in productive infection. Bi-directional transfer of infectious cytomegalovirus between endothelial cells and monocytes has been shown to occur *in vitro*, suggesting that monocytes may be a vehicle for dissemination of infection to distant locations (Waldman *et al.* 1995b). Another proposed mechanism for viral dissemination is via circulating endothelial cells, which also express all three classes of CMV genes (Grefte *et al.* 1993). It is thought that a proportion of the infected endothelial cells detach from the vascular bed due to the cell rounding induced by the loss of fibronectin which takes place following CMV infection (Pande *et al.* 1990), and that these cells disseminate CMV to other areas of the body (Grefte *et al.* 1993).

1.2 HOST RESPONSE TO VIRAL INFECTIONS

The immune response can be divided into mechanisms of innate immunity and acquired immunity. Innate immunity refers to defence systems already in place

in the organism, such as natural killer (NK) cells, phagocytes (neutrophils and macrophages), Type I interferon production and complement. Acquired immunity, which encompasses lymphocyte and antibody responses, is specific for a particular pathogen and must be activated by that pathogen in order to function. Following exposure to a pathogen, specific memory is imprinted on the acquired immune system, leading to rapid responses to a secondary infection. The innate and acquired immune responses are interconnected through cytokine networks.

1.2.1 NK cells

NK cells are highly cytotoxic cells which eliminate NK-targets, which include virally infected cells, without a need for major histocompatibiliy complex (MHC)-restricted antigen presentation or previous sensitisation. NK cells contain cytotoxic granules, including perforin, and can also secrete cytokines, and mediate antibody dependant cellular cytotoxicity (ADCC), due to their expression of Fc γ receptors (Moretta *et al.* 1994). The NK cell response occurs rapidly and takes place early in viral infection, and importantly can occur before replication of the virus has taken place (Welsh, 1986). 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. The mechanisms and molecular interactions between NK cells and their targets are still poorly understood. It appears that NK cells must recognise a specific (unidentified) structure on the target cell, in the absence of an inhibitory signal from class I HLA through the 'killer cell-inhibitory receptor' (Correa *et al.* 1994; Ciccone *et al.* 1996). The NK cell response declines when the specific T cell response is established. It appears that this decline is controlled by cytokines such as TGFβ and IL-4, which have suppressive effects. In addition, T cells have higher affinity IL-2 receptors than NK cells, so compete favourably for IL-2 (Su *et al.* 1993).

1.2.2 Type I Interferons

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'-oligoadenylate synthetase, which activates

endonucleases and leads to degradation of viral mRNA. Interferon also enhances the expression of class I HLA, leading to increased recognition by CD8+ve T cells, although several viruses, including CMV, counteract this effect by downregulating class I HLA expression on infected cells.

1.2.3 Macrophages

Tissue macrophages develop from circulating blood monocytes, and are concentrated in the lung, liver, lymph nodes and spleen. They play a central role in cell-mediated immunity in their capacity as professional antigen presenting cells in tissues, controlling activation of specific T cell clones. They also function by phagocytosis of foreign material, and contain cytoplasmic granules to digest this material. Macrophages possess Fc receptors and can thus function in ADCC. Cytokine-activated macrophages are non-specific in their target recognition and can potentially cause tissue damage in areas of inflammation.

1.2.4 Humoral responses

Neutralising antibodies are important in protection from viral infections. Neutralisation of the virus is generally achieved by blocking an epitope of a viral protein required for infection of a new host cell. The infection may be blocked at the stage of attachment to a receptor, fusion with the host cell, penetration, uncoating of the virus, or release of the genome for transcription. Antibodies which bind to infected cells can also reduce viral load by lysing infected cells in conjunction with complement or by ADCC (Dimmock, 1987).

1.2.5 MHC-restricted T cell responses

T lymphocytes specific for virus-infected cells recognise viral peptides presented by self major histocompatibility antigens (MHC) expressed on infected cells. The specific recognition is achieved by the presence of the T cell receptor (TCR) on the T cell surface. CD8+ve and CD4+ve T cells recognise virally infected cells by the presentation of viral peptides by class I and class II MHC molecules respectively. CD8+ve T cells are the principal cytolytic cells of the immune response, while CD4+ve cells can be cytolytic (Lindsley *et al.* 1986), or can regulate the cellular immune response by the production of

cytokines (helper cells). CD4+ve helper cells can be functionally divided into Th1 and Th2 cells. Th1 cells enhance the immune response by secreting predominantly interferon γ (IFNγ) and interleukin-2 (IL-2), which are also secreted by NK and CD8 cells. Th2 cells secrete IL-4, IL-5, and IL-10, and function in inhibition of the Th1 subset, and hence the arms of the immune response stimulated by it.

1.2.6 Antigen presentation

The classic pathway of MHC class I restricted antigen presentation involves the processing of endogenously synthesised viral proteins for presentation by class I HLA to CD8+ve T cells (Townsend *et al.* 1989). However, there are exceptions to these rules, which allow proteins from viral particles entering the cell to be presented by class I HLA, as is the case for both murine CMV and CMV (Reddehase *et al.* 1984; Riddell *et al.* 1991). Following synthesis of endogenous viral proteins, and their export to the cytosol, or uptake of structural viral proteins by the cell, both undergo the same processing resulting in presentation on class I HLA molecules (Figure 1.1). The proteins are cleaved into smaller peptides by the MHC-encoded LMP proteosome, and are transported by TAP proteins (transporter proteins associated with processing) into the lumen of the endoplasmic reticulum (ER). The MHC encoded heavy chain of the class I molecule, and the $\beta_2$microglobulin molecule, are also transported to the ER, where formation of the class I dimer, consisting of these two proteins and a peptide bound in a groove of the heavy chain, takes place. The complex is then transported to the Golgi apparatus, where further glycosylation takes place, resulting in an endoglycosidase H resistant molecule which is expressed on the cell surface. Proteins which are acquired by the cell from an exogenous source enter an endosomal pathway of antigen processing resulting in presentation by class II HLA (Neefjes *et al.* 1990). Some viruses, such as measles virus, enter the cell by an endosomal pathway and thus stimulate class II restricted cytotoxic responses (Jacobson *et al.* 1989). Class II mediated cytotoxicity by CD4+ve lymphocytes also occurs in CMV infection (Lindsley *et al.* 1986).

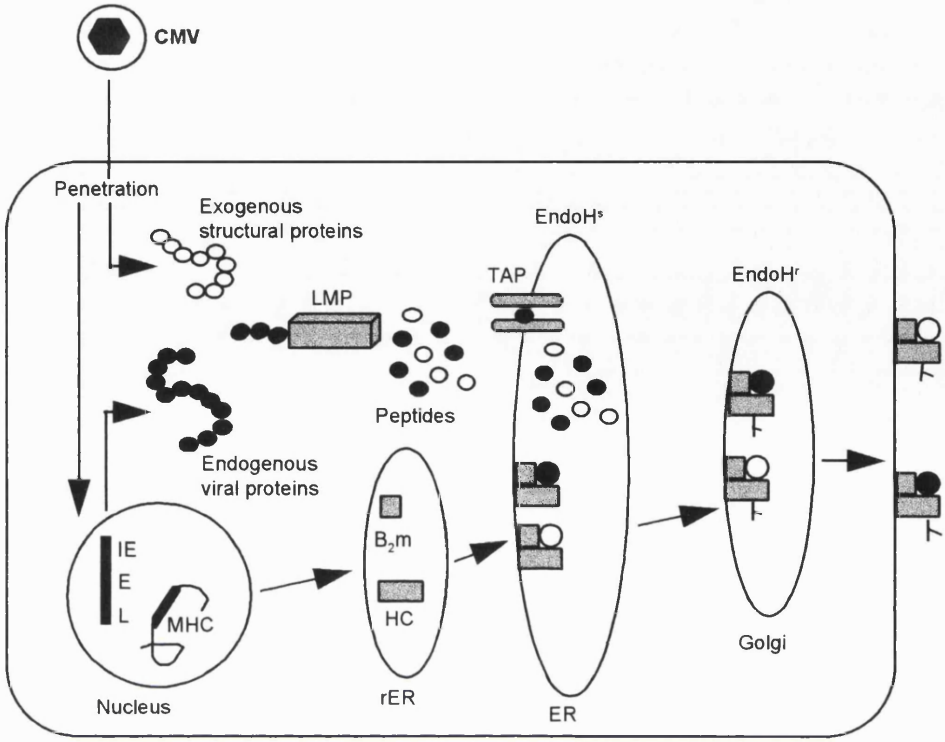


Figure 1.1. Antigen processing and presentation of CMV antigens by class I HLA.

CMV proteins are delivered to the cytosol either exogenously after viral penetration, or endogenously after viral gene expression. Proteins from both sources then enter the same pathway of antigen presentation. The proteins are cleaved into smaller peptides by the LMP proteasome complex and transported by Transporters Associated with Processing (TAP) into the lumen of the endoplasmic reticulum (ER). The peptides are then assembled into a trimolecular complex with the class I HLA heavy chain and β_2 microglobulin. The complexes pass into the golgi where they are glycosylated to an endoglycosidase H resistant form (Endo H^r), from where they are transported to the cell surface. (Adapted from Reddhase and Lucin, 1993)

1.2.7 T cell activation

Following ligation of the TCR by the appropriate MHC-peptide complex, signals are generated which lead to the activation of protein kinase C (PKC) and mitogen activated protein (MAP) kinase, and the activation of the IL-2 gene. The important features of the T cell activation pathway are shown in Figure 1.2. These processes can be mimicked *in vitro* by ligation of CD3 or CD2 with particular monoclonal antibodies. Activation of T cells using phorbol esters directly activates PKC, and requires calcium ionophores to open calcium channels and elevate intracellular calcium levels for efficient activation. CD28 costimulation appears to have a positive effect on the MAP kinase required for activation of IL-2 transcription factors.

The mode of interaction between the TCR and the MHC-peptide complex has not yet been established by structural studies. The affinity of the TCR for the MHC molecule is low, and the proportion of MHC molecules expressing the appropriate peptide on the target cell is low (<0.1%). 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 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, which then detach and form a 'contact cap', which transduces signals when a threshold number of TCRs are assembled (Valitutti *et al.* 1995).

MHC molecules presenting subtly altered peptides can interact with TCRs in a suboptimal way, and cause either partial activation (e.g. cytokine production without proliferation) or anergy of the T cell (Kersh and Allen, 1996). Naturally occurring viral peptides have been identified which can act as antagonists of the cytotoxic T lymphocyte (CTL) response, inhibiting the activity of a CTL which would otherwise lyse the infected cell. Generation of such peptides may be an escape mechanism used by viruses to stay ahead of the immune response (Bertoletti *et al.* 1994b; Klenerman *et al.* 1994).

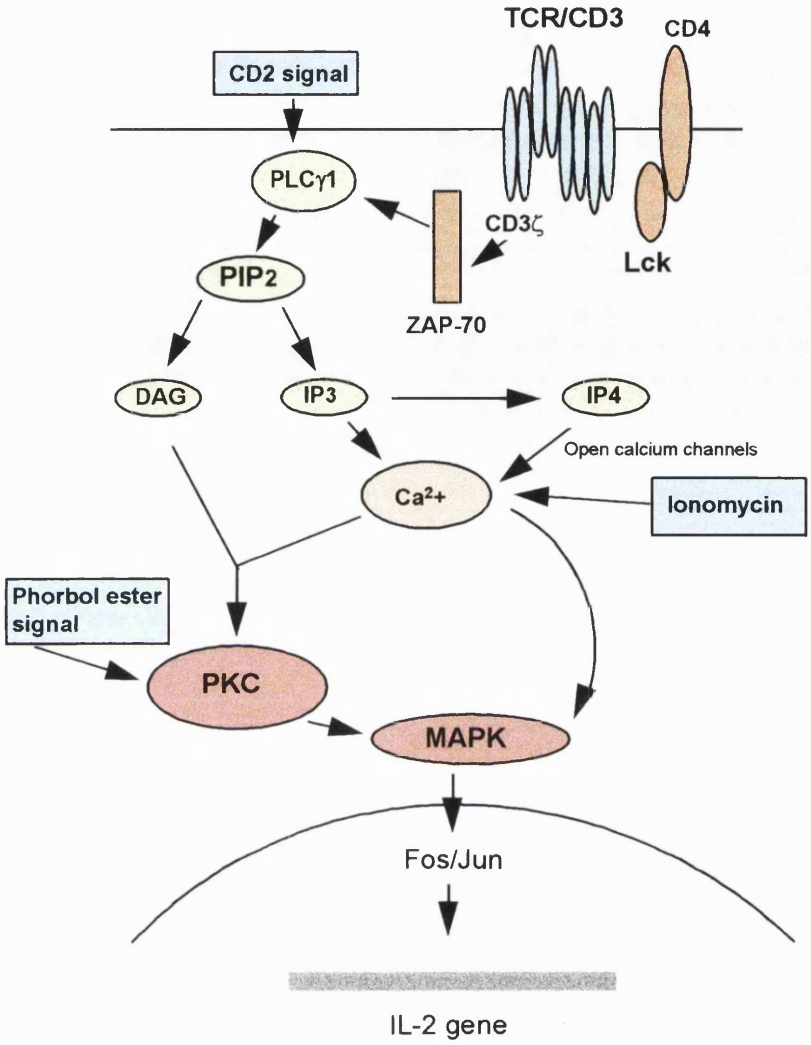


Figure 1.2. T cell activation mediated by the TCR/CD3 complex, CD2 ligation, and phorbol ester and ionomycin stimulation.
TCR ligation leads to the phosphorylation of the CD3ξ chain, perhaps by the CD4 and 8 associated kinase *lck*. CD3ξ then activates the kinase ZAP-70, leading to phosphorylation of phospholipase Cγ1 (PLCγ1). CD2 ligation also signals through PLCγ1. PLCγ1 catalyses the hydrolysis of phosphoinositide 4,5 diphosphate (PIP_2) to generate inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) thereby mobilising intracellular calcium and stimulating protein kinase C (PKC). Phorbol ester acts at the level of PKC, while ionomycin opens calcium channels to elevate intracellular calcium. PKC activates mitogen-activated protein kinase (MAPK), leading to phosphorylation of the transcription factors *c-Fos* and *c-Jun*, and the activation of the interleukin-2 (IL-2) gene.

1.2.8 Cell mediated cytotoxicity

Lymphocyte mediated cytotoxicity (by CTLs or NK cells) of infected cells or tumour cells is a complex, multistep mechanism, involving binding of the lymphocyte to its target, delivery of the lethal hit, pre-lytic fragmentation of the target cell DNA (apoptosis), lysis of the target cell and recycling of the cytotoxic cell to attack another cell. The mechanisms for binding of the CTL to the target cell are discussed in the following section. For CTL lysis to take place the TCR/CD3 complex must be engaged. The cytotoxic event may occur by two independent mechanisms; a secretory pathway involving the release of cytotoxic proteins such as perforin and granzymes from preformed granules, and a receptor mediated pathway involving the ligand-induced triggering of surface receptors, such as the Fas-ligand and the TNF receptor, leading to apoptosis.

Cytoplasmic granules are present in all NK cells and most activated CTLs. The granules localise to the contact site between the target cell and the lymphocyte, fuse with the plasma membrane, and release their contents into the zone of contact. The perforin then forms pores in the target cell membranes by a calcium dependent mechanism, allowing passage of proteins such as granzymes into the target cell, where they induce cell death by apoptosis. The ligation of Fas (CD95), or TNFα receptor 1 which are expressed on target cells, by Fas-ligand or TNFα on activated CTLs constitutes the non-secretory pathway of cell mediated cytotoxicity. These pathways lead to apoptotic cell death within hours, although the mechanisms are not clear (Berke, 1991; Tschopp and Hofmann, 1996).

1.2.9 Adhesion molecules as accessory molecules in CTL killing

The cell to cell interactions required for the induction of an antigen-specific immune response can be divided into three stages. The first is adhesion, where T cells and antigen presenting cells (APCs) randomly interact via adhesion molecules on both cells, such as LFA-1 and CD2 on T cells, and their ligands ICAM-1 and LFA-3 on APCs. If the APCs present sufficient quantities of a specific peptide in the context of MHC molecules, this is followed by recognition of the APC by the T cell via its TCR, and stabilisation of the interaction by CD4 or CD8 molecules, which bind to a separate part of the MHC class II or class I molecule respectively. At physiological densities the interaction between the

TCR and MHC, and between CD4 or CD8 and MHC class II or I are low affinity interactions. Signalling through the TCR is necessary but not sufficient to induce antigen specific activation. The cell requires a costimulatory signal which is essential for the subsequent activation of the lymphocytes. Molecules capable of providing costimulatory signals include LFA-3, ICAM-1, and B7 on the target cells, and their ligands CD2, LFA-1 and CD28 on the T cells. (Figure 1.3) Each of these molecule pairs has distinctive properties which are discussed below.

1.2.9.1 LFA-3 and CD2

LFA-3

It is now well established that the ligand for CD2 is the structurally related molecule CD58, or LFA-3, both of which are members of the immunoglobulin superfamily of molecules, and possess two immunoglobulin domains (Shaw *et al.* 1986; Selvaraj *et al.* 1987; Peterson and Seed, 1987; Wallner *et al.* 1987). The binding between the two molecules takes place via the NH_2 terminal domain of each protein (Somoza *et al.* 1993; Osborn *et al.* 1995). It has also been reported that an unrelated molecule, CD59, is an additional ligand for CD2, but direct interaction between the two molecules has not been demonstrated (Deckert *et al.* 1992; Hahn *et al.* 1992a). LFA-3 is a 40-70 kD glycoprotein which is expressed on a wide range of haematopoetic and non-haematopoetic cells, including epithelium, endothelium and stromal cells. It is also present on monocytes and T cells, and is present on these cells at increased levels following activation. The molecule exists in two membrane anchorage isoforms, a transmembrane form, and a glycophosphatidylinositol (GPI) linked form. which are generated by alternative splicing (Dustin *et al.* 1987b). The GPI linked form is more laterally mobile in the membrane, and this is thought to be important in the formation of contact zones between T cells and target cells, at which LFA-3 molecules accumulate (Hollander, 1992). The gene encoding LFA-3 has been cloned, but the promoter region has not been defined and the mechanisms of regulation of LFA-3 expression are not known (Seed, 1987).

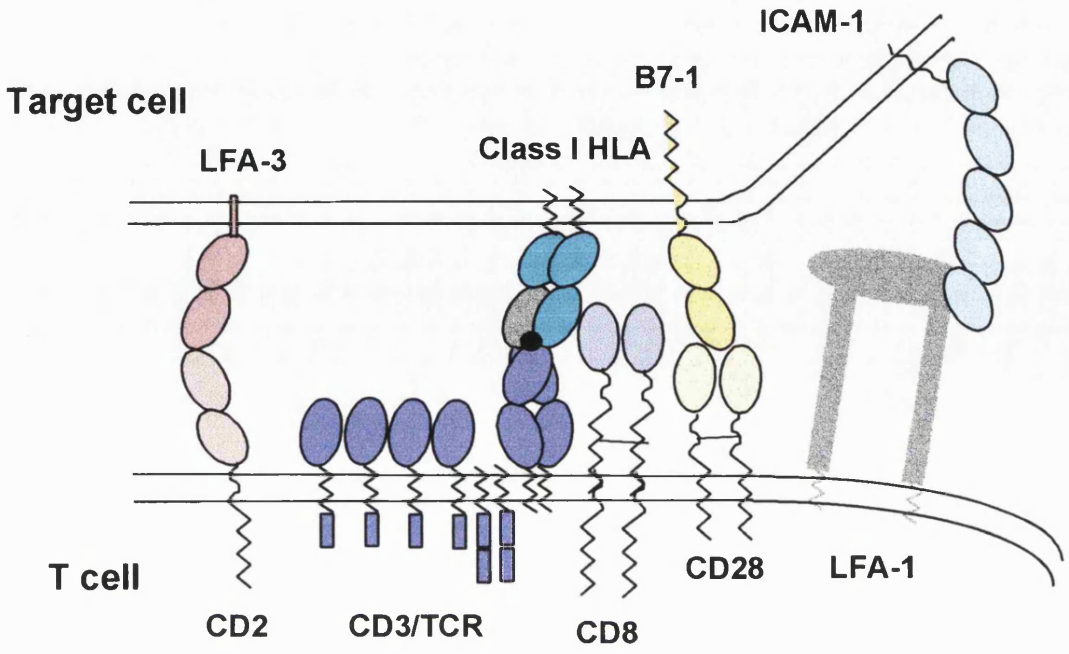


Figure 1.3. Interactions between a CD8+ve T cell and a virally infected target cell.

The basic structures and ligand interactions of the molecules mediating adhesion and recognition of virally infected cells by CD8+ve T cells are shown. Class I HLA interacts with the T cell receptor (TCR) while the interaction is stabilised by the CD8 molecule binding to another part of the class I HLA molecule. LFA-3 and CD2 interact via their membrane distal immunoglobulin domains. ICAM-1 interacts with the α subunit of LFA-1 via its 5th immunoglobulin domain. The molecules are drawn approximately to scale. (Adapted from Davis and van der Merwe, 1996).

CD2

CD2 is a member of the immunoglobulin superfamily which is expressed on all T lymphocytes and NK cells. The molecule is associated with signalling function, due to the positive effect of anti-CD2 antibodies on CD3 mediated T cell activation, and the activation of T cells by certain combinations of anti-CD2 antibodies. One of these antibodies must recognise the CD2R epitope which is poorly expressed on resting T cells but is induced following binding of a second CD2 antibody specific for another epitope (Hunig *et al.* 1987). On T cells CD2 forms a loose association with the TCR complex, and the presence of CD3 is required for activation to take place, unless CD2 is artificially over-expressed. The regulation of the interaction between CD2 and LFA-3 is regulated by the density of expression of each molecule, and by increased avidity of CD2 following engagement of CD3 (Hahn *et al.* 1992b; Hahn and Bierer, 1993).

The CD2/LFA-3 interaction

The CD2/LFA-3 interaction enhances class I and class II MHC-restricted T cell antigen recognition (Sanchez-Madrid *et al.* 1982; Krensky *et al.* 1984), and is also important in non-MHC restricted killing by NK cells and lymphokine activated killer (LAK) cells (Siliciano *et al.* 1985; Bolhuis *et al.* 1986; Zarcone *et al.* 1992). Two important features of the interaction between LFA-3 and CD2 have been identified which may be responsible for enhancing CTL killing: initiation and stabilisation of the interaction between the infected cell and the lymphocyte by adhesion, and the delivery of costimulatory signals to the T cell via the CD2 molecule (Tiefenthaler *et al.* 1987; Dustin *et al.* 1987a; Hahn *et al.* 1992b). It is thought that the adhesion function is of principal importance in enhancing the responsiveness of the cell to antigen. It has been shown that a non-signalling mutant of the CD2 molecule enhances antigen responsiveness of T cells, but only if expressed on the same cell as the TCR/CD3 complex (Moingeon *et al.* 1989). The requirement for both molecules to be expressed on the same cell was confirmed by others, showing augmentation of the proliferative response to HLA-DR transfectants by co-transfection of LFA-3 molecules (Greenlaw *et al.* 1992). The molecular dimensions of the CD2 and LFA-3 molecules are similar to those predicted for the TCR and MHC molecules (Bjorkman *et al.* 1987; Davis and Bjorkman, 1988; Jones *et al.* 1992). It is thus proposed that the CD2/LFA-3 interaction positions the membranes at the appropriate distance to favour MHC interactions with the TCR-CD3 complex (van der Merwe *et al.* 1995; Dustin *et al.* 1996), providing a

mechanism by which the small proportions of MHC molecules expressing the appropriate peptide can be sampled by the TCRs.

In addition to reports of enhancement of T cell responsiveness by CD2 ligands, there have also been reports of inhibition of T cell function by the binding of certain anti-CD2 antibodies (Palacios and Martinez-Maza, 1982; Yssel *et al.* 1987). Inhibition of T cell function by recombinant LFA-3 fusion proteins has also been shown, but this may be due to the nature of the recombinant proteins used, and may not be relevant *in vivo* (Guckel *et al.* 1991; Miller *et al.* 1993). It has been shown that the CD2 molecule expressed on anergised cells underwent a temporary conformational change, which prevented LFA-3 binding. The reversion of CD2 to its former state following IL-2 treatment facilitated the binding of LFA-3, and the re-acquisition of the ability to respond to alloantigen in the presence of LFA-3 (Boussiotis *et al.* 1994; Bell and Imboden, 1995). CD2 may therefore have a further unique role in reversal of anergy which is not shared by other adhesion molecules.

CD2 knockout mice have been constructed, and develop apparently normal T cell dependent immune responses, suggesting that CD2 does not have any unique, essential functions which cannot be compensated for by other molecules (Killeen *et al.* 1992). However, it is unlikely that a gene would have survived in a functional form, and with such a high degree of sequence conservation between species if it was truly redundant (Marshall *et al.* 1994). It is therefore likely that the phenotypic change due to the loss of CD2 is so subtle that it is not detectable in the knockout mouse system. A role in the reversal of anergy as has been suggested is a possibility. It is also possible that a decrease in the size of the T cell repertoire, due to non-selection of lower affinity T cell clones during thymic selection, will become apparent in further studies, as has been shown for CD5 deficient mice (Tarakhovsky *et al.* 1994).

1.2.9.2 ICAM-1 and LFA-1

ICAM-1

ICAM-1 derives from a protein precursor of around 55kD in its non glycosylated form, and is processed via an intracellular precursor of 73kD, to a final molecular weight of 90-114kD. The ICAM-1 sequence has 7 potential glycosylation sites, which accounts for this heterogeneity in molecular weight seen between different cell types (Simmons *et al.* 1988). The ICAM-1 gene was cloned by two separate groups in 1988, and was identified as a member of the immunoglobulin superfamily based on similarities with other members of this

family (Yang *et al.* 1986; Simmons *et al.* 1988). It possesses 5 immunoglobulin domains of which the domain most distal from the membrane interacts with the α subunit of LFA-1 (Staunton *et al.* 1990). The majority of cell surface ICAM-1 molecules are expressed in a dimeric form, which has a greatly enhanced affinity for LFA-1 compared to the monomeric molecule (Reilly *et al.* 1995; Schulz *et al.* 1995). ICAM-1 has a wide tissue distribution, and is found on both haemopoietic and non-haemopoietic cells. It is induced by IL-1β, TNFα and IFNγ on various cell types (Dustin *et al.* 1986b; Rothlein *et al.* 1988; Simmons *et al.* 1988), and by IL-4 on dermal fibroblasts (Piela-Smith *et al.* 1992). The induction of surface expression of ICAM-1 requires *de novo* mRNA and protein synthesis (Dustin *et al.* 1986b). A single NF-κB site is essential for the ICAM-1 promoter to respond to inflammatory cytokines, lipopolysaccharide, or phorbol esters in human endothelial cells (Voraberger *et al.* 1991; Ledebur and Parks, 1995). ICAM-1 is important in many aspects of the immune response, as it is a ligand for LFA-1 (present on all leukocytes) and Mac-1 (present on neutrophils).

LFA-1

LFA-1 (CD11a/CD18) is a member of the β2 family of integrins, and consists of an α and β chain which are non covalently linked (Hynes, 1987). The ligands for LFA-1 are ICAM-1, -2 and -3 (Marlin and Springer, 1987; Staunton *et al.* 1989; De Fougerolles *et al.* 1991; De Fougerolles and Springer, 1992). T cells typically express from 10^4 to 10^5 LFA-1 molecules, but do not spontaneously adhere to cells or surfaces bearing ICAM-1. This is because LFA-1 is present in an inactive form on resting leukocytes and becomes activated following activation through the CD3 molecule, crosslinking of the TCR, or by phorbol ester stimulation. Integrin activation occurs rapidly (minutes) and is transient (30 minutes to 2 hours), providing a mechanism for adhesion and de-adhesion of leukocytes and other cells (Rothlein and Springer, 1986; Dustin and Springer, 1989). The interaction of the cytoplasmic domain of LFA-1 with cytoskeletal elements appears to be important in both the maintenance of a low avidity state, and the generation of a high avidity state (Peter and O'Toole, 1995). Clustering of integrins increases the adhesiveness of the cell to integrin ligands (Detmers *et al.* 1987). This appears to be modulated by calcium binding (van Kooyk *et al.* 1991). However, the individual LFA-1 molecules also exist in high and low affinity states, which are regulated by the availability of particular divalent cations (Dransfield *et al.* 1992; Lollo *et al.* 1993; van Kooyk *et al.* 1994). Magnesium is particularly important in the regulation of LFA-1 affinity at the single molecule level (Dransfield and Hogg, 1989; Dransfield *et al.* 1992).

Activation of cells with phorbol ester, or crosslinking of TCR/CD3 complexes, while producing equivalent increases in the binding of cells to immobilised ICAM-1, did not result in mAb 24 expression or soluble ICAM-1 binding. Adhesion by these processes was found to be increased compared to resting cells due to increased cell spreading and cytoskeletal changes, in contrast to the induction of a high avidity state of LFA-1 (Stewart *et al.* 1996). The cytoplasmic domain of the β subunit of LFA-1 is associated with the cytoskeleton, a factor which may be important in the maintenance of a high or low affinity state, as aggregates of adhesion molecules have higher affinity for their ligands (Pardi *et al.* 1992; Peter and O'Toole, 1995). However, as none of these activation stimuli are physiological stimuli, the relevance of these differences *in vivo* is not clear.

The ICAM-1/LFA-1 interaction.

The interaction between ICAM-1 and LFA-1 is important in many leukocyte functions, including T cell mediated cytotoxicity, T cell proliferation in the mixed lymphocyte response, and T cell dependent B cell activation (Davignon *et al.* 1981; Makgoba *et al.* 1988; Krensky *et al.* 1984). The expression of ICAM-1 on HLA-DR transfectants is critical for effective class II restricted and allospecific T cell activation (Altmann *et al.* 1989). It is also critical for NK cell lysis or MHC-unrestricted lysis of target cells (Zarcone *et al.* 1992; Chong *et al.* 1994). Adhesion and migration of NK cells is also reliant on the interaction of ICAM-1 with both LFA-1 and Mac-1 (Allavena *et al.* 1991; Somersalo *et al.* 1992). ICAM-1 expressed on a separate cell provides costimulatory function in the activation of naive CD4+ve T cells, demonstrating that the costimulatory signal is not required to be on the same cell, as is thought to be the case for LFA-3 costimulation (Dubey *et al.* 1995). The LFA-1 molecule has signalling function, and becomes phosphorylated upon T cell activation, but the main function is thought to be mediation of adhesion during both leukocyte migration and interactions with target cells (Haverstick and Gray, 1992; Pardi *et al.* 1992). Mutational analysis has shown that phosphorylation of LFA-1 is not required for binding to ICAM-1 (Hibbs *et al.* 1991).

Studies in patients lacking the β_2 integrin chain have shown the importance of LFA-1 in the immune response. The main feature of this condition, leukocyte adhesion deficiency is the lack of neutrophil infiltration into tissues, leading to recurrent life threatening bacterial and fungal infections. These patients do not suffer form severe viral infections, suggesting that an alternative pathway compensates for the lack of LFA-1 expression. However, *in vitro* studies have

shown that such patients have diminished allospecific CTL responses and NK cell activity, which correlated with the degree of severity of the lack of LFA-1 expression in different patients (Kohl *et al.* 1984; Krensky *et al.* 1985).

1.2.9.3 CD28 and the B7 family

Crosslinking of CD28 following a TCR signal or non-specific activation of T cells augments proliferation and cytokine secretion (June *et al.* 1990; Linsley *et al.* 1991; Sansom *et al.* 1993). Interactions between CD28 and members of the B7 family (B7.1,CD80 and B7.2,CD86) appear to be essential for the activation of resting T cells (Azuma *et al.* 1992). Both B7.1 and B7.2 are expressed on a variety of cell types, including dendritic cells, B cells, macrophages and T cells. Resting T cells which do not recive a signal through CD28 become anergic (Boussiotis *et al.* 1993). The signal transduction pathway has not been elucidated but is thought to involve phosphatidylinositol 3-kinase (Robey and Allison, 1995). The importance of the interaction has been confirmed by studies in CD28 deficient mice, in which T cell antibody dependent responses were absent, and proliferation of isolated T cells to mitogens and antigen presenting cells was also greatly reduced (Shahinian *et al.* 1993; Guinan *et al.* 1994). A second ligand for the B7 molecules, CTLA-4, is thought to play a role in negative regulation of TCR signalling (Walunas *et al.* 1994; Gribben *et al.* 1994).

Although CD28 ligation is required for the initial activation of resting cells (Sansom *et al.* 1993), and for proliferation of CD4+ve cells, it appears that this pathway is not required for cytotoxicity by effector cells (Azuma *et al.* 1993a). The fact that CD28 is expressed by most CD4+ve T cells but only 50% of CD8+ve T cells may also signify that this pathway is not important in the killing of virally infected cells (McMichael and Gotch, 1987). The CD28-ve cells are described as large and granular, express cytotoxic granules and are considered to be the most activated effector cells (Borthwick *et al.* 1994; Azuma *et al.* 1993b). During some viral infections, and during rejection episodes, the CD28-ve population is expanded, and it has been suggested that these cells represent the virus-specific memory cells (Azuma *et al.* 1993b).

The CD2/LFA-3 or the LFA-1/ICAM-1 pathway augments cytotoxicity by virus specific CD8+ve CTLs, while the CD28/B7 pathway does not (de Waal Malefyt *et al.* 1993). The CD2/LFA-3 pathway also supports IL-2 production and proliferation of CD8+ve lymphocytes in response to allogeneic endothelial cells, whereas there was no role for LFA-1/ICAM-1 or CD28/B7 in this system

(Hughes *et al.* 1990; Epperson and Pober, 1994). The importance of accessory molecules for a particular T cell clone may depend upon the affinity of the TCR for the MHC molecule, as *in vitro*, strong signals such as high concentrations of immobilised anti-CD3 antibody cannot be augmented by accessory signals (Geppert and Lipsky, 1987).

1.2.10 Chemokines

Chemokines appear to regulate the specificity of the leukocyte subsets which migrate into particular tissues. They are a group of structurally related molecules, 8-10kD in size, contain 4 cysteine residues, and possess sites for binding heparin and glycosaminoglycans. There are two major families of chemokines, termed CXC and CC based on the presence or absence of an amino acid between the two cysteine residues proximal to the amino terminus of the protein (Baggiolini *et al.* 1993) . Another chemokine, lymphotactin, exists which has only one cysteine residue, and has been placed in a separate family of C chemokines (Kelner *et al.* 1994). Sequence searches have not yet revealed any potential additions to this family. There is approximately 20-40% homology between chemokines in the three families. The members of these families are listed in Table 1.1. Chemokines are produced by most cell types, including leukocytes, platelets, eosinophils, fibroblasts, endothelial cells and smooth muscle cells, and are produced in response to a variety of stimuli, including viruses, lipopolysaccharide, interleukin-1 (IL-1), tumour necrosis factor α (TNFα) and interferons.

The molecular characterisation of cellular chemokine receptors in terms of their cellular expression and chemokine ligands has been useful in understanding the leukocyte subsets which respond to a particular chemokine. A new nomenclature for the chemokine receptors has recently been applied. Receptors for CC chemokines are designated CCR1 to CCR5, while those for CXC chemokines are designated CXCR1 to CXCR4 (Mackay, 1996). Their cellular expression and ligand binding characteristics are summarised in Table 1.2. In addition to these cellular receptors, functional chemokine receptors are also encoded by viruses, including CMV. These viruses appear to have hijacked the cellular genes, and the functional significance of this is currently a subject of speculation (Ahuja *et al.* 1994; Smith, 1996). The chemokine receptors, like classical chemoattractant receptors, are seven membrane spanning receptors which signal through G protein interactions. Activation signals such as raised cAMP and intracellular calcium levels take place following chemokine binding, and appear to be required for migration.

Table 1.1. Members of the CXC, CC, and C families of chemotactic cytokines.

CXC chemokines (α chemokines)

- Interleukin-8 (IL-8)
- Epithelial neutrophil activating protein-78 (ENA-78)
- Growth related oncogene α, β, γ (GRO α, β, γ)
- Neutrophil activating protein-2 (NAP-2)
- Platelet factor-4 (PF4)
- Interferon γ inducible protein-10 (IP-10)
- Monokine induced by interferon γ (Mig)
- Stromal cell derived factor (SDF-1)

CC chemokines (β chemokines)

- Monocyte chemotactic protein 1,2,3,4 (MCP-1,2,3,4)
- Macrophage inflammatory protein-1 α, β (MIP-1 α,β)
- Regulated on activation normal T cell expressed and secreted (RANTES)
- Eotaxin

C chemokine

- Lymphotactin

Table 1.2 Chemokine receptors, their ligands, and predominant expression on leukocytes

Chemokine receptor	Previous names	Ligands defined[a]	Cellular expression	Reference
CCR1	CC CKR 1	MIP-1α,β RANTES MCP-3	monocytes, T cells	(Gao *et al.* 1993; Neote *et al.* 1993; Combadiere *et al.* 1995b)
CCR2a,b	MCP-1Rα,β	MCP-1,3	monocytes, memory T cells, basophils	(Charo *et al.* 1994; Combadiere *et al.* 1995b; Franci *et al.* 1995)
CCR3	CC CKR-3	eotaxin MCP-3 RANTES	eosinophils, basophils	(Combadiere *et al.* 1995a)
CCR4	none	RANTES MIP-1α MCP-1	basophils, T cells	(Power *et al.* 1995)
CCR5	CC CKR5	RANTES MIP-1α,β	Monocytes, T cells	(Samson *et al.* 1996)
CXCR1	IL-8R1, α	IL-8	Neutrophils, NK cells	(Holmes *et al.* 1991; Murphy and Tiffany, 1991; Qin *et al.* 1996)
CXCR2	IL-8R2, β	IL-8 GROα NAP-2 ENA-78	Neutrophils, NK cells	(Lee *et al.* 1992; Qin *et al.* 1996)
CXCR3	none	IP-10 Mig	Activated T cells, NK cells	(Loetscher *et al.* 1996)
CXCR4	Fusin/LESTR	SDF-1	T cells	(Feng *et al.* 1996)
Duffy antigen		Promiscuous	Erythrocytes, certain endothelial cells	(Neote *et al.* 1994)
BLR-1			B cells, memory T cells	(Forster *et al.* 1994)
CMV US28		MCP-1 MIP-1α MIP-1β RANTES	CMV infected cells ?	(Gao and Murphy, 1994; Neote *et al.* 1993)

[a]The full names of these chemokines can be found on Table 1.1

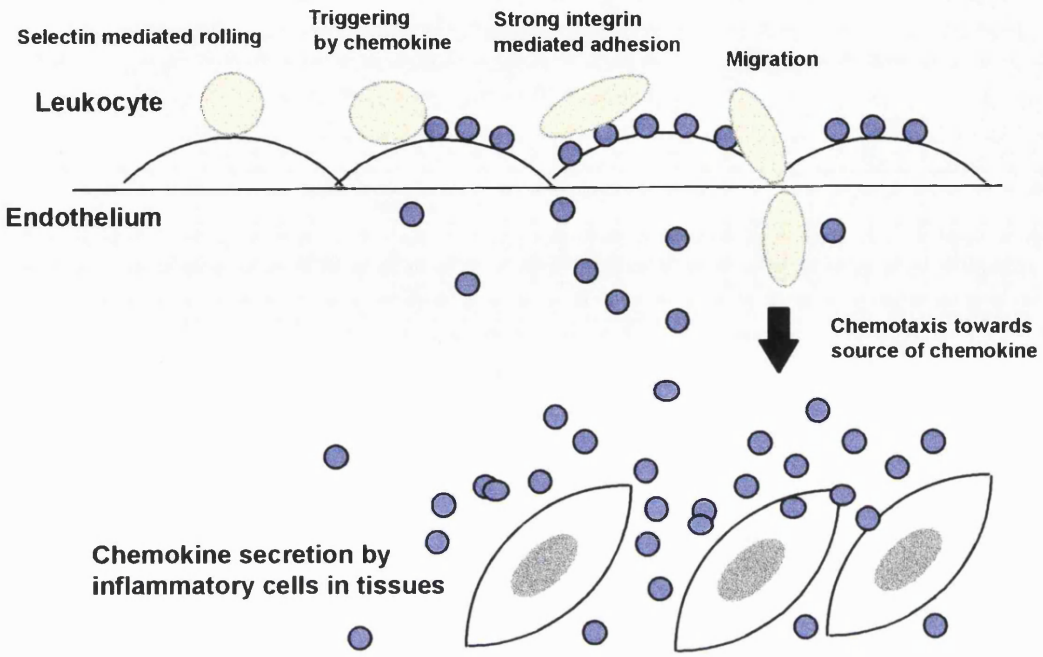


Figure 1.4. The current model for the mechanism of leukocyte migration at sites of inflammation.

The flowing leukocyte is first tethered by selectin mediated interactions, and then rolls along the endothelium. The triggering of integrins to a high affinity state leads to strong adhesion of leukocytes to the vessel wall. The stimulus which activates the integrins is provided to neutrophils by chemokines which are bound to endothelial cell proteoglycans, and this model is also thought to apply to lymphocytes and monocytes. The triggering leads to cell spreading and extravasation into the tissues. The leukocyte moves through the extravascular tissue by a haptotactic mechanism, towards a concentration gradient of chemokines produced at the site of inflammation. (Adapted from Adams and Shaw, 1994).

1.2.11 Leukocyte migration into tissues.

Migration of leukocytes into tissues takes place throughout the body and is essential for immune surveillance. Neutrophils and monocytes migrate rapidly into areas of inflammation in response to inflammatory signals, but cannot recirculate. T cells are recruited more slowly and selectively to sites of inflammation than monocytes and neutrophils, but continuously migrate and recirculate through normal tissues in their function of immune surveillance. The general model for leukocyte migration which is currently accepted applies to all these leukocyte subsets (Adams and Shaw, 1994; Springer, 1995). The 'adhesion cascade' can be divided into four sequential but overlapping steps of tethering, triggering, strong adhesion, and extravasation. A model showing the main stages of the migration process is shown in Figure 1.4

Tethering

Tethering is the initial contact of the flowing leukocyte with the vessel wall, and is mediated by selectins. The three selectin molecules, L-selectin (on leukocytes), and E, and P-selectin (on endothelial cells) interact with specific carbohydrate sequences in a transient interaction which allows the leukocyte to roll along the endothelium allowing it to search for trigger factors which are required for the next stage of adhesion to take place (Sebok *et al.* 1993).

Triggering

This is the process by which integrin molecules on the leukocytes are converted to an active state which enables them to mediate strong adhesion to the endothelium. All leukocytes express LFA-1 ($\beta_2\alpha_L$ integrin), and neutrophils and NK cells express Mac-1 ($\beta_2\alpha_M$ integrin). T cells and monocytes also express VLA-4, an β_1 integrin, which binds to VCAM-1 on the endothelium, as well as various extracellular matrix components (Hynes, 1992). The presentation of chemokines by proteoglycan molecules on the endothelium is believed to be the mechanism for the triggering of leukocyte integrins and the induction of strong adhesion to the endothelium (Rot, 1992; Tanaka *et al.* 1993b). The most convincing evidence for this comes from the neutrophil model, where the chemokine interleukin-8 (IL-8) has been shown to activate Mac-1 on neutrophils (Detmers *et al.* 1990). IL-8 and other neutrophil chemoattractants also induce neutrophil shape change and shedding of L-selectin, which is required for extravasation to take place (Huber *et al.* 1991). Chemokines which attract T cells and monocytes are also found immobilised on

endothelium, suggesting a similar role in integrin activation (Tanaka *et al.* 1993a).

Although it is currently accepted that this model applies to all leukocytes, recent data disputes the role of chemokines in the activation of LFA-1 on T cells, showing that its adhesion to ICAM-1 is not increased by chemokine stimulation, but that the adhesion of the α_4 integrin VLA-4 to fibronectin is the major effect of chemokine stimulation. The enhanced adhesion of VLA-4 to fibronectin by chemokine activated T cells argues for a major role for chemokines in the later stages of migration of T cells through tissues, rather than the triggering of strong adhesion (Campbell *et al.* 1996; Carr *et al.* 1996). Based on these findings, it has been suggested that strong adhesion of T cells to endothelium can be mediated by the interaction of VLA-4 with VCAM-1, although earlier data suggests that VCAM-1 is not involved in lymphocyte transmigration (Oppenheimer-Marks *et al.* 1991).

Strong adhesion

Strong adhesion is mediated by the binding of activated integrin molecules to their ligands on endothelium. Following cellular activation a proportion (approximately 10%) of Mac-1 (Diamond and Springer, 1993) or LFA-1 molecules (Lollo *et al.* 1993) are in an active state, and can thus bind to their ligands. The association of integrin molecules with the cytoskeleton, along with the transient nature of the interaction between LFA-1 and ICAM-1, which allows cycles of adhesion and de-adhesion to take place, is thought to facilitate leukocyte motility across ligand bearing surfaces, by the generation of a trailing edge and the constant formation of new interactions at the leading edge (Pardi *et al.* 1992; Peter and O'Toole, 1995).

Transmigration

Following strong adhesion, lymphocytes move along the endothelium and migrate through intercellular junctions. The endothelial expression of PECAM-1 (CD31), which adheres homotypically to leukocyte expressed PECAM-1, is concentrated in these areas, and is reported to be essential for leukocyte transmigration (Albelda *et al.* 1991; Muller *et al.* 1993). PECAM-1 is expressed on most leukocytes, with the exception of a population of T cells, which nevertheless migrate across endothelial monolayers, so the function of PECAM-1 in this process is not clear (Bird *et al.* 1993). Ligation of PECAM-1 activates β_1 and β_2 integrins, so it may be important in the activation of LFA-1 or VLA-4 expressed on the pseudopodia of lymphocytes which extend into

endothelial junctions (Berman *et al.* 1996). In the extravascular tissue, the interactions between immune cells and the extracellular matrix are mediated primarily by β_1 and β_2 integrins. Chemokines stimulate the adhesion of these molecules to adhesion molecules, or to fibronectin and other extracellular matrix components, and provides a mechanism for haptotactic migration of immune cells to sites of inflammation (Gilat *et al.* 1994; Lloyd *et al.* 1996). The concentration gradient of chemokines generated from the site of inflammation also maintains the subset specificity of the migrating cells.

1.3 HOST RESPONSE TO CMV

Acute CMV infection in normal immunocompetent hosts rarely results in clinically apparent disease, but is instead associated with a lifelong, persistent asymptomatic infection. In contrast, infection in immunocompromised individuals is often associated with in manifestations of CMV disease of varying degrees of severity. It is therefore clear that the immune system has an important role in the control of CMV infection. CMV disease is common in patients who are immunosuppressed 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 immunity

The physiological function of antibodies during CMV infection is not clearly defined, but a wealth of information has been provided by the murine model of CMV infection which uses murine CMV (MCMV). It has been shown in a B cell deficient mouse that the absence of antibodies does not alter the clearance kinetics, the establishment of latency, or the burden of latent viral DNA during primary MCMV infection. However, during reactivation of latent virus the presence of neutralising antibody significantly affected viral spread (Jonjic *et al.* 1994). Administration of neutralising antibodies prophylactically has been shown to have a protective function (Shanley *et al.* 1981; Farrell and Shellam, 1991).

Findings in humans suggest that humoral immunity does not prevent infection but ameliorates the severity of CMV disease. It is known that antibodies do not

protect against reinfection, as reinfection with new strains has been reported in AIDS patients (Spector *et al.* 1984) and renal transplant patients (Grundy *et al.* 1988b; Chou, 1986). However, beneficial effects of pre-existing humoral immunity are evident in many forms of CMV infection e.g. seropositive renal transplant recipients are much less likely than seronegative individuals to sustain serious CMV disease (Smiley *et al.* 1985); passive treatment with IVIG reduces the severity of CMV infection in certain subsets of allograft recipients (Snydman *et al.* 1987; Snydman *et al.* 1993); increased levels of neutralising antibodies modulate the progression of CMV retinitis in AIDS patients (Boppana *et al.* 1995); a deficiency in gH antibody in AIDS patients was associated with increased an severity of CMV retinitis (Rasmussen, 1994); a lack of CMV specific IgM antibodies was associated with more severe disease in cardiac transplant recipients (Rasmussen *et al.* 1982); maternal immunity before conception could reduce the damage caused by foetal infection, and lower the rate of maternal to foetus transmission (Fowler *et al.* 1992; Stagno *et al.* 1986).

These observations, alongside an increasing understanding of the viral antibody responses which are important for protective immunity, and of the patient groups who would benefit, have provided the basis for the design and testing of vaccines against CMV disease, which have met with some success in limiting the severity of disease (Plotkin *et al.* 1991; Adler *et al.* 1995). The envelope glycoproteins gB and gH are the most attractive potential components of a subunit vaccine, as these elicit a strong antibody response in natural infection. Animal models have provided evidence that vaccination with a single CMV envelope glycoprotein may be sufficient to confer protective immunity (Rapp *et al.* 1993; Harrison *et al.* 1995), although it is not yet known if this is the case in humans.

1.3.2 Cellular immunity

The MCMV model has also yielded important information regarding the importance of the cellular immune response, although how relevant these findings are to human CMV is not clear, as there are many differences between the two viruses. Using the severe combined immunodeficiency mouse, which has no acquired immunity due to a defect in the rearrangement of antigen specific receptors, it was shown that the mice were not able to control MCMV replication, and succumbed to disease, and that depletion of NK cells accelerated the development of lethal disease (Welsh *et al.* 1991). Mice which are depleted of NK cells by antibody treatment are more susceptible to MCMV

infection, and adoptive transfer of NK cells induced resistance to the virus in susceptible mice (Bukowski *et al.* 1983; Bukowski *et al.* 1994). The transfer of T cells from an immunocompetent mouse to a mouse which was immunosuppressed by γ irradiation conferred protection against a lethal MCMV viral challenge. Transfer of the CD8+ve subset in the absence of CD4+ve cells was sufficient for protection, whilst the CD4+ve subset did not confer protection when transferred alone (Reddehase *et al.* 1988). However, in another study, CD4+ve T cells were essential for the clearance of virus from salivary glands, a function which could not be achieved by, and did not require, CD8+ve T cells (Jonjic *et al.* 1989; Jonjic *et al.* 1990). The production of IFNγ by CD4+ve T cells was an important factor in the function of control of replication in the salivary glands (Lucin *et al.* 1992). CD4+ve cells are thus important in the control of horizontal spread of infection, as saliva is an important route of transmission of the virus.

In the BALB/c mouse, up to 50% of the MCMV specific cytotoxic T cell precursors are specific for the IE1 gene product (pp89) (Reddehase and Koszinowski, 1984; Koszinowski *et al.* 1987), and immunisation with this protein alone could protect mice from lethal virus challenge (Jonjic *et al.* 1988). A single nonameric peptide from this protein has been identified as the dominant epitope, which confers protection against lethal MCMV disease when administered as a recombinant vaccine (Del-Val *et al.* 1991).

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), are risk factors for disease (Portela *et al.* 1995). Studies evaluating the role of cell mediated immunity in humans have suggested that CD8+ve CMV-specific class I HLA restricted T cell responses are important for the successful resolution of infection in immunocompromised hosts. After allogeneic BMT the recovery of CD8+ CTL responses has correlated with improved outcome and protection from the development of disease (Quinnan *et al.* 1982). The role of virus specific CTLs was confirmed by an analysis of CTL development in bone marrow transplant (BMT) recipients. Patients who developed a CMV specific CTL response did not develop CMV interstitial pneumonia, whereas the majority of those patients with a delayed recovery of CTL responses died of the disease (Reusser *et al.* 1991). The development of an efficient CD8 response also depends upon the presence of adequate CD4 helper function (Reusser *et al.* 1991). The CMV-specific CTLs which develop in

BMT patients are specific for structural viral proteins, and are similar to those found in healthy CMV-seropositive individuals (Yakushijin *et al.* 1992). It appears that the early development of a specific CTL response is essential for limiting disease, but there is evidence to suggest that the cellular immune response may also contribute to the pathology of the disease under certain conditions. The evidence linking the development of CMV interstitial pneumonitis and allograft rejection with the cellular immune response is discussed in a later section.

After primary infection, infected individuals develop and maintain a high precursor frequency of CMV specific CTL (Schrier and Oldstone, 1986a; Borysiewicz *et al.* 1988a; Riddell *et al.* 1991). Structural viral proteins delivered to the cell in the incoming virions can be presented on class I HLA molecules and thus sensitise the cell for CTL recognition before the onset of viral protein synthesis. This pathway provides an opportunity for the immune system to attack infected cells before the onset of viral replication (McLaughlin-Taylor *et al.* 1994). 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 proteins comprise the dominant response to CMV in healthy seropositive individuals (Riddell *et al.* 1991). The most abundant constituents of the virion are pp65 and pp150. The host CTL response in normal seropositive individuals, and in allogeneic BMT recipients, is predominantly focused on these structural proteins. Both are efficiently presented in the absence of *de novo* protein synthesis, unlike other CTL targets, gB and CMV IE antigen (McLaughlin-Taylor *et al.* 1994). The envelope glycoprotein gB has a low CTL precursor frequency, and the precursor frequency for gH is even lower, suggesting a limited role for CTLs specific for viral glycoproteins in CMV infection. Cells expressing recombinant gB or gH are poor targets for polyclonal CMV-specific CTL lines (Borysiewicz *et al.* 1988b). The precursor frequency of CMV IE specific clones is also low (Gilbert *et al.* 1993).

The identification of specific CTL targets is important for the design of therapies to restore the components of the host immune response essential for protective immunity for CMV in immunocompromised patients. The use of adoptive transfer therapy is currently being investigated, in which T cells are isolated pre-transplantation from the CMV-positive MHC matched bone marrow donor, and expanded *in vitro* for use in therapy (Riddell and Greenberg, 1990). The

cells are infused into the patients at 30 to 40 days post bone marrow transplantation. The cells are only reactive against CMV infected cells, therefore the risk of graft versus host responses is reduced. Adoptive therapy has been shown to achieve protective levels of anti-CMV CTL activity in the recipients, although CMV-specific CD4 helper cells were essential for the maintenance of the response. Neither CMV viraemia nor disease occurred in any of the treated allogeneic bone marrow transplant patients (Riddell *et al.* 1992; Walter *et al.* 1995). The ability to reconstitute an effective immune response at the appropriate time is an important step forward in the management of CMV disease.

1.4 THE EFFECT OF CMV ON THE HOST IMMUNE RESPONSE

1.4.1 Class I HLA downregulation by CMV.

CMV, like several other viruses, has evolved mechanisms to evade the host immune response (Rinaldo, 1994). To evade the cellular immune response, class I HLA is downregulated on the surface of infected cells (Barnes and Grundy, 1992b). A series of studies have elucidated some of the characteristics of class I HLA synthesis and transport in CMV infected cells, and principally show that heavy chains are synthesised at normal or enhanced rates, but are rapidly broken down while in an Endo-H sensitive form ie. in a pre-golgi compartment (Yamashita *et al.* 1993; Beersma *et al.* 1993; Yamashita *et al.* 1994; Warren *et al.* 1994). A CMV gene which causes the downregulation of class I HLA when transfected into cells in the absence of other viral gene products has recently been identified (Jones *et al.* 1995). This gene, US11, is expressed as a CMV early gene, and encodes a 32kD ER resident glycoprotein which dislocates newly synthesised class I HLA proteins from the ER to the cytosol where they are degraded (Wiertz *et al.* 1996). A further region of the CMV genome, encompassing US2 to US5 has also been shown to affect class I HLA transport (Jones *et al.* 1995). This function has been attributed to the US2 gene product by the same group (Jones and Sun, 1996), and to the US3 gene product (an ER resident immediate early protein), by another group (Ahn *et al.* 1996). It is possible that a second gene in addition to US11 may be required for downregulation of class I HLA on cells already expressing high levels due to cytokine exposure (Hengel *et al.* 1995). In addition, it has recently been shown that the CMV protein pp65 from the incoming virions selectively abrogates the presentation of CMV IE proteins by class I HLA by phosphorylating the major immediate early protein, which was suggested to restrict access of the protein

to the antigen processing machinery, or divert the protein to a different degradative pathway to that resulting in class I HLA presentation (Gilbert *et al.* 1996).

Decreased cell surface expression of class I antigens in murine fibroblasts infected with MCMV also takes place (Del-Val *et al.* 1992; Campbell and Slater, 1994). This occurs by a different mechanism than that described for CMV, involving reduced synthesis, and a defect in transport to the golgi compartment leading to the accumulation of endo-H sensitive MHC molecules in the ER or *cis*-golgi. In the murine system, increased degradation of the mature molecules does not appear to occur. A MCMV early gene product, m152, which is found in a position in the MCMV genome homologous to the human US11, is involved in the downregulation, but other MCMV genes are also involved (Thale *et al.* 1995; Koszinowski *et al.* 1996).

1.4.2 Cytokine induction by CMV

Various studies have shown the upregulation of IL-1β mRNA and protein following CMV infection of monocytes (Moses and Garnett, 1990; Iwamoto *et al.* 1990). However, as production of the IL-1 receptor antagonist (IL-1ra) is also upregulated, the activity of IL-1β released into the supernatant is often decreased (Rodgers *et al.* 1985; Kapasi and Rice, 1988; Loetscher *et al.* 1996b). CMV infection has been reported to both decrease (Kapasi and Rice, 1988), or increase (The *et al.* 1995) IL-2 production by monocytes, and has also been shown to override the inhibitory effect of cyclosporin on IL-2 transcription (Geist *et al.* 1992). There is no evidence for an IL-2 antagonist, so the reason for these disparate reports is not clear. Another important inflammatory cytokine stimulated by CMV infection is TNF-α, which is upregulated in response to the CMV IE genes, both following infection and transfection of monocytes or macrophages (Smith *et al.* 1992; Geist *et al.* 1994).

Induction of mRNA for IL-6, platelet derived growth factor, oncostatin, and melanoma growth stimulating activity takes place in CMV infected fibroblasts (St.Jeor *et al.* 1993). Increased expression of IL-6 transcripts have also been detected in bronchoalveolar lavage specimens from patients with CMV pneumonitis (Devergne *et al.* 1991; Humbert *et al.* 1993). Increased production of TGF-β1 mRNA and protein have also been shown in infected fibroblasts, induced by the action of the CMV IE proteins (Michelson *et al.* 1994). CMV

infected fibroblasts also produce an endothelial cell growth factor identified as basic fibroblast growth factor (Alcami *et al.* 1991; St.Jeor *et al.* 1993). TGFβ1 stimulates CMV replication, while basic fibroblast growth factor inhibits replication (Alcami *et al.* 1993).

1.4.3 Immunosuppressive effects of CMV

CMV infection is itself immunosuppressive, inducing disturbances of both cellular and humoral immunity. In vitro evidence of this includes suppression of antigen-specific cytotoxic T cell activity and suppression of T cell and NK cell proliferation, which occurs in response to clinical isolates of CMV, but is not evident in response to laboratory strains (Schrier and Oldstone, 1986b; Schrier *et al.* 1986). Lymphocyte proliferative responses measured *in vitro* upon stimulation with recall antigens, mitogens, and allogeneic stimuli are decreased during CMV infection (Hirsch and Felsenstein, 1984; Roenhorst *et al.* 1985; Timon *et al.* 1993). Reports have shown that patients with CMV mononucleosis have elevated levels of suppressor/cytotoxic T cells (CD8+ve), as well as inverted levels of helper to suppressor T cells compared to normal individuals (Bukowski *et al.* 1983; Carney *et al.* 1983). A role for a defect in the accessory function of monocytes in CMV infected individuals has also been proposed, suggested to be due to decreased production of IL-1 by CMV infected monocytes (Carney and Hirsch, 1981). However, others have shown that infection of peripheral blood lymphocytes has a greater inhibitory effect on peripheral blood mononuclear cells (PBMC) proliferation than infection of monocytes (Kapasi and Rice, 1988), which may be due to defects in IL-2 production. The defect appears to be restricted to the T cell receptor/CD3 activation pathway, involving a signalling event prior to PKC activation, as activation signals transduced through surface costimulatory molecules such as CD28, and phorbol ester and ionomycin-induced signals, elicited normal proliferative responses (Timon *et al.* 1993; van den Berg *et al.* 1995). CMV infection was also associated with an increased incidence of apoptosis of peripheral blood lymphocytes (van den Berg *et al.* 1995), which is a common mechanism for the reduction of T lymphocyte numbers following the resolution of viral infections (Akbar *et al.* 1993). Although apoptosis is thought important for the reduction of lymphocyte numbers following resolution of viral infection, memory T cells specific for other antigens may also be primed for apoptosis, leading to anergy to recall antigens during and following infection, as has been

shown in the murine lymphochoriomeningitis (LCMV) model (Razvi and Welsh, 1993).

1.4.4 Induction of IgG Fc receptors by CMV infection.

CMV infection induces the expression of a receptor for the Fc portion of IgG, which is distinct from the cellular Fc receptors Fc γ receptors I, II and III (Sakuma *et al.* 1977; Xu-Bin *et al.* 1989; MacCormac and Grundy, 1996). In the case of murine CMV this is a viral gene product (Thale *et al.* 1994), but the origin of the CMV induced Fc receptor is not known. The Fc receptor may protect the infected cell from antibody mediated destruction by binding non-virus specific antibody and impeding the attachment of specific antiviral IgG. It may also prevent functional engagement of the Fc portion of the antibody, thus preventing its participation in antibody-dependent cell mediated cytotoxicity or complement mediated lysis. The Fc receptor may also contribute to tissue injury by contributing to immune complex deposition, an early stage in the formation of atherosclerotic plaques, with which CMV infection has been associated (Grattan *et al.* 1989).

1.4.5 CMV genes and proteins with homology to components of the host immune system.

Antigenic mimicry or crossreactivity of CMV antigens and cellular proteins may be involved in the perturbation of host immune function following CMV infection. Antibodies raised against a peptide from the CMV IE2 protein cross react with a conserved domain of HLA DR (Fujinami *et al.* 1988). Antibodies generated against CMV virion epitopes have also been shown to crossreact with the TCR/CD3 complex (Yang *et al.* 1991), and a reading frame with homologies to regions of the TCRγ chain has been identified (Beck and Barrell, 1991). The CMV genome includes a class I HLA homologue gene, although the protein has not been detected in infected cells (Beck and Barrell, 1988)

CMV encodes 3 proteins which are homologues of G protein-coupled receptors (Chee *et al.* 1990b; Welch *et al.* 1991). All three genes are transcribed in the late phase of infection, but expression of the proteins on infected cells has not been shown. One of these proteins, the US28 gene product, has a high degree of homology with cellular β chemokine receptors (>30%), and has 56% homology with the amino terminal segment of the cellular chemokine receptor

CCCR1 (Neote *et al.* 1993; Gao and Murphy, 1994). Expression of the protein by transfection into human kidney cells showed that it functions as a high affinity β chemokine receptor with more promiscuous binding characteristics than other known chemokine receptors at the time. US28 was also shown to transduce signals in response to chemokine binding in transfected cell lines (Neote *et al.* 1993; Gao and Murphy, 1994). The expression of a chemokine receptor may be a method of immune evasion, but its function has not been determined.

1.5 PATHOLOGY ASSOCIATED WITH CMV INFECTION

The pathology of CMV disease can be caused either by direct viral damage of infected cells or by indirect tissue damage caused by the host immune system, or by both mechanisms. Direct viral damage 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 transplant recipients. The production of inflammatory cytokines such as TNF-α, IL-2 and IFNγ, and the action of T lymphocytes, are thought to be central to this type of pathology.

1.5.1 CMV disease in HIV infected individuals

CMV retinitis is the most common sight-threatening infection of 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, with disease due to cellular infiltration a rare occurrence. 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; Rene *et al.* 1989). The most frequent primary lesion is ulceration, possibly due to vasculitis resulting from the presence of infected endothelial cells in the walls of small vessels (Tatum *et al.* 1989). In the absence of such lesions, inclusions of CMV accompanied by a non-specific inflammatory response are present in affected tissues (Webster *et al.* 1989). CMV pneumonitis is much less common in AIDS than in other immunocompromised patients, although CMV is recovered from 30 to 50% of

cultures of bronchoalveolar lavage from AIDS patients. CMV in the lung is frequently associated with the presence of other organisms, such as Pneumocystis carinii, but survival rates in patients with Pneumocystis carinii pneumonia are not affected by the presence or absence of CMV (Bower *et al.* 1990; Millar *et al.* 1990). Findings from other large clinical studies have similarly not supported the importance of CMV as a cause of pneumonia in AIDS patients (Jacobson *et al.* 1991; 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.* 1987a).

1.5.1.1 CMV as a cofactor for HIV

It is not clear whether the association between rapid progression of HIV disease and CMV infection is due to severely compromised cellular immune function allowing the development of invasive CMV disease, or whether CMV is a cofactor in the pathogenesis of AIDS (Webster *et al.* 1989; Webster *et al.* 1992). Many mechanisms have been proposed for the apparent positive effect of CMV infection on HIV replication which takes place *in vitro* (Davis *et al.* 1987; Lathey *et al.* 1994). Direct interaction of the CMV IE proteins with regulatory elements of the HIV long terminal repeat was shown to increase HIV transcription (Barry *et al.* 1990a; Barry *et al.* 1990b). The induction of TNFα by CMV infection was also shown to induce HIV replication (Peterson *et al.* 1992). It has recently been proposed that CMV infection results in the expression of a superantigen which activates a subset of T cells expressing specific Vβ genes, which form a reservoir for HIV throughout the course of the disease (Dobrescu *et al.* 1995). A chemokine receptor, CCCR 5, has recently been identified as a co-receptor for HIV (Deng *et al.* 1996; Drajic *et al.* 1996). β chemokines have also been identified as having a suppressive effect on the replication of HIV (Cocchi *et al.* 1995; Paxton *et al.* 1996). It is thus possible that the expression of the CMV-encoded β chemokine receptor may affect HIV replication by the removal of HIV suppressive β chemokines.

1.5.2 CMV infection in allograft recipients

CMV disease in organ transplant recipients can arise 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 is reactivated from latency. Symptomatic CMV disease more commonly results from primary infections and reinfection than from reactivation, and is more severe (Smiley *et al.* 1985; Grundy *et al.* 1987b; Grundy *et al.* 1986; Chou, 1989). 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). Pre-existing immunity is important in limiting the severity of CMV disease, although the effect of this diminishes with increased immunosuppression (Smiley *et al.* 1985). However, other donor factors are also important in the risk of transmission, as the transplantation of cadaver kidneys from the same donor to two different recipients have shown that either both recipients, or neither, contracted CMV disease (Chou and Norman, 1988). The use of more powerful immunosuppressants, such as anti-lymphocyte immunoglobulins, either as induction therapy or as treatment for rejection, increases the risk of symptomatic CMV infection and the severity of disease in renal transplant recipients, particularly in seropositive individuals (Oh *et al.* 1988; Portela *et al.* 1995). CMV preferentially attacks the transplanted organ, particularly in liver, heart, or lung transplantation (Dummer *et al.* 1986; Gonwa *et al.* 1989; Stratta *et al.* 1989), suggesting that either allografts are particularly vulnerable to infection, or that there is local reactivation of CMV in the latently infected allograft. CMV infection at sites other than the transplanted organ are rare in liver and lung transplant patients. This may suggest that the host immune response can control infection in tissues other than the transplanted organ, but not within the transplanted organ, due to reduced CTL recognition of the infected cells because of HLA mismatches (Smyth *et al.* 1990; Arnold *et al.* 1992). This is supported by the observations from a rat CMV model of allogeneic lung transplantation, where virus titres were increased in animals receiving an allogeneic lung in comparison to those animals which received a syngeneic lung, suggesting that viral infection in the allogeneic lung had escaped the self-MHC restricted immunological control (Steinhoff *et al.* 1996).

CMV is proposed to be a cofactor in the development of allograft rejection, although the existence of an association between the two is controversial. The most convincing data comes from studies in cardiac transplantation. 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, independently of other risk factors (Grattan *et al.* 1989;

McDonald *et al.* 1989; Loebe *et al.* 1990). CMV infection has been shown to be a risk factor for the vanishing bile duct syndrome, a type of chronic rejection which occurs in 5% of recipients of liver allografts. While persistent CMV infection in the hepatocytes was associated with the vanishing bile duct syndrome, acute systemic CMV infection which was cleared by the host immune system was not (O'Grady *et al.* 1988; Arnold *et al.* 1992). However, other groups have not found associations between CMV infection and the vanishing bile duct syndrome (Paya *et al.* 1992). CMV is associated with decreased survival of heart-lung transplant recipients (Duncan *et al.* 1991). Obliterative bronchiolitis is the most significant long term complication following pulmonary transplantation, and is thought to be a form of chronic rejection. CMV seropositivity was found to be a risk factor for the development of this condition post transplantation, and CMV seropositive patients developed the condition earlier post transplantation than CMV seronegative patients (Keenan *et al.* 1991; Sharples *et al.* 1996). Finally, CMV infection is associated with a poor outcome in renal transplantation (Rubin *et al.* 1985). CMV infection in renal transplant patients is associated with a histologically distinct glomerulopathy, involving deposits of immunoglobulin, increased presence of CD8+ve T cells and activated macrophages, in the glomeruli, and increased expression of class I HLA on the glomeruli (Herrera *et al.* 1986; Richardson *et al.* 1981). Other investigators have confirmed the presence of this distinct lesion, but were unable to associate its presence with CMV infection (Herrera *et al.* 1986; Boyce *et al.* 1988). The underlying mechanisms by which CMV is associated with allograft rejection are not clear, but virus-induced inflammation of the endothelium and vascular wall, mediated by increased expression of MHC antigens and adhesion molecules might be contributory factors.

1.5.3 Pathology of CMV interstitial pneumonitis

CMV is the most common infectious cause of death following allogeneic bone marrow transplant (BMT), due primarily to CMV pneumonitis (Meyers *et al.* 1986). In contrast, autologous or syngeneic bone marrow transplantation is associated with a very low incidence of pneumonitis (Appelbaum *et al.* 1982; Wingard *et al.* 1988a; Ljungman *et al.* 1994). Patients who are seropositive pretransplantation have a higher incidence of CMV infection and disease than those who are seronegative, due to a high incidence of reactivation of latent infection (Meyers *et al.* 1986; Wingard *et al.* 1988b). Other factors influencing the development of CMV pneumonitis include the occurrence and severity of graft-versus-host disease, and the use of anti-thymocyte globulin for treatment

of graft versus host disease (GVHD)(Meyers *et al.* 1986; Miller *et al.* 1986). The histopathological characteristics of CMV pneumonitis include thickening of the alveolar membranes, with a mononuclear cell perivascular infiltrate (Beschorner *et al.* 1980)

The incidence of CMV pneumonitis has declined in recent years, possibly due to better control of GVHD (possibly by CMV IVIG), or to T-cell depletion of the donor marrow (Wingard *et al.* 1988b; Sullivan, 1996; Guglielmo *et al.* 1994). Although the mechanism by which CMV IVIG reduces disease is not clear, such treatment is known to reduce graft-versus-host disease (Sullivan, 1996; Guglielmo *et al.* 1994). The use of seronegative marrow and blood products has greatly decreased the incidence of the disease in seronegative recipients (Bowden *et al.* 1991; Sayers *et al.* 1992). CMV pneumonitis in allogeneic BMT recipients was historically associated with very high mortality rates, of 80 to 90%,(Shepp *et al.* 1985), which were reduced in the short term with the combination of ganciclovir therapy and CMV IVIG (Schmidt *et al.* 1988; Reed *et al.* 1988; Emanuel *et al.* 1988). However, more recent data has indicated that the mortality of patients surviving an episode of CMV pneumonitis is compromised. Mortality rates of 60 to 70% at 12 months post transplantation have been reported from several centres in the USA and in Europe (Ljungman *et al.* 1992; Emanuel, 1993). CMV pneumonitis thus remains a major determinant of the outcome of allogeneic BMT.

1.5.4 The contribution of immune mediated pathology in CMV pneumonitis

Several clinical observations have lead 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.* 1992). CMV can be detected in bronchoalveolar lavage fluids at day 35 post bone marrow transplantation, but the peak of onset of pneumonitis occurs between 70 and 120 days post transplantation, suggesting that a further stimulus is required to initiate disease (Wingard *et al.* 1988b; Schmidt *et al.* 1991).The initial trials of ganciclovir treatment showed no improvement in survival rate above untreated patients, despite reduction of viral titres in the lungs (Shepp *et al.* 1985).

The association of CMV pneumonitis with allogeneic, but not autologous, bone marrow transplantation, suggests that the allogenicity of the graft is an important factor (Wingard *et al.* 1988a). The occurrence of graft-versus-host disease is a risk factor for CMV pneumonitis, further suggesting that the immune response may be involved (Miller *et al.* 1986). The low incidence of CMV pneumonitis in AIDS patients has been discussed in a preceding section, and is thought to be due to the absence of an alloreactive immune response in these patients.

These finding have provided two important pieces of information about the pathology of CMV pneumonitis, firstly, that the severity of disease is not related to the degree of viral replication, and secondly that the disease only manifests itself in the presence of the appropriate immune response. Alongside these clinical findings, data from the MCMV model have lead to the hypothesis that the pathology of CMV pneumonitis appears to be mediated in part by components of the host immune response. Experiments carried out in the MCMV model have been instrumental to our understanding of the pathology of this disease.

CMV pneumonitis has been shown to develop only in mice both infected with MCMV and given a graft-versus-host challenge, although there were no differences in the virus titres (Grundy *et al.* 1985). The pneumonitis was characterised by an increase in lung weight, inflammatory changes in the interstitial and perivascular structures, and an increase in lung T lymphocytes of donor origin. Treatment of these mice with ganciclovir reduced virus replication in the lung to undetectable levels, but did not reduce the histological characteristics of pneumonitis (Shanley *et al.* 1987). Depletion of T cells using antibodies protected against the development of CMV pneumonitis, while reconstitution with syngeneic cells resulted in pneumonitis (Erlich *et al.* 1989). Infection of T cell deficient mice resulted in viral replication in the absence of pneumonitis until late stages of the disease (Grundy *et al.* 1987a). These findings support the hypothesis that the infiltration of T cells contributes to the pathology of CMV disease (Grundy *et al.* 1987a).

In mice with persistent MCMV infection of the salivary gland, but in the absence of replication in other organs, activation of T cells using an anti-CD3 antibody results in death from MCMV pneumonitis, while CD3 activation had no such effect on mice without MCMV infection. The serum levels of TNFα and IFNγ were elevated in the MCMV infected mice following T cell activation. It was

suggested that CMV infection resulted in the presence of primed T cells which produced high levels of cytokines upon activation (Tanaka *et al.* 1994). This may relate to the relationship between graft-versus-host disease and the onset of interstitial pneumonitis in humans, as the graft-versus-host disease may provide the T cell activation stimuli required to trigger disease.

The balance between the T cell response as an essential component of the host immune response to limit disease, and as an immunopathological component of the disease, is likely to be an important factor in the outcome of CMV infection in BMT patients.

1.6 AIMS OF THE THESIS

The outcome of CMV infection appears to be determined by the cellular immune response, which prevents the development of disease in normal individuals, but which may be responsible for some of the pathology associated with CMV disease in the immunocompromised populations at risk. The control of interactions between the host immune system and cells in infected tissues may thus be an important factor in the development of CMV-induced pathology. It has been shown that the production of specific chemokines and adhesion molecules determines the migration patterns of leukocytes from the blood into tissues, and that the expression of MHC molecules and adhesion molecules on target cells determines the outcome of the interaction between leukocytes and target cells. In the present study the changes which take place in the expression of class I HLA, adhesion molecules, and chemokine production following CMV infection of fibroblasts have been investigated. The functional effects of such changes on leukocyte recruitment, adhesion and activation have also been addressed.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2. Materials and Methods

2.1 CELL CULTURE

2.1.1 Media

Minimal Essential Medium (MEM)

Minimal essential medium with Earle's salts and containing 2mM L-glutamine was supplemented with penicillin at 100U/ml and streptomycin at 100mg/ml (Gibco, Paisley, UK). For propagation of fibroblasts, the medium was supplemented with 10% foetal calf serum (FCS) (MEM/10% FCS). For maintenance of cells following virus infection, MEM was supplemented with 4% FCS (MEM/4%FCS).

RPMI 1640

For experiments involving T cells, RPMI 1640 containing 2mM glutamine (Gibco) and penicillin and streptomycin as described above, was supplemented with 10% FCS (RPMI/10% FCS).

Foetal Calf Serum

Two batches of FCS were used for all experiments (Advanced Protein Products). The batches used were tested by the Department of Virology, Royal Free Hospital School of Medicine (RFHSM), for suitability for growth and maintenance of fibroblasts. FCS was also tested for the ability to support lymphocyte activation and the absence of endogenous mitogens in tritiated thymidine incorporation assays. The FCS was heat-inactivated by incubation at 56°C for 1 hour, and stored in aliquots at -20°C until use.

2.1.2 Isolation and propagation of Human Embryonic Lung (HEL) fibroblasts.

HEL fibroblasts were isolated from the lungs of 12-17 week gestation foetuses which were obtained from the MRC Tissue Bank, Hammersmith Hospital, London. The tissue was cut into small pieces using a scalpel, and digested with trypsin(0.05%) /EDTA(0.01%) (Gibco) for 15 minutes at 37°C. The tissue was then disaggregated using a pasteur pipette, washed in phosphate buffered saline (PBS) by centrifugation at 500 x g for 5 minutes, resuspended in MEM supplemented with 20% FCS, and transferred to a 75cm^2 flask. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. To passage

cells, monolayers were removed from the flasks by trypsinisation for 5 mins at 37°C, and split into further flasks at a ratio of 1:2 or 1:3. All experiments were conducted using fibroblasts between passages 5 and 15.

2.1.3 Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment (0.1% in PBS, Sigma, Poole,U.K.) and grown to confluence in fibronectin coated (2μg/cm^2, Sigma) tissue culture flasks. HUVEC growth medium consisted of Iscove's Modified Dulbecco's Medium (Gibco) containing with 20% FCS, endothelial cell growth factor (25μg/ml, Sigma) and heparin (25 U/ml; Multiparin, Wrexham, UK). The cells were passaged using trypsin/EDTA (Gibco) and were used at passages 1-4. Culture of endothelial cells was carried out by Dr. K. Yong, Dept. of Haematology, RFHSM.

2.1.4 Mycoplasma testing

The Genprobe mycoplasma detection kit (Eurogenetics, Twickenham, U.K.), based on the detection of mycoplasma RNA in cell supernatants, was used to routinely test cell lines and virus stocks for mycoplasma contamination. The instructions supplied with the kit were followed. Briefly, cells to be tested were passaged twice in antibiotic-free medium, and a 2ml aliquot of supernatant was removed at least 3 days after the second passage. Cell debris was removed by centrifugation at 500 x g for 5 minutes. The supernatant was then incubated with a radiolabelled probe specific for mycoplasma RNA for 18 hours at 42°C. The sample was then incubated at 42°C with the separation suspension provided, followed by 3 washes in the wash solution provided. The resulting pellet was resuspended in scintillation fluid, and the amount of radioactivity in each sample was determined by scintillation counting. The percentage of bound probe was calculated in relation to the amount of probe added, with values of over 2.4% scored as positive. Virus stocks were tested in the same way.

2.2 VIRAL INOCULUM

2.2.1 Preparation of virus inoculum.

Human CMV strain AD169 (American Type Culture Collection, Rockville, Maryland, USA) was harvested from the culture medium of infected fibroblasts grown in MEM /4% FCS, clarified by centrifugation at 500 x g for 10 min, and stored at -80°C. Stocks of virus inoculum were screened for mycoplasma using the RNA kit as described above, and were all found to be negative.

2.2.2 Plaque assay of virus stocks.

Solutions used:

Methyl cellulose overlay: A 2% methyl cellulose solution was made by autoclaving 10g methyl cellulose (Sigma) and 500ml distilled water in separate bottles. These were then combined while still hot, and left stirring to allow the powder to dissolve. The methyl cellulose overlay was made by combining 10ml 10x MEM, 30ml L-15 medium, 5 ml FCS, 3ml 7.5% sodium bicarbonate, 1 ml antibiotic/antimycotic solution, and 0.5 ml L-glutamine. This was combined with 50 ml 2% methyl cellulose and mixed well by inversion several times. All tissue culture medium components except FCS were supplied by Gibco.

Formal saline: 50 ml formalin, 4g sodium chloride and 450 ml distilled water were combined at room temperature.

Procedure

HEL fibroblasts were seeded into 48 well plates (Falcon, Marathon Laboratory Products, London, UK) at $5x10^4$ cells/well. The virus stocks to be tested were diluted serially in half log dilutions in MEM + 2% FCS and 100µl/well was inoculated onto the monolayers when they were subconfluent. All experiments were carried out in triplicate or quadruplicate. After 1 hour, the monolayers were washed and overlaid with 0.5 ml of methyl cellulose overlay. Plates were incubated at 37°C in a humidified incubator for 10-14 days. The fibroblasts were then fixed with formal saline for 30 minutes at room temperature, washed thoroughly in distilled water, and stained with 0.03% methylene blue for 1 hour at room temperature. The plates were then washed in water and allowed to dry. Plaques appeared as dark blue areas, and were counted under a light microscope. The number of plaque forming units per ml (pfu/ml) was calculated

by counting the number of plaques in wells containing approximately 50 plaques, and applying the equation:

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

2.2.3 Preparation of virus free supernatants.

To remove the minimal amount of virus which was present in supernatant harvested from infected cells at 2 and 3 days post infection, or from viral inoculum, supernatant or inoculum was ultracentrifuged at 100,000 x g for 1 hour in a Beckman TLA-100 tabletop ultracentrifuge, using a TLA-100.3 fixed angle rotor. The supernatant was then transferred to a fresh tube and ultracentrifuged for a further hour. The resulting solutions were found to be free of infectious virus by the lack of CMV IE antigen expression when inoculated onto fibroblast monolayers (see below).

2.2.4 UV inactivation of viral inoculum

Tissue culture plates containing CMV inoculum were treated with UV light at 254nm for 10 minutes using a hand held UV light (Genetic Research Instrumentation, Essex, UK). The process was carried out at 4°C to prevent heating of the inoculum. The resulting inoculum was found to be free of infectious virus by the lack of CMV IE antigen expression when inoculated onto fibroblast monolayers (see below).

2.3 FLOW CYTOMETRY

2.3.1 Double and triple staining of lymphocytes for flow cytometric analysis.

Aliquots of $2x10^5$ cells were stained using the microplate technique (Janossy and Amlot, 1987). Antibody incubations carried out in PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (PBSA) at room temperature for 15-30 minutes, followed by 5 washes in PBSA. Directly conjugated primary antibodies were added at their saturating concentrations, as determined by prior titration. The antibodies used for the staining of

lymphocytes are shown in Table 2.2. In the case of biotin-conjugated antibodies the second layer was a streptavidin-tricolour conjugate (Caltag, San Francisco, USA). Unconjugated primary antibodies were detected using the isotype specific secondary antibodies detailed in Table 2.3. Stained cells were fixed in 1% paraformaldehyde (To prepare 8% paraformaldehyde, 8g paraformaldehyde was added to 100 ml PBS and heated to 70°C in a fume hood for 2 hours. The solution was filtered (0.22μm) and stored at 4°C. This was diluted to 1% strength in PBS before use). Directly conjugated purified mouse immunoglobins of the appropriate isotype were used at identical concentrations to the primary antibodies as negative controls (Sigma).

2.3.2 Flow cytometric analysis of lymphocyte phenotypes.

For each sample, 5000 to 20,000 events were acquired using the Becton Dickinson FACSCAN, and analysed using LYSIS II software. Computer graphics shown in this thesis were generated using WinMDI software. A gate was placed around the lymphocyte population based on forward and side light scatter characteristics prior to analysis. In experiments involving mixtures of fibroblasts and lymphocytes, the T and NK cell population was further identified by expression of the CD2 marker. Most of the data presented in this thesis refers to the percentage of lymphocytes positive for a particular surface antigen. Positive and negative populations for each surface antigen were calculated by the placing of a marker at the point considered to be positive, which was determined by the maximum signal from cells stained with an irrelevant antibody.

2.3.3 Flow cytometric analysis of fibroblast and endothelial cell surface markers.

Aliquots of $2x10^5$ cells were stained by indirect immunofluorescence using the microplate technique. All staining was carried out in 96 well round bottomed plates (Greiner, Stonehouse, Gloucestershire, UK) which had been siliconized using Sigmacote (Sigma), to prevent cells sticking to the plate. All incubations were carried out in PBSA at 4°C for 1 hour, in a 50μl volume, followed by 5 washes in 200μl PBSA. Cells were first blocked with a Fc fragment of human IgG at 10μg/ml (Calbiochem, Nottingham, UK), in order to prevent possible antibody binding to the CMV induced Fc receptor. Primary antibodies were then added at their saturating concentrations, as determined by prior titration,

followed by the secondary antibodies at their optimum concentration, and fixation in 2% formaldehyde. Purified mouse immunoglobins of specific isotypes were used as controls, at identical concentrations to the primary antibodies (Sigma). The primary and secondary antibodies used are detailed in Tables 2.2 and 2.3. 5,000 to 20,000 events were collected using a logarithmic amplifier and analysed using LYSIS II software. The data was converted from logarithmic values (Mean fluorescence intensity, MFI) to channel values using LYSIS II software, and subsequently to fluorescence intensity units (FIU) for presentation (see below).

2.3.4 Conversion of flow cytometry data to fluorescence intensity units (FIU).

At a resolution of 1024 channels, using 4 log decades, an increase of 256 channels (1024/4) theoretically represents a 10 fold increase in brightness, but in practice this value varies between different machines. Electronic test pulses were used to determine the shift in channel values generated by a 10-fold increase in signal brightness. Briefly, this was achieved by recording the mean fluorescence intensity (MFI) of a given test signal, amplifying the signal by a factor of 10 using the manually controlled amplifier on the machine, and recording the MFI of the amplified signal. This provided a measure of the increase in MFI which represented a 10 fold increase in brightness of the signal, which was then converted to channel values. For our machine, using the FL1 detector at 1024 channels resolution, this value was found to be 235 channels using the above method. The machine could also be calibrated using fluorescent beads of known 'brightness', but the disadvantages of fading of fluorescence over a period of time, and discrepancies between different batches of beads led to our decision to use electronic test signals.

For each sample, the data was converted to channel values by the LYSIS II software. Background fluorescence of identical cells stained with an irrelevant antibody were subtracted from the values for the stained samples. The channel values were then converted to fluorescence intensity units (FIU) using the relationship $FIU=10^{(x/235)}$, where x is the change in median channel value of the sample from that of the control cells (Watson, 1992). In this study, control cells were uninfected or unstimulated cells stained for the molecule under investigation, and FIU values thus represent fold increases or decreases from

this value. Statistical analysis was performed on these values using the two-tailed Student's t-test.

An example of the method used to calculate FIU for each sample is shown:

	Median channel value (class I HLA expression)	**Median channel value- irrelevant control value**
Interferon treated cells	700	600
Uninfected cells	522	422
Change in channels (x)		178
FIU (calculated from $10^{(x/235)}$)		**5.72 fold increase**

Examination of stained cells by fluorescence microscopy.

In order to check the staining pattern of cells analysed by flow cytometry, aliquots of cells were applied to a poly-L-lysine (Sigma) coated glass microscope slides. Slides were soaked in poly-L-lysine for 1 minute, washed, and allowed to dry in air. Stained cells were applied to the slides and allowed to dry, before mounting in Citifluor® (Citifluor, Canterbury, UK) (PBS-glycerol with *p*-phenylenediamine to retard fading). Fluorescein labelled cells were examined under an Olympus BH2 fluorescence microscope.

2.4 DETECTION OF CMV ANTIGENS BY IMMUNOFLUORESCENCE

To confirm that the viral inoculum used resulted in 100% infection of fibroblast monolayers, or to confirm the absence of virus from virus free inoculum or supernatant, staining for CMV antigens was carried out, either by immunofluorescence or flow cytometry. The antibodies used are shown in Table 2.1.

2.4.1 Determination of percentage of infection by flow cytometry.

To determine the percentage of infection in a particular experiment, the cells were removed from the plate at 24 hours post infection by trypsinisation, and an aliquot of $2x10^5$ cells was stained for CMV immediate early antigen (CMV IE) using the monoclonal antibody E13. Trypsinised cells were washed in PBS, and fixed at 4°C for 15 minutes in 66% acetone/34% PBS. The cells were then

washed in PBS and a final concentration of 10μg/ml of fluorescein conjugated monoclonal antibody specific for CMV IE diluted in PBS/1% BSA was added, and incubated for 1 hour at 37°C. The cells were then washed twice in PBS/1% BSA. For flow cytometric analysis 5000 or 10,000 events were collected on a Becton Dickinson FACSCAN. After the exclusion of cell debris and dead cells by application of a viable gate, two distinct peaks, representing cells positive and negative for CMV IE antigen were seen. The percentage of infected cells in the gated population was determined by placing markers around each of the peaks using LYSIS II software.

2.4.2 Determination of the percentage of infection by immunofluorescence.

Preparation of slides

Fibroblasts for immunofluorescent staining were either grown in 8 well slide chambers (Gibco), applied to poly-L lysine coated glass slides from cell suspensions, or cytospin preparations were prepared from cell suspensions. Slides were coated with poly-L-lysine (Sigma) by treatment for 10 minutes at room temperature, to provide an adherent surface to which suspensions of cells could be directly applied. Cytospin preparations were prepared using the Shandon cytocentrifuge. Cells were resuspended in PBS at 1 x 10^6/ml, placed in cytocentrifuge buckets, and centrifuged onto glass slides at 500 x g for 2 minutes.

Acetone fixation and staining

The slides were fixed in ice-cold acetone for 20 minutes at 4°C, and washed in PBS for 5 minutes before the addition of antibody. Primary and secondary antibody incubations were carried out in a dark humidified chamber at 37°C for 1 hour, followed by washing for 3 x 5 minutes in a bath of PBS between each stage of staining. The Fc fragment of human IgG was used at 10μg/ml for 1 hour at 37°C to block possible reactivity of antibodies with the CMV induced IgG Fc receptor. Murine monoclonal antibodies were used at their optimum concentrations, as determined by prior titration. These were detected with a FITC-labelled $F(ab)_2$ fragment of goat IgG specific for mouse IgG (Sigma). The staining was examined and photographed using the Olympus BH2 fluorescence microscope, or the Biorad confocal scanning microscope. Assistance in the use of this microscope was provided by Luci MacCormac, Department of Clinical Immunology, RFHSM. This method was used for detection of CMV IE antigens

or the CMV late antigen gB. The monoclonal antibodies used are listed in Table 2.1.

2.5 RECOMBINANT CYTOKINES AND NEUTRALISING ANTIBODIES

IL-8, IL-6 and MCP-1 were purchased from R&D Systems (Abingdon, UK). TNFα and IL-1β were purchased from Sigma. IFNβ was purchased from Serotec (Kidlington, Oxford, UK). The details of neutralising antibodies specific for cytokines are shown in Table 2.4. The neutralising capacity of these antibodies was tested against recombinant cytokines and is shown in the table.

2.6 CYTOKINE ELISA ASSAYS

2.6.1 Preparation of samples

Supernatant was removed from fibroblasts at a range of time points, and stored at -80°C until all samples had been collected, when the cytokine assays were performed. Separate wells (in duplicate or triplicate) were used for each time point, thus the results presented represent cumulative amounts of cytokine produced between the starting time and the time point indicated.

2.6.2 Sources and procedures of ELISA assays

The assays used for the detection of IL-6, IL-8, MIP-1α, MCP-1, RANTES, IL-4, TNFα, IL-1β, G-CSF and GM-CSF were purchased from R&D Systems. The assays were carried out following the instructions provided with the kits. A plate was provided coated with the appropriate monoclonal antibody. Samples or standard was added to each well and incubated at room temperature for 2 hours. Each well was then washed 3 times using a semi-automatic plate washer. Horseradish peroxidase (HRP) linked secondary antibody was then added to each well and incubated for 2 hours at room temperature, followed by 3 washes. Tetramethylbenzidine (TMB) and hydrogen peroxide were mixed together immediately before use and added to each well. After 30 minutes the reaction was stopped using sulphuric acid.

IFNβ and IFNγ immunoassay kits were purchased from Medginex (Fleuris, Belgium). Assays were carried out according to the manufacturer's instructions.

Briefly, supernatants were added to microplates precoated with affinity purified goat anti-human IFNβ or IFNγ. Samples or standards were added to the wells together with HRP labelled monoclonal antibody to human IFNβ or IFNγ. The plate was incubated at room temperature for 2 hours on a microplate shaker. The plate was then washed and colour was developed by addition of TMB solution. The reaction was stopped using sulphuric acid. As IFNβ is particularly easily denatured by heat or physical forces, care was taken in the storage and handling of the standards and specimens. Supernatants for testing were stored at +4°C for no longer than 2 days prior to the assays.

For all assays the optical density at 450nm was determined using a microplate reader (ICN Flow, Thame, Oxfordshire), with a reference wavelength of 595nm. The concentrations of cytokines present in the samples were calculated using Soft 2000 analysis software (ICN Flow), which linearized the data from the standard curve by plotting a log/log graph, and determined the cytokine concentrations in each sample from this curve.

2.7 USE OF ANTIVIRAL AGENTS

2.7.1 Antiviral agents

Ganciclovir (Cymvene, Syntex) and foscarnet (Foscavir, Astra) were obtained from the pharmacy of the Royal Free Hospital. Ganciclovir was supplied as a lyophilised powder which was reconstituted in saline to a concentration of 50 mg/ml, and was stored at room temperature for up to 1 month. Foscarnet was supplied as a solution at 24mg/ml, and was stored at room temperature until the expiry date printed on the package. Working dilutions of the above (in MEM/4% FCS) were prepared immediately prior to use. The concentrations used in this study were determined by the following experiments:

2.7.2 Titration of ganciclovir and foscarnet.

The concentration of each drug required to block late protein synthesis was assessed by immunofluorescent staining of the treated infected cells using a monoclonal antibody specific for the CMV late protein, gB, at three days post infection. At this stage of infection, CMV late proteins are present only at low levels in some cells, and cannot yet be detected in others. This time was chosen as it coincides with the peak of adhesion molecule expression following

CMV infection. It can be seen from Figure 2.1 that concentrations of both ganciclovir and foscarnet from 1-100μg/ml substantially reduced glycoprotein B expression in the culture, and detectable expression was totally inhibited by ganciclovir at 10μg/ml, and foscarnet at 100μg/ml.

For experiments requiring ganciclovir treatment of cells for periods of up to 7 days, it was necessary to determine whether a single dose of ganciclovir would be effective over this time period. Prevention of gB synthesis was used as a measure of ganciclovir efficiency, alongside a measurement of production of infectious virus (see below). Infected cells were treated with 50μg/ml of ganciclovir immediately following infection, and stained for both CMV IE antigen and gB expression at 7 days post infection. As can be seen from Figure 2.2 all infected cells were expressing CMV IE antigen, either in the presence or absence of ganciclovir. In contrast ganciclovir treatment of infected cells completely abrogated gB expression at 7 days post infection.

As a second measure of the efficacy of one initial treatment with ganciclovir, a plaque assay was used to compare the amount of infectious virus produced by ganciclovir-treated cells compared to untreated infected cells. Supernatant was harvested from infected and ganciclovir-treated cells at 2, 4, 6, and 9 days post infection, and the amount of infectious virus was determined as described in section 2.7. The production of infectious virus over a nine day period was almost totally abrogated by one initial treatment with ganciclovir at 50μg/ml (Figure 2.3), and this concentration was subsequently used for experiments involving treatment of the cells for up to 7 days.

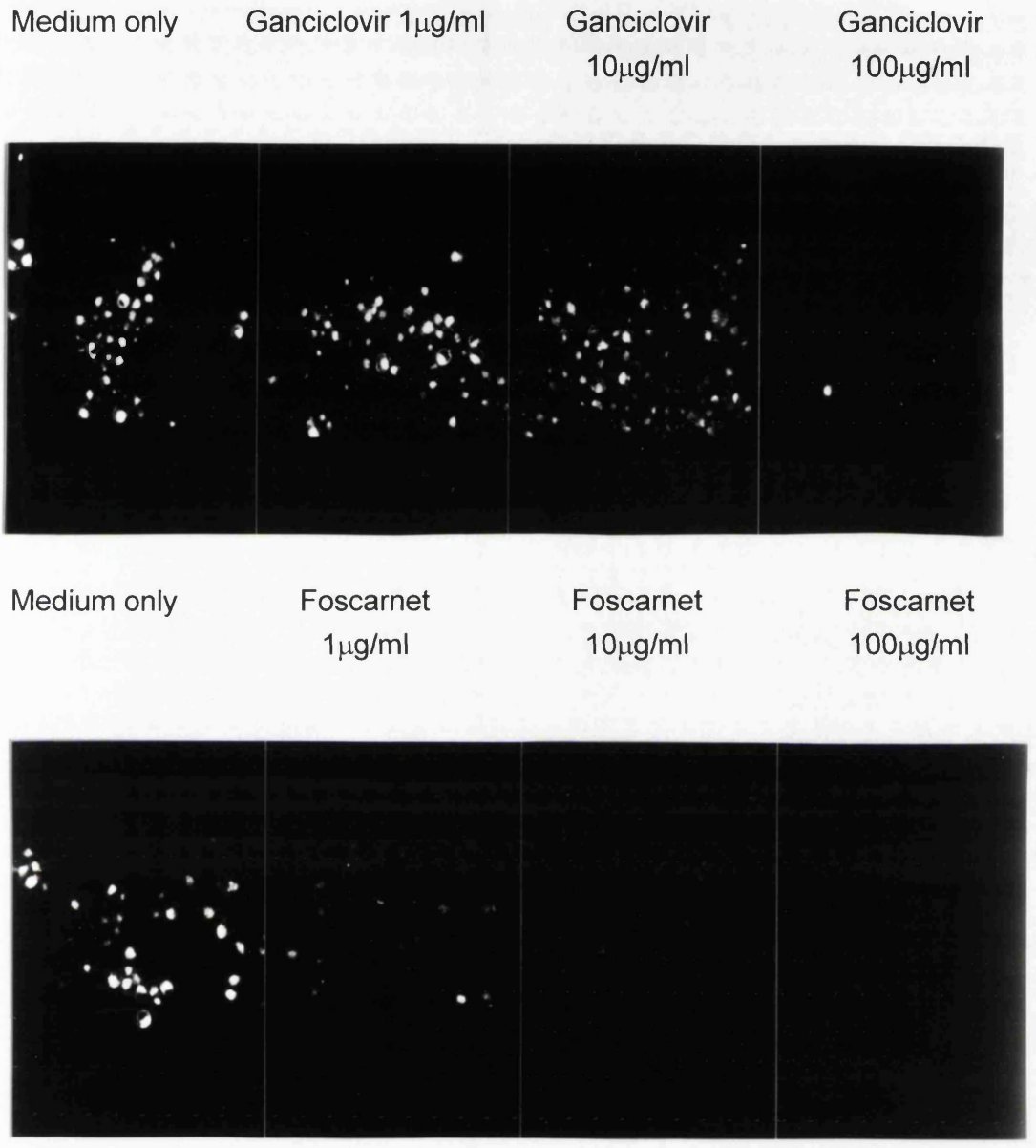


Figure 2.1. Titration of ganciclovir and foscarnet. Fibroblasts were infected with CMV at a multiplicity of infection of 2-4 PFU/cell. Immediately following infection they were treated with ganciclovir at 1, 10 or 100 μg/ml, Foscarnet at 1, 10, or 100 μg/ml, or with medium alone. At 3 days post infection the cells were stained for the expression of the CMV late antigen, gB. The cells were photographed using the Biorad confocal scanning laser microscope. Magnification x400.

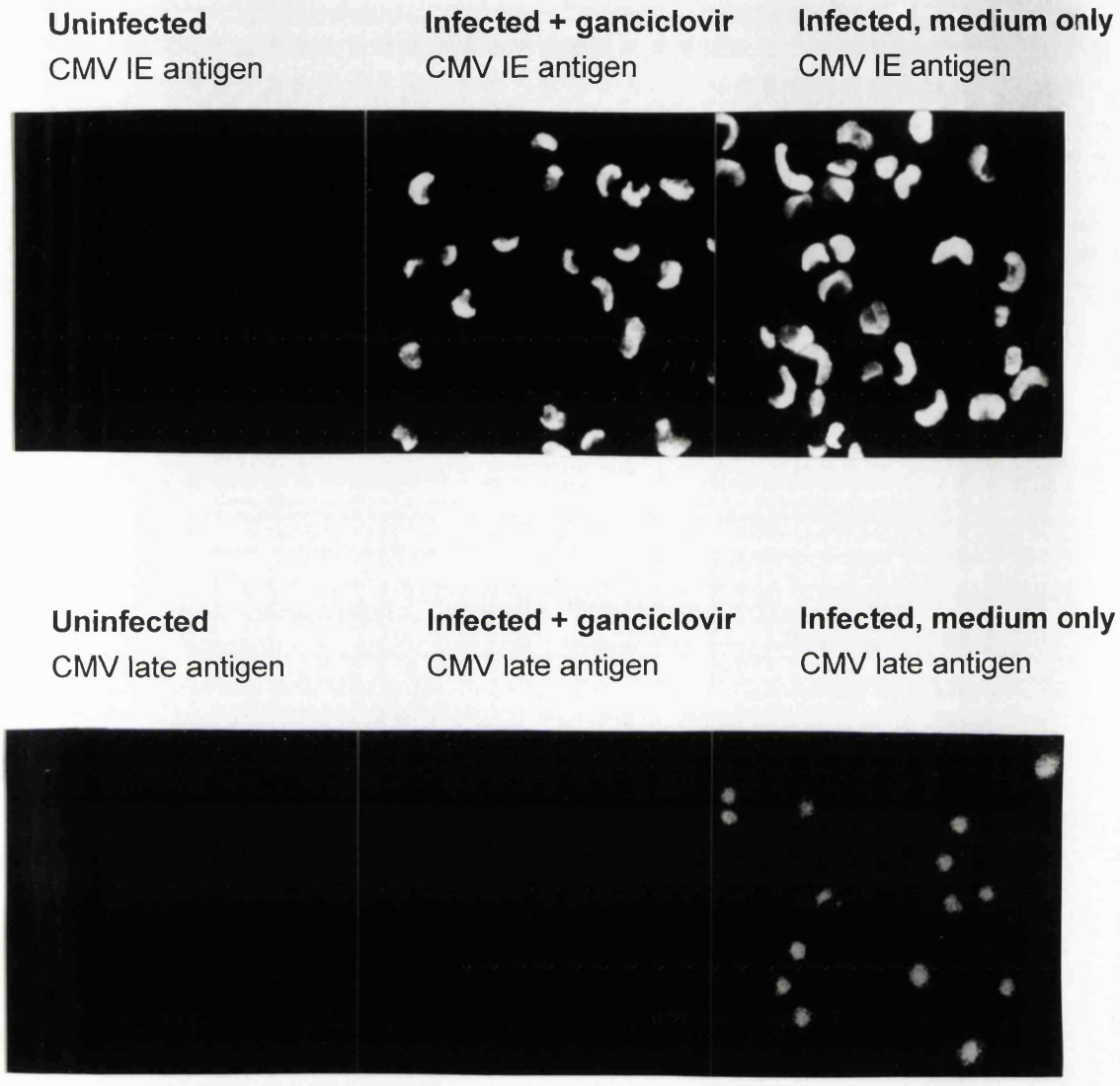


Figure 2.2. Efficacy of ganciclovir over a time period of 7 days. Cells were treated with ganciclovir at 50μg/ml immediately following infection. The cells were stained for the expression of CMV IE antigen, and the CMV late antigen, gB, at 7 days post infection. The cells were photographed using the Biorad confocal scanning laser microscope. Magnification x400.

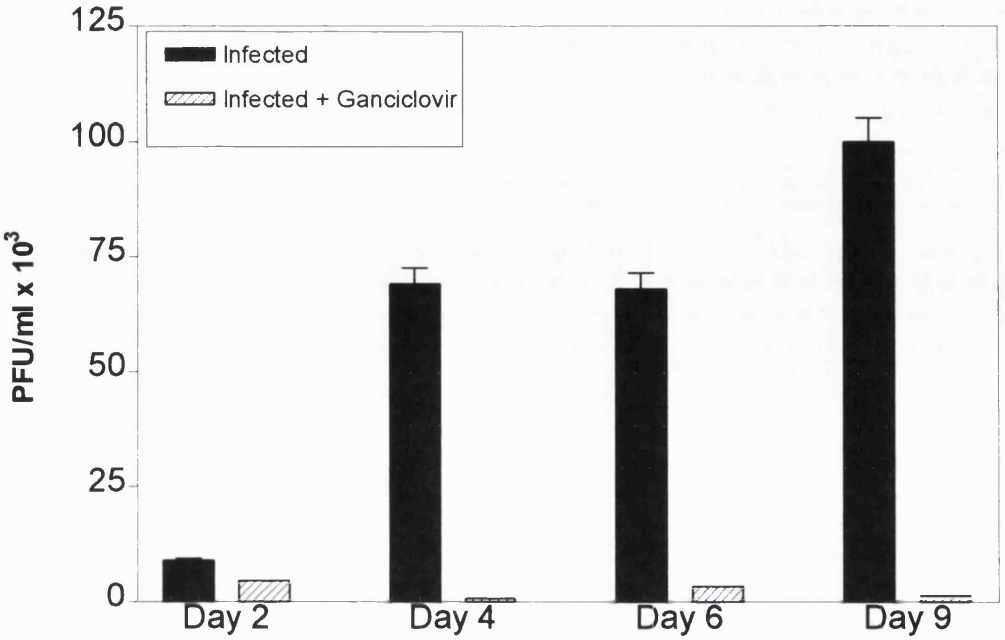


Figure 2.3. The production of infectious virus in the presence of ganciclovir.

Cells were ganciclovir treated, or incubated with medium alone, as in Figure 2.2. The cell supernatants were harvested at 2, 4, 6 and 9 days post infection. The amount of infectious virus present was measured using a plaque assay. The bars represent the mean ± SD from triplicate wells from a representative experiment.

2.8 ADHESION ASSAYS

2.8.1 Separation of peripheral blood mononuclear cells (PBMC)

Venous blood was collected from normal volunteers into heparinized tubes. This was then mixed with an equal volume of Hank's Balanced Salt Solution (HBSS), layered onto an equal volume of Lymphoprep (Gibco), and centrifuged at 800 x g for 30 minutes. Lymphoprep is a solution comprising 9.6% (w/v) sodium metrizoate, and 5.6% (w/v) Ficoll, resulting in a density of 1.077 g/ml and osmolality of 280 mOsm. This permits sedimentation of erythrocytes, and retention of the mononuclear cells at the interface. PBMC were harvested from the interface and washed twice in HBSS. The cells were >99% viable by trypan blue exclusion.

2.8.2 Removal of monocytes by adherence

PBMCs were resuspended at 5 $x10^6$ cells/ml in RPMI + 20% FCS, and placed in a tissue culture flask. This was incubated for 2 hours at 37°C, which allowed the monocytes to adhere to the base of the flask. The non-adherent cells were removed by gentle swirling of medium over the base of the flask, and were washed and resuspended in medium.

2.8.3 CD2+ve lymphocyte purification by negative depletion.

The Dynabead negative depletion technique was used to deplete the non-adherent PBMC population of B cells, activated T cells, and any residual monocytes not removed by the adherence stage. Cells were incubated with a cocktail of anti-CD19 and anti-HLA DR antibodies, both in the form of hybridoma supernatants. Dynabeads M-450 coated with goat antibodies specific for mouse IgG (Dynal, Oslo, Norway) were used to remove the conjugates using the Dynal Magnetic Particle Concentrator (MPC).

Antibody preparations were added to PBMCs at previously defined saturating concentrations, and incubated for 30 minutes at room temperature. Unbound antibody was removed by washing, and Dynabeads were added at a 2:1 bead/cell ratio in a total volume of 2ml RPMI + 2% FCS. To bring the beads into close contact with the antibody coated cells, the tubes were spun at 800 g for 2

minutes, and resuspended in the same supernatant. This was repeated 4 times. RPMI was then added to a total volume of 5 mls and the tube placed on the MPC for 2 minutes. The supernatant was removed to a new tube containing fresh beads, and a second depletion was carried out in the same way. The resulting cells were washed in RPMI, and resuspended at the required concentration. This technique routinely produced >95 % pure CD2 +ve cells when tested by flow cytometry.

2.8.4 Preparation of fibroblast monolayers for quantitative adhesion assays.

The use of CMV infected cells in the adhesion assays posed additional problems over adhesion assays using uninfected fibroblasts or transfected cells, as the cells have a rounded morphology, and detach more easily from the wells during the removal of non-adherent cells. Very careful washing of the wells was necessary to minimise this as much as possible. The seeding density of the cells was also important, and it was essential to seed infected cells at the required density one day prior to the assay rather than infecting the cells when already seeded in the 96 well plate. This was because efficient infection with CMV monolayers requires subconfluent cells, which resulted in incomplete coverage of the wells by the fibroblasts at three days post infection when the adhesion assays were carried out. Seeding of infected and uninfected cells into 96 well plates one day prior to the assay allowed the formation of fully confluent monolayers.

Fibroblasts in $25cm^2$ tissue culture flasks were infected with CMV AD169 at a multiplicity of infection (MOI) of 2-4 pfu/cell, or left uninfected. After 2 days they were trypsinised and seeded into 96 well flat bottomed plates at a concentration of $4x10^4$ cells/well, in 200µl of MEM/10%FCS. The plates were used for quantitative adhesion assays on the following day ie. At 3 days post infection. The cells formed confluent monolayers when seeded at this density.

2.8.5 Leucine labelling of lymphocytes.

Lymphocytes were washed and resuspended at 5×10^7 cells/ml in leucine-free RPMI/1% FCS (ICN Flow). Tritiated leucine (L-leucine, [2,3,4,5-^{3}H]; Amersham International, Bucks.) was added at 50µCi/ml and incubated for 1 hour in a 37°C waterbath with occasional shaking. The cells were then washed twice in

large volumes of RPMI and resuspended at the required concentration for use in the adhesion assay.

2.8.6 Quantitative adhesion assay

Confluent fibroblast monolayers in 96 well plates were washed twice in warm HBSS/ 1%FCS. The appropriate number of lymphocytes, in a 100μl volume of RPMI (without FCS), was added to the fibroblast monolayers. The plates were then centrifuged at 20 x g for 5 minutes, and incubated for 10 minutes at 37°C. The wells were then gently washed 5 times with warm HBSS/1% FCS, and centrifuged, the plate inverted, at 500 x g for a few seconds before a final wash. The contents of the wells were solubilized with 50μl of 1% Nonidet P 40, and the contents transferred to a filter mat for the counting of bound radioactivity using the Wallac 1205 β plate counter. The radioactivity in aliquots of the original T cell suspension was also counted, and the percentage of the original population which had bound to each well was calculated. Triplicate or quadruplicate wells were used for each condition. Statistical analysis was performed on these values using the two-tailed Student's t-test.

2.8.7 Visual counting of the number of adherent lymphocytes.

The quantitative adhesion assay was carried out as described above. Following removal of the non-adherent lymphocytes from the plate by washing, each well was photographed in order to count the number of adherent lymphocytes in each randomly chosen field of view. The area counted was 1/4 of the total area of each photograph, which contained approximately 100 fibroblasts. Triplicate readings were obtained by counting three separate fields of view.

2.8.8 Activation of T cells for use in adhesion assays

Quantitative adhesion assays were carried out using lymphocytes which were activated using three different stimuli. The lymphocytes were ^{3}H leucine labelled as described previously and were then divided into four equal aliquots, three of which were activated by the stimuli described below, and one of which was used in a resting state.

Resting T cells

The resting CD2+ve lymphocytes were stored on ice during the activation of the T cells for other parts of the experiment. They were resuspended at $5x10^6$ cells/ml in RPMI with no serum added for use in the adhesion assay.

Cation-free T cells

Resting T cells, following ^3H leucine labelling, were washed twice in HEPES/glucose assay buffer (20mM HEPES, 140mM sodium chloride, 2mg/ml glucose, pH7.4) containing 1mM EGTA, and resuspended in this buffer at 5 x 10^6/ml for use in the adhesion assay.

CD3 activation.

^3H leucine labelled resting T cells were resuspended at $5x10^6$ cells/ml in RPMI/10% FCS. They were incubated with an anti-CD3 monoclonal antibody (Clone OKT3) at optimal concentrations (as determined by prior titration in tritiated thymidine incorporation assays) for 30 minutes on ice, and washed twice in RPMI/10% FCS. The cells were again resuspended at $5x10^6$ cells/ml, incubated on ice for 10 minutes with goat anti mouse IgG at a final concentration of 2μg/ml (Serotec), to cross link the CD3 antibodies, thus allowing activation of the cells to take place. Following a further wash in RPMI/10%FCS, the cells were resuspended at a final concentration of $5x10^6$/ml in RPMI containing 1% normal mouse serum. This was necessary to saturate unoccupied binding sites of the goat anti mouse antibodies, and thus prevent crosslinking of the T cells to the mouse monoclonal antibodies used for blocking in the adhesion assay.

Magnesium activation of integrins.

The ^3H leucine-labelled resting T cells were washed in HEPES/glucose assay buffer. They were then resuspended at 5x106/ml in assay buffer containing 5mM Mg2+ and 1mM EGTA, and kept on ice throughout the procedure.

Phorbol ester activation.

The ^3H leucine labelled resting T cells were washed in HEPES/glucose assay buffer and resuspended at $5x10^6$/ml in the same assay buffer containing 1mM Ca^{2+}, 0.3mM Mg^{2+}, and 50nM phorbol 12,13 dibutyrate (PdBu), a phorbol ester (Calbiochem). The lymphocytes were stored on ice throughout the procedure.

The phorbol and magnesium activations were carried out immediately prior to the addition of the lymphocytes to the fibroblast wells.

2.8.9 Antibody blocking of fibroblast adhesion molecules.

Blocking antibodies specific for ICAM-1 (clone 15.2, IgG1) or LFA-3 (Clone TS/9, IgG2a), or IgG isotype controls were incubated with the fibroblasts for 1 hour at 37°C prior to the assay, and the excess antibody was removed by washing the monolayers 3 times with warm RPMI, or HEPES/glucose assay buffer for experiments using magnesium activation. The antibodies were previously titrated in adhesion assays, and were found to have optimal blocking function at 10μg/ml.

2.8.10 Qualitative adhesion assay

Larger scale adhesion assays using unlabelled lymphocytes were carried out to facilitate the phenotypic analysis of the adherent and non adherent populations. Fibroblasts were seeded in 6 well plates at $3x10^5$ cells/well, and infected at a MOI of 2-4 pfu/cell when subconfluent. CD2+ve lymphocytes were purified and added to the fibroblasts at 5×10^6 cells/well. The plates were then incubated at 37°C for one hour after which the non adherent cells were removed by washing the wells 3 times with HBSS containing 1% FCS. The adherent cells were then detached from the fibroblasts by treatment with 0.53mM EDTA, and incubation at 4°C for 15 to 30 minutes, followed by mechanical disaggregation of remaining adherent cells. The lymphocytes were then stained and analysed by flow cytometry as described in section 2.3.

2.9 LYMPHOCYTE PROLIFERATION ASSAYS

2.9.1 Establishment of ganciclovir and mitomycin C concentrations for the suppression of fibroblast proliferation and viral DNA synthesis

In addition to mitomycin C treatment to arrest the growth of the fibroblasts, it was necessary to use ganciclovir to prevent the synthesis of viral DNA by infected cells. Ganciclovir is known to suppress the proliferation of lymphocytes, so preliminary experiments were carried out in order to establish

a concentration of ganciclovir which would suppress viral DNA synthesis but would not interfere with lymphocyte proliferation.

Infected or uninfected fibroblasts were treated with mitomycin C (Kyowa Hakko UK Ltd, London, UK) at 0, 1, 10 or 100 μg/ml at 37°C for 30 minutes, washed three times, and seeded at 1 x 10^4 cells/well in 96 well flat-bottomed tissue culture plates in RPMI/10% FCS. At each mitomycin C concentration, ganciclovir was included in the medium at 0, 1, 10 or 50μg/ml. Following a four day incubation, the plates were pulsed for 18 hours with 0.5 Mbq (13.5μCi) per well of tritiated thymidine (Amersham International). The plates were harvested using a Tomtec automatic harvester, and incorporated radioactivity was measured using a Wallac 1205 β plate reader. The results are shown in Figure 2.4. Mitomycin C was subsequently used at 10 μg/ml as this reduced the proliferation of uninfected fibroblasts to basal levels. Thymidine incorporation of infected fibroblasts was reduced to basal levels by ganciclovir at 1μg/ml.

The effect of this concentration of ganciclovir on lymphocyte proliferation was then investigated. PBMCs were seeded at 1 x 10^5 cells per well in 96 well, flat-bottomed plates in RPMI/10% FCS. Ganciclovir was included at 1μg/ml. Lymphocytes were activated by CD3 antibody (Clone OKT3) at its optimal concentration, or with phytohaemagglutinin (PHA) at 0.5μg/ml, or were unstimulated. The wells were pulsed with tritiated thymidine at days 2, 3, 4, and 5 post stimulation and the incorporation measured, as described above (Figure 2.5). The inclusion of ganciclovir at 1μg/ml did not alter the proliferative response to CD3 or PHA activation. Based on the results on fibroblast and lymphocyte thymidine incorporation it was decided to use ganciclovir at 1μg/ml for subsequent experiments.

2.9.2 Conditions for proliferation assays

Infected or uninfected fibroblasts were trypsinised at 3 days post infection, treated with mitomycin C at 10μg/ml as described above, and seeded in 96 well plates at 1 x 10^4 /well in RPMI/10% FCS containing 1μg/ml ganciclovir. Control wells contained medium only. PBMCs were added at 1 x 10^5 cells/well in the same medium. Anti-CD3 antibodies were added at their optimal concentrations to the appropriate wells. Cells were pulsed with 0.5MBq/well for 18 hours at the time points stated in the text and the amount of thymidine incorporation measured.

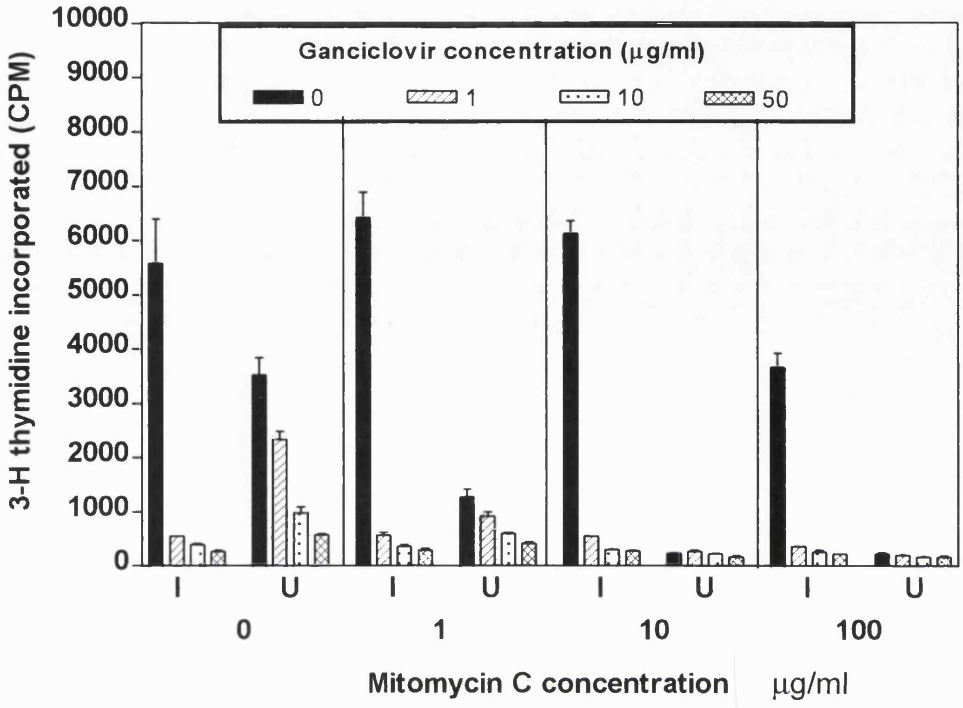


Figure 2.4. Titration of ganciclovir and mitomycin C.

Infected (I) or uninfected (U) fibroblasts were treated with mitoycin C at the concentrations stated on the X axis and seeded into tissue culture plates. Cells treated with each mitomycin C concentration were incubated in the presence of ganciclovir at the concentrations shown on the legend. The plates were pulsed with tritiated thymidine at 4 days post incubation and the amount of incorporated thymidine determined. The bars represent the mean ± SD of triplicate wells.

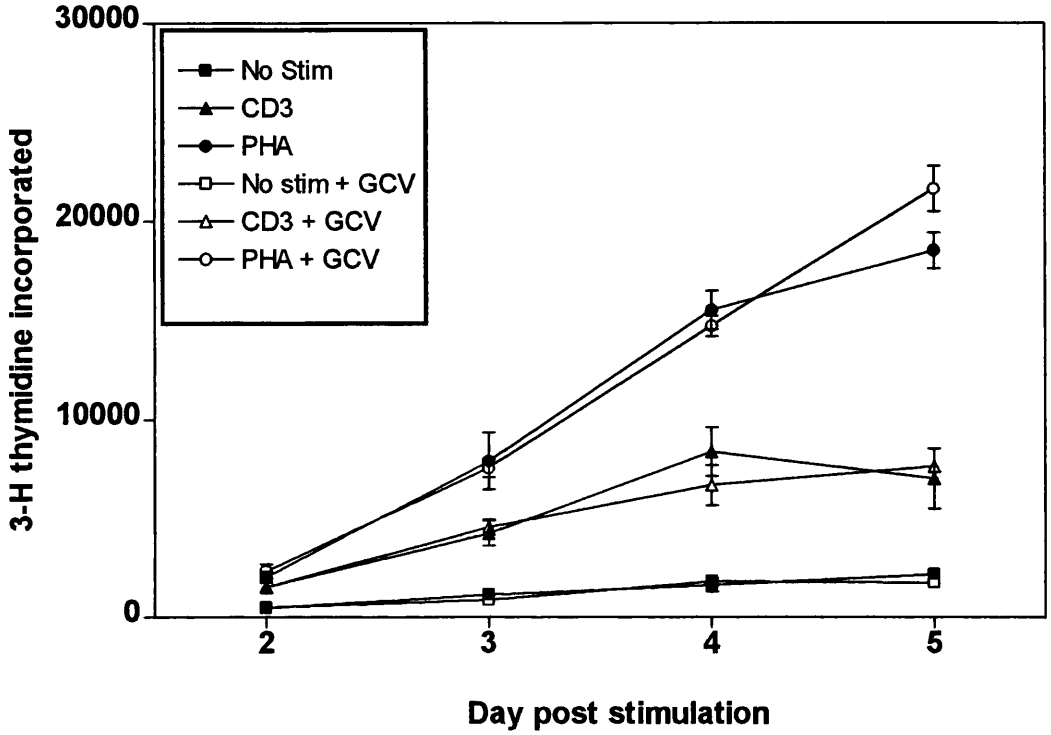


Figure 2.5. The effect of ganciciovir on lymphocyte proliferation.
PBMCs were stimulated with either anti-CD3 antibodies, or PHA, or remained resting. Cells were incubated either in the presence or absence of ganciclovir (1μg/ml) for the times shown on the X axis, after which they were pulsed with tritiated thymidine for 18 hours. Incorporated thymidine was then measured. Each data point represents the mean ± SD of triplicate samples.

2.10 NEUTROPHIL MIGRATION ASSAYS

2.10.1 Preparation of endothelium coated cell culture inserts.

Endothelial cells were seeded onto fibronectin coated 6.5mm diameter millipore (3 μm pore size) cell culture inserts (Falcon). The formation of confluent monolayers was verified the following day by microscopic examination. The ability of the monolayers to act as a barrier was tested by measuring the permeability to ^{125}I-labelled BSA placed in the upper chambers and incubated in parallel with the filters used for the transmigration assays. Equilibration of I^{125}-BSA across endothelium covered filters was consistently <5%, while that across filters alone was >95% over a 2 hour period. The endothelial cell coated inserts were prepared by Dr. K.Yong, Department of Haematology, RFHSM.

2.10.2 Neutrophil purification

Neutrophils were purified from venous blood taken into preservative free heparin by double density centrifugation (Histopaque 1119 and 1077, Sigma). The gradients were prepared by underlaying first HBSS, then Histopaque 1077, then Histopaque 1119, all in 10 ml volumes. The blood was then applied to the top of the gradient. The tubes were centrifuged at 1800 x g for 45 minutes. Neutrophils sedimented to the interface between the two density gradient solutions, while PBMC were retained at the interface of the Histopaque 1077 and the plasma. The neutrophils were harvested from the interface and washed twice in HBSS containing 2% FCS and 5mM glucose, at 700 x g and then at 400 x g for 10 minutes. Neutrophils obtained by this method were >95% pure by microscopic examination and >99% viable by trypan blue exclusion.

2.10.3 Labelling of neutrophils

Neutrophil suspensions (5-10 x 10^6 cells/ml) were labelled with ^{51}Cr (2mCi/10^6 cells; Amersham International) at 37°C for 60 minutes with occasional agitation, washed four times in large volumes of HBSS/FCS/glucose, and resuspended at 2-4 x 10^6 cells/ml in RPMI/10% FCS, for use in transmigration assays.

2.10.4 Transendothelial migration assays

Confluent endothelial monolayers on cell culture inserts were washed 3 times in warm medium, and placed in wells with fresh medium (RPMI/10% FCS), supernatants from CMV infected or uninfected fibroblasts, or fMLP (10^{-7}M). Anti-IL-8 neutralising antibodies (R&D Systems) were used at 20 μg/ml where stated in the text, and were incubated for 30 minutes at 37°C with the appropriate medium before addition of the filter to the wells. As a positive control, endothelial cells on filters were stimulated with 2ng/ml of IL-1β for 3 hours prior to the assay. ^{51}Cr-labelled neutrophils (2-4 x10^5 cells in 100μl volume/insert) were placed in the upper chamber, and the wells incubated for 2 hours in a humidified incubator at 37°C. Duplicate or triplicate wells were used for each data point. At the end of the incubation, the cell culture inserts were removed, and the lower surface was swabbed with a cotton wool tip, which was counted together with the contents of the lower chamber, using a gamma counter. The percentage of migrated cells was calculated by dividing the radioactivity harvested from each well by the counts obtained from 100μl of the original radiolabelled neutrophil suspension. Statistical analysis was performed on these values using the two-tailed Student's t-test. Data from different experiments were normalised such that the % migration of the control wells in each experiment was designated as 100%.

2.11 T CELL MIGRATION ASSAYS

Endothelium coated cell culture inserts were prepared as described above. For quantitative assays, 6.5mm inserts were used in 24 well tissue culture plates. For phenotypic analysis of the migrated lymphocytes 25mm diameter inserts were used in 6 well plates. For studies using 24 well plates, 5 x 10^5 lymphocytes in 100μl of medium were added to each insert, while for 6 well plates this was increased to 3 x 10^6 cells in 2mls of medium per insert. Duplicate or triplicate wells were used for each data point. At the end of an 18 hour incubation the migrated cells were harvested and counted using a Cytoron Absolute flow cytometer (Ortho Diagnostics Ltd, High Wycombe, U.K.). The counting was carried out by Dr. Nicola Borthwick, Department of Clinical Immunology, RFHSM. The results are expressed as the percentage of the starting population which migrated. Phenotypic analysis of lymphocytes was carried out as described in section 2.3.

2.12 RNA ISOLATION AND NORTHERN BLOT ANALYSIS.

2.12.1 Working with RNA

RNA is very sensitive to degradation RNAses which are present in the environment. For this reason all reagents used were prepared in diethylpyrocarbonate (DEPC) treated MilliQ water, or solutions were DEPC treated when made up. This was prepared by adding 1ml of DEPC/litre of water or prepared solution, and incubating at 37°C for at least 4 hours, before autoclaving to remove residual DEPC. This treatment destroys RNAses. Wherever possible, equipment such as gel apparatus and boxes for incubations were used for RNA work only, or were rinsed with a dilute solution of hydrogen peroxide followed by DEPC treated water before use. Sterile plasticware, which is RNAse free, was used wherever possible for the preparation of solutions.

2.12.2 Extraction of RNA

For the purification of sufficient mRNA for northern blotting, a $25cm^2$ flask of fibroblasts was used for each stimuli or time point measured. Cells were infected in the usual way, or treated with cytokines as detailed in the appropriate experiments. RNA was purified using RNAzol B™ (Biotecx Laboratories, Houston, Texas), a method which reduces the guanidium isothyocyanate extraction and phenol chloroform extraction to a single step. The method used was essentially as described in the protocol supplied by the manufacturers. Cells were homogenised with RNAzol B solution before addition of chloroform and centrifugation to separate the phenol chloroform and aqueous phases. The aqueous phase containing the RNA was added to isopropanol and centrifuged, resulting in the pelleting of the precipitated RNA. The pellet was washed in 75% ethanol and dried under vacuum, before resuspension in DEPC treated water. The absorbance at 260 and 280 nm was measured to determine the amount of RNA present and its purity. A 260:280 ratio of 2 indicates pure RNA, free of protein contamination. The amount of RNA present in the sample (in mg) was calculated from the formula:

$$A_{260} = \text{dilution factor} \times \text{volume of RNA} \times 40$$

2.12.3 Denaturing gel electrophoresis

A 1% agarose gel was made by combining 3g agarose with 230 mls DEPC water. When the agarose was melted, 15 ml 20X MOPS running buffer (0.4M MOPS, 0.02M EDTA, 0.2M sodium acetate, pH 7), and 54 ml formaldehyde solution were added. The samples were adjusted so that between 7.5 and 10μg RNA was loaded per lane of the gel, following dilution in RNA sample buffer (7μl formaldehyde solution, 4μl 5X MOPS running buffer, 2μl ethidium bromide solution from 1mg/ml stock, 20μl formamide, 2.5μl bromophenol blue). The gel was run using precooled 1X MOPS, at 130 volts until the bromophenol blue had migrated about 3 cm. The gel was then observed under UV light, and the 28S and 18S bands could be seen. The gel was photographed to demonstrate equal loading of samples. (Polaroid CU5 88-46 Land Camera). The gel was then agitated in DEPC treated water for 30 minutes to remove formaldehyde.

2.12.4 Transfer of RNA to membranes

The RNA was transferred, overnight, to the nylon membrane (Boehringer Mannheim, Lewes, East Sussex, UK) by capillary action. The gel apparatus was used as a blotting apparatus, with the reservoirs filled with 20X SSC (3M Sodium Chloride, 0.3M Sodium citrate, pH 7). The RNA was fixed to the membrane by baking at 120°C for 20 minutes. The blot was then cut into pieces for hybridising with individual probes, and sealed into hybridisation bags.

2.12.5 Principles of the digoxigenin (DIG) detection system.

The DIG detection system is based on the steroid hapten digoxigenin, which is a plant product and thus avoids problems with background activity which are encountered with other haptens. DIG labelled DNA probes are hybridised to mRNA which is immobilised on a nylon membrane during northern blotting. The hybrids are detected with an anti-DIG antibody (Fab fragment) conjugated to alkaline phosphatase. The signal is detected using a chemiluminescent substrate, CSPD®, followed by autoradiography. The DIG system was chosen because of the safety of the technique in comparison to radioactive detection, and the equally high sensitivity.

2.12.6 Hybridisation with DIG-labelled oligonucleotides

The following reagents were used for the hybridisation stages:

- **Buffer 1**:- 0.1M maleic acid, 0.15M sodium chloride, adjusted to pH 7.5 with solid sodium hydroxide)
- **Blocking stock solution**:- 10g Boehringer Mannheim blocking reagent, 100ml buffer 1, heated to dissolve.
- **Buffer 2**:- Blocking stock solution diluted 1:10 in buffer 1
- **Buffer 3**:- 0.1M Tris, 0.1M sodium chloride, 50mM magnesium chloride, adjusted to pH 9.5 with hydrochloric acid.
- **Hybridisation solution**:- 5X SSC (0.75M sodium chloride, 0.075M sodium citrate, pH 7.0; DEPC treated), 1% blocking reagent (1:10 dilution from stock), 0.1% Sarkosyl, 0.02% sodium dodecyl sulphate (SDS).

The membrane was prehybridised by incubation at 42°C for 1 hour in hybridisation solution. The solution was replaced with the DIG-labelled DNA probe for the appropriate chemokine in the same buffer, and hybridised overnight at 42°C. The blot was then washed in 2X SSC + 0.1% SDS for 2 x 5 minutes, and in 0.1X SSC + 0.1% SDS for 2 x 5 minutes. All steps were carried out at 42°C.

2.12.7 DIG labelled probes.

DIG-labelled DNA probes (30 or 31-mer) specific for IL-8, MCP-1, RANTES, MIP-1α, and β-actin mRNA were used at 10ng/ml (R&D Systems).

2.12.8 DIG luminescence detection.

This was carried out using components from the Boehringer Mannheim kit No. 1363524, essentially as detailed in the manufacturers instruction sheet. All steps were carried out at room temperature. The membrane was washed for 5 minutes in washing buffer (0.3% Tween-20 in buffer 1). The membrane was blocked for 30 minutes in buffer 2, then incubated for 30 minutes in anti-DIG antibody conjugated to alkaline phosphatase, diluted 1/10,000. The membrane was then washed in washing buffer for 2 x 15 minutes, then equilibrated for 5 minutes in buffer 3. The lumingen was then diluted 1/100 in buffer 3. The membrane was placed on a plastic sheet, and the substrate, CSPD®, added (1ml/membrane). The membrane was covered with the plastic sheet, sealed to

make a bag, and incubated at 37°C for 15 minutes. This was then exposed to X-ray film for 1-2 hours. Further exposures were done depending on the initial exposure. The signal was seen as a dark band on the X-ray film.

Ethidium bromide was included in each sample, enabling confirmation of equal loading by densitometric analysis. IL-8 specific bands on X-ray film were quantified by densitometric analysis (Biorad imaging densitometer) using the Molecular Analyst program. Corrections were made for small differences in loading by comparison with the β-actin control.

Table 2.1 Murine monoclonal antibodies specific for CMV antigens

Specificity	Clone	Isotype	Source
CMV IE antigens 1 and 2	E 13[1]	IgG1	Biosoft, Paris, France
CMV IE antigens 1	63.27[2]	IgG1	Dr. W. Britt, Alabama, USA.
glycoprotein B (gB) (CMV late antigen)	7.17[1]	IgG3	Dr. W. Britt, Alabama, USA.

[1] Suitable for flow cytometry or immunofluorescent staining on slides.
[2] Not suitable for flow cytometry.

Table 2.2. Primary murine monoclonal antibodies used in this study[1].

CD number	Alternative names	Clone	Isotype	Proposed function	Main expression	Source
	class I HLA (Heterodimer)	W6/32	IgG2a	Antigen presentation	Macrophages, dendritic cells, fibroblasts, endothelial cells	ATCC[2]
	class I HLA (Heterodimer)	PA 2.6	IgG1	Antigen presentation	As above	ATCC
	class II HLA	RFDR2	$IgG2_a$	Antigen presentation	B cells, monocytes, dendritic cells	RFHSM[3]
CD2	E rosette receptor,TII	RFT11	$IgG2_a$	Adhesion molecule, T cell activation	T cells, NK cells	Prof. PCL Beverley[4]
CD3	T3, Leu4	OKT3	IgG2a	Signal transduction	T cells	ATCC
CD4	T4, Leu3	RFT4	IgG1	Signal transduction	T cell subset	RFHSM
CD8	T8, Leu2	RFT8	IgG1	Signal transduction	T cell subset	RFHSM
CD11a	LFA-1 α subunit	MEM-25	IgG1	Adhesion to ICAM-1,2,3	All leukocytes	Caltag
CD16	FcγRIII	B.E16	$IgG2_a$	Low affinity Fcγ receptor, ADCC	NK cells, granulocytes	Serotec[6]
CD19		RFB9	IgG1	Signal transduction	B cells	RFHSM
CD28	Tp44	L293	IgG1	Ligand for B7.1, B7.2, costimulation	Most CD4+, subset of CD8+ T cells	Becton Dickinson[7]
CD31	PECAM-1	9G11	IgG1	Adhesion molecule	Endothelial cells, platelets	R&D Systems[8]
CD45RO	Low MW form of CD45	UCHL1	$IgG2_a$	Signal transduction (Tyrosine phosphatase)	Memory T cells, monocytes	Prof PCL Beverley
CD45RA	CD45R, high MW form of CD45	SN130	IgG1	As above	Naive T cells, B cells	RFHSM
CD45RB	T200	PD7	IgG1	As above	Memory T cells, B cells, monocytes	Dako[9]

Table 2.2 continued.

CD number	Alternative names	Clone	Isotype	Proposed function	Main expression	Source
CD54	ICAM-1	BB1G-1	IgG1	Adhesion to LFA-1	Widespread	R&D Systems
CD54	ICAM-1	15.2	IgG1	As above	As above	Novacastra
CD56	Leu 19, NCAM	NKH1A	IgM	Adhesion	NK cells	Coulter[10][11]
CD57	Leu 7	HNK1	IgM	Unknown	NK cells, T cell subset	ATCC
CD58	LFA-3	TS2/9	IgG2a	Adhesion to CD2	Widespread	ATCC
CD62E	E-Selectin, LECAM-2	BBIG-E4	IgG1	Adhesion molecule	Activated endothelial cells	R&D Systems
CD62L	L-selectin, LECAM-1	SK11	IgG2a	Adhesion molecule	Leukocytes	Becton Dickinson
CD69	Activation inducer molecule (AIM)	L78	IgG1	Activation	Act. T and B cell, NK cells, Act. Macrophages	Becton Dickinson
CD106	VCAM-1	BBIG-V1	IgG1	Adhesion to VLA-4	Endothelial cells	R&D Systems

[1] The information in this table was taken from the 3rd, 4th, and 5th International Workshops on Human Leukocyte Differentiation Antigens

[2] American Type Culture Collection, Rockville, Maryland, USA

[3] Produced by the Department of Clinical Immunology, Royal Free Hospital School of Medicine

[4] Prof. PCL Beverley, University College London.

[5] Caltag, San Francisco, USA

[6] Serotec, Kidlington, Oxford, UK

[7] Becton Dickinson UK Ltd., Cowley, Oxford, UK

[8] R&D Systems, Abingdon, UK

[9] Dako Ltd, High Wycombe, UK

[10] Novacastra, Newcastle upon Tyne, UK

[11] Coulter Immunology, Luton, UK

Table 2.3. Secondary antibodies used for fibroblast and lymphocyte staining

Specificity	Antibody type	Fluorochrome	Source
anti mouse IgG	goat IgG $F(ab)_2$ fragment	FITC[3]	Sigma[1]
anti mouse IgG1	sheep IgG $F(ab)_2$ fragment	FITC, PE[4]	SBA[2]
anti mouse IgG2a	sheep IgG $F(ab)_2$ fragment	FITC, PE	SBA
anti mouse IgG3	sheep IgG $F(ab)_2$ fragment	FITC	SBA
anti mouse IgM	sheep IgG	PE	SBA

[1]Sigma Chemical Company, Poole, UK

[2]Southern Biotechnology Associates, Birmingham, Alabama, USA

[3]Fluorescein isothyocyanate.

[4]Phycoerythrin

Table 2.4 Neutralising antibodies specific for cytokines

Cytokine	Clone	Antibody	Neutralising activity	Source
IFNβ		Rabbit polyclonal	96 units neutralised 90% of the activity of 10 IU of IFNβ, 960 units neutralised all the activity of 10 IU IFNβ[1]	Lee Biomolecular
IL-1β	B-A15	Mouse IgG1	1μg neutralised 200pg IL-1β[2]	Serotec
TNF-α	B-C7	Mouse IgG1	1μg neutralised 50pg TNFα[2]	Serotec
IL-8		Goat polyclonal	1μg neutralises 50% of the activity of 5ng IL-8[3]	R&D Systems

[1] 10 IU/ml of IFNβ was added to fibroblasts and the induction of class I HLA was measured by flow cytometry. The addition of anti-IFNβ antibodies at the same time abrogated this increase as stated.

[2] 200pg/ml IL-1β or 50pg/ml TNFα were added to fibroblasts and the induction of ICAM-1 was measured by flow cytometry. The addition of the appropriate neutralising antibodies at the same time totally abrogated this increase.

[3] This figure was stated by the manufacturers.

CHAPTER 3

THE EFFECT OF CMV INFECTION ON THE EXPRESSION OF CLASS I HLA: DIFFERENTIATION BETWEEN INFECTED CELLS AND BYSTANDER UNINFECTED CELLS

Chapter 3. The effect of CMV infection on the expression of class I HLA: differentiation between infected ceils and bystander uninfected cells.

3.1 INTRODUCTION

CMV is epidemiologically linked with allograft atherosclerosis, allograft rejection, and decreased patient survival following transplantation (Lopez *et al.* 1974; Rubin *et al.* 1985; Grattan *et al.* 1989). It is suggested that CMV infection may contribute to these processes by the induction of MHC molecules in the graft, which is associated with increased rejection (von-Willebrand *et al.* 1986; Tuder *et al.* 1994). In support of this proposal, increased expression of class II HLA in CMV infected human kidney allografts (von-Willebrand *et al.* 1986), and in rat kidney allografts (Ustinov *et al.* 1994) have been reported. However, *in vitro* evidence suggests that CMV does not induce class II HLA expression, and may inhibit its induction by IFNγ (Sedmak *et al.* 1990; Sedmak *et al.* 1994a). The same group have also shown low class II HLA expression in the lungs of patients suffering from CMV pneumonitis (Ng-Bautista and Sedmak, 1995). There have been conflicting reports regarding the effect of CMV infection on class I HLA expression *in vitro*, with some investigators reporting upregulation in a range of cell types (van-Dorp *et al.* 1989; Tuder *et al.* 1991; Ustinov *et al.* 1991; van Dorp *et al.* 1993b; Tuder *et al.* 1994), and others reporting downregulation, generally in fibroblasts (Barnes and Grundy, 1992b; Beersma *et al.* 1993; Warren *et al.* 1994). It has recently been established that CMV infection downregulates the expression of class I HLA in infected cells, due to the expression of a CMV early gene product (US11) which promotes the degradation of newly formed class I HLA molecules (Jones *et al.* 1995; Wiertz *et al.* 1996). These findings suggest that CMV infection should reduce the potential of the graft to present alloantigen rather than contribute to graft rejection.

Despite the reduction of class I HLA expression and the absence of class II induction, CMV infected endothelium stimulates proliferation of allogeneic T cells *in vitro* (Waldman *et al.* 1992). It appears that the induction of inflammatory cytokines produced by activated T cells mediates increased class II HLA and adhesion molecule expression on uninfected endothelial cells and it is suggested that production of cytokines by activated T cells may be responsible for upregulation of class II HLA which has been shown in allografts in CMV infected individuals (Waldman *et al.* 1993; Waldman *et al.* 1995a). Class I HLA expression is also induced by cytokines produced by activated T

cells, and additionally by IFNβ, which is produced by infected cells in the absence of lymphocyte derived cytokines (Lapierre *et al.* 1988; Grundy *et al.* 1988a). Induction of class I expression in the infected allograft may therefore be a direct mechanism for the induction of alloreactivity.

The aforementioned studies reporting enhancement of class I HLA expression following CMV infection derived their results from populations of cells containing a large proportion of uninfected cells. In many cases this was unavoidable due to the low percentage of infection which can be achieved in cells other than fibroblasts using laboratory strains of CMV. In the light of the recent results confirming the downregulation of class I HLA expression by CMV infection, it is likely that the measurements of class I HLA in the studies where class I HLA was upregulated were distorted by the presence of large numbers of uninfected cells. However, it has also been reported that the immediate early genes of HCMV transactivate the HLA-A2 promoter and increase gene expression in the Jurkat cell line (Burns *et al.* 1993), so it is possible that a direct increase in class I HLA expression takes place in other cell types, particularly those which undergo only an abortive infection and thus do not express early genes such as US11.

In the light of discrepant findings from different groups, it was important to define the patterns of class I HLA expression on both infected cells and uninfected bystander cells. This was achieved by investigating the CMV status of infected and uninfected fibroblasts separated from mixed cultures by cell sorting. Studies using neutralising antibodies specific for IFNβ were also carried out to determine the contribution of this cytokine to the upregulation of class I HLA which was evident on 'uninfected bystander' cells. Although the downregulation of class I HLA appears to be important in evasion of the host immune response (Warren *et al.* 1994), the expression of class I HLA on uninfected bystander cells is equally interesting, as the majority of cells in an infected organ are uninfected.

3.2 RESULTS

3.2.1 The relationship between CMV infection and class I HLA expression.

To provide some information about the patterns of class I HLA expression in infected and uninfected fibroblasts, populations of fibroblasts consisting of varying proportions of infected and uninfected cells were generated. The class I HLA expression and the expression of CMV IE antigen were determined by single colour flow cytometry.

Subconfluent fibroblast monolayers on duplicate plates were infected with a virus inoculum which infected a high percentage of cells (5 pfu/cell, >95% infection), and with two-fold dilutions of this inoculum (neat to 1/64) to a minimum virus dose of 0.078 pfu/cell (16% infection). Cells from one plate were stained for the expression of CMV IE antigen at 24 hours post infection. This time point was chosen for CMV IE staining so that only cells infected by the initial CMV inoculum were included, not cells infected by a second round of infection. Cells from the second plate were stained for the expression of class I HLA at 2 days post infection, as this was the first time point at which discrete populations of class I HLA_{high} and class I HLA_{low} expression could consistently be detected. The cells were analysed by flow cytometry.

The expression of class I HLA decreased dramatically following CMV infection in almost all cells in the population infected with the highest virus dose. In cultures with lower levels of infection, the decrease in class I HLA on a proportion of the cells was accompanied by an increase in class I HLA expression on the remaining cells in the culture. The percentage of infection in each population was determined by analysis of CMV IE antigen expression (Figure 3.1a). The change in class I HLA expression on both populations of cells resulted in the development of two distinct peaks of fluorescence (Figure 3.1b). The percentage of cells which were class I HLA_{high} or class I HLA_{low} was determined for each population. The percentage of HLA_{low} cells was plotted against the percentage of infected cells (Figure 3.2). A high degree of correlation (r^2=0.991) between the percentage of infection and the percentage of cells which were class I HLA_{low} was found.

3.2.2 The expression of CMV antigens in the HLA_{high} and HLA_{low} cell populations.

The previous experiments suggested that the class I HLA_{low} cell population were infected cells, while the class I HLA_{high} population were uninfected. In order to confirm this, it was necessary to measure both surface class I HLA levels and CMV status in the same cell. It was not possible to perform simultaneous double staining for surface class I HLA and CMV antigens, as conditions could not be established whereby the detection of the CMV IE antigen, which is expressed in the nucleus, could be carried out without affecting the levels of cell surface molecules. Although CMV glycoproteins can be detected on the cell surface and in the cytoplasm at later stages of infection, these are not present at the time points at which HLA downregulation initially occurs, so could not be used as a marker of infection in these experiments. Cells expressing high and low levels of class I HLA were therefore separated using a fluorescence activated cell sorter (FACSORT, Becton Dickinson), and the separated populations subsequently stained for the expression of CMV IE or late antigens. The use of monoclonal antibodies of different isotypes for the staining of class I HLA, CMV IE antigen and glycoprotein B allowed this to be carried out using isotype specific secondary antibodies.

Mixed populations of infected and uninfected cells were stained at 3 days post infection for surface expression of class I HLA, using the monoclonal antibody W6/32 (IgG2a), followed by detection using PE conjugated isotype specific secondary antibodies. The cells were then separated into two populations based on either high or low expression of class I HLA. The sorting was carried out by Dr. Mark Lowdell, Department of Haematology, RFHSM. Populations of >90% purity in the case of HLA_{low} cells, and >95% purity in the case of HLA_{high} cells were purified using this method, as determined by subsequent flow cytometric analysis. Profiles from a representative experiment are shown in Figure 3.3. Cytospin preparations were made from each population of cells, and stained with monoclonal antibodies specific for CMV IE (63.27, IgG1) or late proteins (gB, 7.17, IgG3), which were detected by FITC conjugated, isotype specific secondary antibodies (Table 2.3). Visual examination showed that the population of HLA_{low} cells was composed of cells expressing both CMV IE (>90% of cells) and late antigens (approximately 25% of cells). The population of HLA_{high} cells was composed primarily of uninfected cells, but had some contamination by cells expressing IE antigen only (<10%). No cells expressing late antigens were present in this population. The IE positive cells appeared to

be cells which had recently become infected, due to the absence of CMV late antigens, but may also have been present due to inaccuracy of the cell sorting procedure. These results confirmed that the phenotype of class I HLA_{low} represented the infected population, and that class I HLA_{high} represented the 'uninfected bystander' cells.

3.2.3 The intensity of class I HLA expression on 'uninfected bystander' cells from mixed populations.

It can be seen from Figure 3.1 that class I HLA expression on the class I HLA_{high} populations was higher than expression on uninfected fibroblasts. To determine the magnitude of this increase, populations with approximately 50% infected cells were generated, and stained for the expression of class I HLA. The levels of expression on the class I HLA_{high} populations from these mixed populations were compared to the levels on cells from uninfected populations. A profile from a representative experiment is shown in Figure 3.4(a). The average levels of class I HLA on the HLA_{high} population were significantly increased in comparison to uninfected cells (mean fold increase = 4.64 ± 0.67, $p=0.005$, $n=5$ experiments) (Figure 3.4(b)). There were two possible explanations for this increase. Firstly, a soluble factor released from infected cells may have increased class I HLA expression on uninfected bystander cells. Secondly, a component of the viral inoculum may have increased class I HLA expression on those cells which did not become infected.

3.2.4 The effect of virus dose on the intensity of class I HLA expression on infected and uninfected bystander cells.

Having demonstrated that class I HLA expression on 'uninfected bystander' cells was increased in comparison to uninfected cells, it was also of interest to determine the relationship between virus dose and the amount of upregulation on these cells. A soluble factor released by infected cells would be expected to have a greater effect when the proportion of infected cells in the population is higher. Whether viral dose affected the extent of downregulation of class I HLA in the infected population was also investigated.

Cells were infected with virus inoculum at an MOI ranging from 0.078 to 5 pfu/cell. Staining was carried out at day 2 and day 3 post infection. The level of class I HLA expression on the class I HLA_{high} cells was increased as the

proportion of infected cells in the population increased. Class I HLA expression on uninfected bystander cells was higher at 2 days than at 3 days post infection (Figure 3.5). Class I HLA expression on infected cells was decreased in a proportion of cells at all virus doses used, corresponding with the percentage of infected cells. There was little change in class I HLA downregulation on the infected population in response to increasing virus dose (Figure 3.5).

3.2.5 Demonstration of increased class I HLA on uninfected bystander cells using a dual chamber system.

The results from the previous section suggested that the increase in class I HLA expression on uninfected bystander cells may be due to the release of soluble factors by infected cells. To investigate this possibility, a dual chamber system was used, in which uninfected cells growing on a semi-permeable membrane (0.45μm pore size) in a cell culture insert were exposed to soluble factors released from infected cells growing in the same well.

Preliminary experiments were carried out to test the efficiency of transfer of cytokines across the membranes. IL-1β (1ng/ml) or IFNβ (100 IU/ml) was added to fibroblasts in tissue culture plates, and monolayers of fibroblasts on cell culture inserts were added to these wells and incubated for 24 hours. ICAM-1 expression on cells exposed to IL-1β was comparable on cells on the insert or in the plate, indicating efficient transfer of this factor across the membrane. Transfer of IFNβ was tested by the expression of class I HLA in a similar way, and again similar levels were found on cells on both sides of the membrane (Table 3.1).

To investigate the effect of soluble factors released from infected cells on class I HLA expression on uninfected cells, cells growing in the plate were infected for the usual one hour period, or left uninfected, and washed before addition of a cell culture insert coated with uninfected fibroblasts. At 2 days post infection, fibroblasts from the plates and from the cell culture inserts were removed by trypsinization and stained for class I HLA expression (Figure 3.6). Uninfected fibroblasts which had been exposed to infected fibroblasts expressed increased levels of class I HLA in comparison to uninfected fibroblasts (Mean fold increase above uninfected levels = 1.22±0.036, mean±SEM, $p=0.004$, $n=5$ experiments). This suggested that a soluble factor was released by infected cells which increased class I HLA expression on uninfected cells.

3.2.6 The effect of ganciclovir and foscarnet on class I downregulation on infected fibroblasts.

Ganciclovir and foscarnet are antiviral agents used for the treatment of CMV clinically. They allow immediate early and early gene expression, but inhibit the viral DNA polymerase, thus preventing DNA replication and late protein synthesis. We have used these agents to prevent CMV late protein synthesis in infected fibroblasts, in order to determine whether late proteins were required for the downregulation of HLA on infected cells.

Infected and control uninfected fibroblasts were treated with ganciclovir or foscarnet at concentrations of 1, 10 and 100μg/ml, or left untreated. These concentrations of ganciclovir and foscarnet were shown in section 2.7 to abrogate CMV late protein expression, while permitting CMV IE expression. Cell surface levels of class I HLA were analysed at 3 days post infection by flow cytometry. Treatment of infected cells with ganciclovir or foscarnet did not affect the downregulation of class I HLA which occurred following CMV infection. A representative experiment is shown in Figure 3.7. These findings suggested that CMV IE or early proteins were responsible for the downregulation of class I HLA.

3.2.7 The effect of ganciclovir on the upregulation of class I HLA on 'uninfected bystander' cells in mixed populations.

It was important to determine whether ganciclovir treatment abrogated the increase in class I HLA expression on 'uninfected bystander' cells, as this may be important *in vivo*. Mixed populations of infected and uninfected fibroblasts were generated by infection with CMV inoculum at an MOI of approximately 0.5 pfu/cell. Following infection, cells were incubated with ganciclovir (50μg/ml), or with medium alone. Cells were analysed for the expression of class I HLA at 2 days post infection. The cells treated with ganciclovir formed two populations of cells expressing high and low levels of class I HLA similar to the cells incubated in medium alone (Figure 3.8). This showed that immediate early or early events following infection were responsible for both the class I downregulation on infected cells and the upregulation on 'uninfected bystander' cells.

3.2.8 Detection of IFNβ levels in CMV infected cultures.

It was suspected that the soluble factor responsible for class I HLA upregulation on uninfected cells was IFNβ, as this is known to be induced following CMV infection of fibroblasts (Steinmassl and Hamprecht, 1994). To determine the levels of IFNβ produced under the conditions used in this study, IFNβ levels were determined by immunoassay of supernatants harvested from fibroblasts infected at an MOI of 5 pfu/cell, and uninfected fibroblasts, at 6, 12, 24 and 48 hours post infection. IFNβ was detected at 24 and 48 hours post infection in supernatants from infected fibroblasts (3.4 IU/ml; maximum level detected), and was undetectable in supernatants from uninfected fibroblasts (Table 3.2). IFNβ production was also measured in cells infected with 'purified' virus preparations, which were used to produce more concentrated viral inoculum. The maximum amount of IFNβ produced in response to this inoculum at 24 hours post infection was 13.7 IU/ml (Figure 3.9).

3.2.9 The effect of recombinant IFNβ on class I HLA expression.

In order to determine the levels of class I HLA induced by recombinant IFNβ, for comparison with the levels induced by soluble factors released from infected cells, fibroblasts were treated with IFNβ at concentrations of 1, 10 and 100 IU/ml and their expression of class I HLA was analysed by flow cytometry at days 1 to 4 post treatment. Increases in class I HLA expression could be detected from day 1 post treatment at all the concentrations used. Results are shown for day 3 post treatment only, when class I MHC expression had increased to a maximum of 5 fold the levels on untreated uninfected cells in response to 100 IU/ml of IFNβ (Figure 3.10).

The class I HLA levels achieved by treatment with IFNβ were similar to the levels induced in cells exposed to infected fibroblasts in a dual chamber system, but were significantly lower than the levels induced in 'bystander uninfected' cells from infected cultures. It thus appeared that IFNβ or other soluble factors released from infected cells could not account for all the upregulation of class I HLA on 'uninfected bystander' cells.

3.2.10 The role of IFNβ in the class I HLA upregulation on cells exposed to infected cells in a dual chamber system.

To investigate the contribution of IFNβ to the upregulation of class I HLA on cells exposed to infected fibroblasts in a dual chamber system, identical experiments to those in section 3.2.5 were carried out, with the inclusion of neutralising antibodies specific for IFNβ. The antibody added was sufficient to neutralise at least 10 IU of recombinant IFNβ, as tested in Table 2.4. Fibroblasts were stained for the expression of class I HLA at 3 days post infection. Increased surface expression of class I HLA took place on cells from cell culture inserts which were incubated with infected fibroblasts, whereas no increase took place on control cells incubated with uninfected fibroblasts, in accordance with the results in section 3.2.5. The addition of anti-IFNβ antibody completely abrogated the increase in class I HLA which occurred on the cells incubated with infected fibroblasts (Figure 3.12). In contrast, antibodies specific for IL-1β and TNFα, added in sufficient quantity to neutralise 0.5ng/ml of their respective cytokines (see Table 2.4), had no effect on the increase in class I HLA on these cells. This suggested that the soluble factor released by infected cells which was responsible for the increased class I HLA expression on uninfected cells in the dual chamber system was IFNβ.

3.2.11 Abrogation of Increased class I HLA on uninfected bystander cells by blocking the activity of IFNβ.

To determine whether IFNβ was also responsible for the increased class I HLA expression on uninfected cells in mixed populations of infected and uninfected fibroblasts, neutralising antibodies specific for IFNβ were also added to experiments similar to those in section 3.2.3. Mixed populations of infected and uninfected cells were generated, which were subsequently shown to contain 25-35% of infected cells. Neutralising antibodies specific for IFNβ were added during and immediately following the 1 hour infection period. Fibroblasts were stained for the expression of class I HLA at 3 days post infection. The addition of anti-IFNβ antibody significantly reduced the expression of class I HLA on uninfected bystander cells, but not to the levels seen in uninfected cultures (mean decrease = 63.3% ± 3.78%, mean±SEM, $p=0.002$, $n=3$ experiments). A representative experiment is shown in Figure 3.12. The reason for the incomplete abrogation of the effect on class I HLA may have been due to insufficient neutralising activity from the amount of antibody added, but this was

unlikely, as the levels of IFNβ produced by infected cells were never higher than 13.7 IU/ml when measured, and the antibody was sufficient to neutralise this amount of recombinant IFNβ. It was not possible to test whether the addition of a higher concentration of antibody would decrease the class I HLA expression to uninfected levels, as a large quantity of antibody had been used in the previous experiments, and unfortunately the source was no longer available.

The results of these experiments suggested that most of the class I HLA upregulation on 'uninfected bystander' cells was due to the action of IFNβ released by infected cells in the same well, but that some upregulation was caused by other factors. A fundamental difference between this type of experiment and the dual chamber experiments was that the uninfected bystander cells had been exposed to viral inoculum alongside infected cells during the initial infection. This suggested that a component of the inoculum might be responsible for some of the class I HLA upregulation on the uninfected bystander cells. Possible candidates are defective viral particles, which could be present at high levels in viral inoculum, or other cytokines. Either factor could perhaps explain the higher levels of class I HLA expression in 'uninfected bystander' cells from infected wells, in comparison to cells exposed to supernatants released by infected cells or treated with recombinant IFNβ.

3.2.12 The effect of UV inactivated CMV on the expression of cell surface class I HLA.

UV inactivation destroys the ability of the virus to replicate, but virions remain structurally intact. It was not expected that infection with UV inactivated virions would lead to class I HLA downregulation, as this is a function of a viral gene expressed at early times post infection. However, it was possible that UV inactivated virions could induce expression of class I HLA on the treated cells as was suggested in the previous section.

Cells were treated with CMV inoculum at an MOI of 2-4 pfu/cell, with an equivalent aliquot of inoculum which had been UV inactivated, or remained uninfected. Cells were stained for the expression of CMV IE antigen and surface expression of class I HLA at 3 days post infection. Treatment of fibroblasts with UV inactivated virus inoculum resulted in no detectable expression of CMV IE antigen. However, surface expression of class I HLA was significantly enhanced on cells treated with UV inactivated viral inoculum, in

contrast to the decrease in class I HLA expression on cells infected with infectious virus (Figure 3.13). This shows that non infectious viral particles are capable of causing upregulation of class I HLA on uninfected fibroblasts, and supports the idea that defective particles in the inoculum may be partially responsible for the upregulation of class I HLA on 'uninfected bystander' cells.

Table 3.1. Testing the action of recombinant cytokines across semi-permeable membranes[a].

Cytokine	Location of cells	ICAM-1 expression[b]	Class I HLA expression[b]
None	In well	1	1
IL-1β 1ng/ml	In well	5.04	Not done
IL-1β 1ng/ml	On cell culture insert	5.83	Not done
IFNβ 100 IU/ml	In well	Not done	2.28
IFNβ 100 IU/ml	On cell culture insert	Not done	2.21

[a]Fibroblasts were seeded in tissue culture plates or onto the semi-permeable membranes of cell culture inserts. Recombinant cytokines were added to the medium in the wells, and the cell culture inserts placed in the wells. Cells from the wells and the inserts were removed by trypsinisation after 24 hours, and the expression of ICAM-1 or class I HLA was measured by flow cytometry.

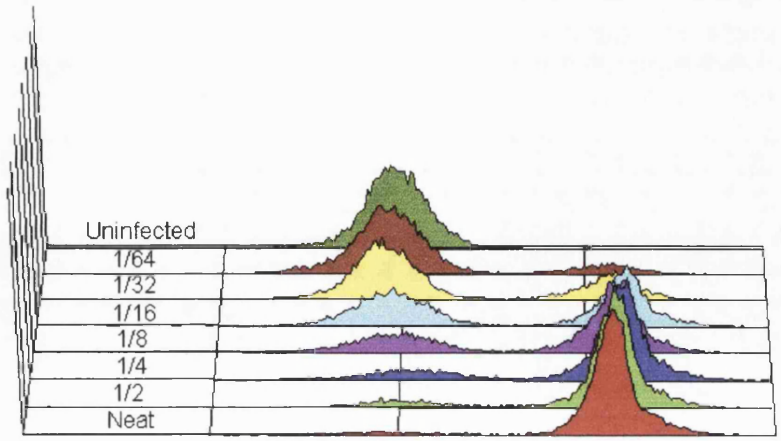
[b]Values represent the intensity of either ICAM-1 or class I HLA expression in fluorescence intensity units in relation to the expression of each molecule on uninfected, untreated cells, which has been assigned a value of 1.0. The results shown are mean values from duplicate wells.

Table 3.2. IFNβ production following CMV infection[a].

Hours post infection	Infected	Uninfected
6	-ve	-ve
12	-ve	-ve
24	3.484 IU/ml	-ve
48	2.743 IU/ml	-ve

[a] Fibroblasts were infected with CMV at a multiplicity of infection of 2-4 pfu/cell, or remained uninfected. Supernatants were harvested at the times post infection indicated on the table, and assayed for the presence of IFNβ by ELISA. The results shown are mean values from duplicate wells for a representative experiment.

(a) CMV E13 expression



(b) Class I HLA expression

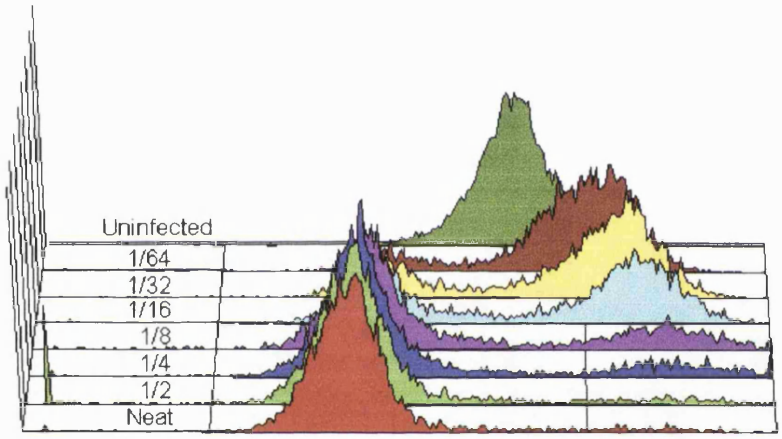


Figure 3.1. The effect of virus dose on the expression of class I HLA.

Duplicate plates of subconfluent fibroblast monolayers were infected with neat viral inoculum, or two-fold serial dilutions of that inoculum, corresponding to an MOI of 5 pfu/cell at the highest dose to 0.078 pfu/cell at the 1/64 dilution, or remained uninfected. At 24 hours post infection cells from one set of plates were removed by trypsinisation and stained for the expression of CMV IE antigen (a). At 2 days post infection cells were stained for the surface expression of class I HLA (b). The flow cytometric profiles from a representative experiment are shown. Dead cells and cell debris were excluded by gating using forward and side light scatter. Each axis is labelled with the dilution of viral inoculum used for infection.

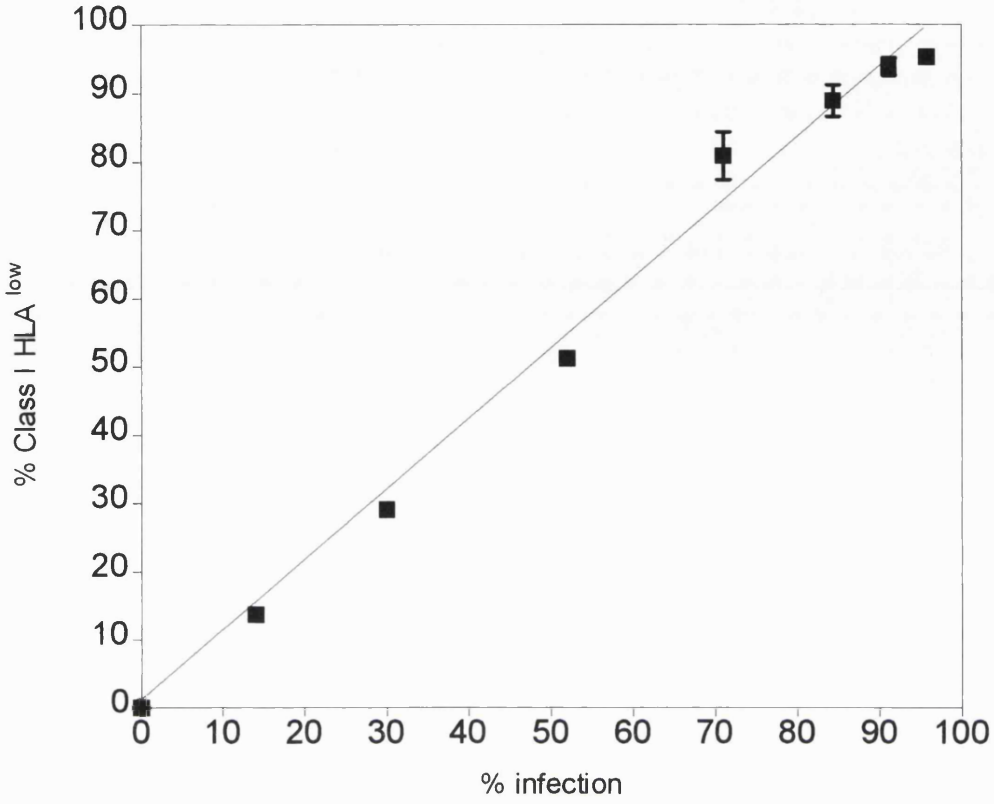


Figure 3.2. The correlation between class I HLA downregulation and CMV IE gene expression.

The percentage of cells positive or negative for CMV IE antigen, and high or low for class I HLA expression, at each virus dilution shown in Figure 3.1 was determined by placing markers around the separate peaks. The percentage of cells expressing CMV IE antigen has been plotted against the percentage of cells which have low class I HLA expression. The data represents means of triplicate values ± SD for a representative experiment similar to that shown in Figure 3.1.

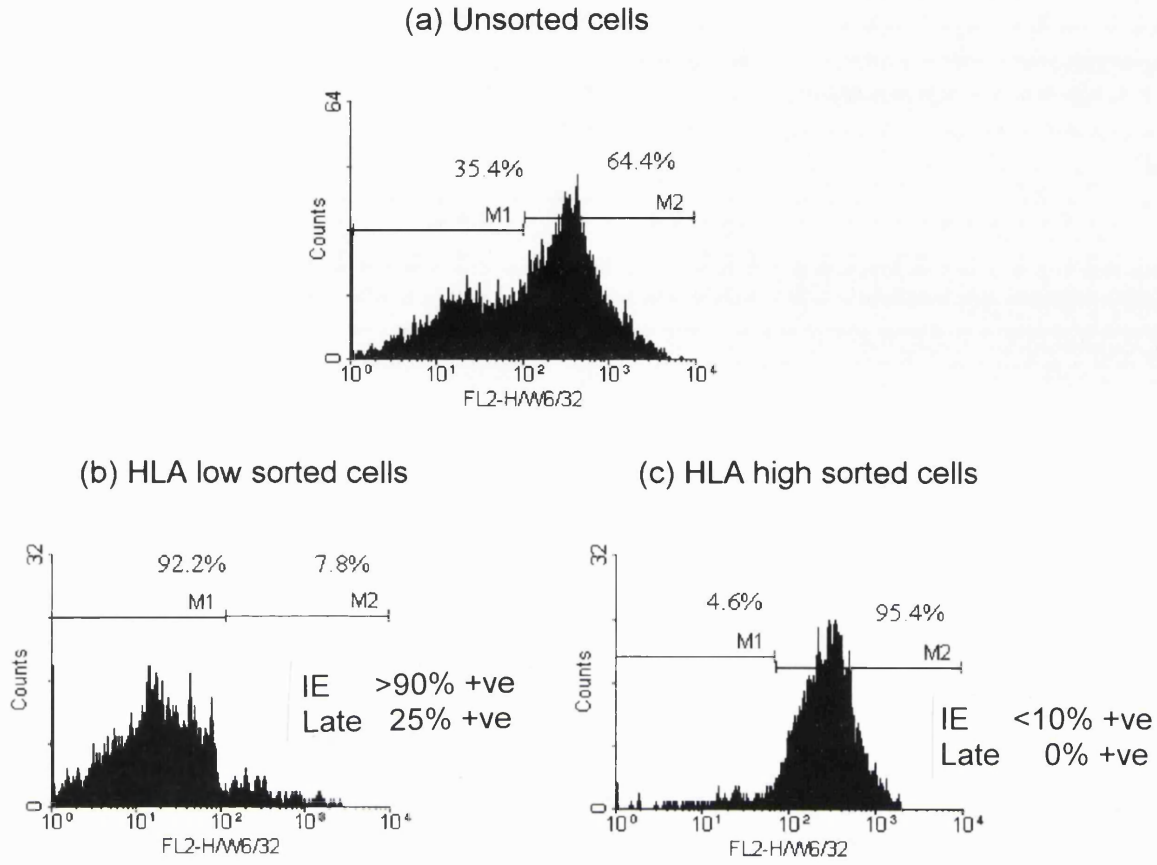


Figure 3.3. Cell sorting of class I HLA high and low populations.

Subconfluent fibroblast monolayers were infected with a virus dose insufficient to infect all the cells in the culture. At 2 days post infection the cells were removed by trypsinisation and stained for the surface expression of class I HLA using the monoclonal antibody W6/32, and a PE conjugated isotype specific secondary antibody for detection. 35% of the cells were found to express low levels of class I HLA (a). The cells were then sorted on the basis of the class I HLA expression using a Becton Dickinson FACSORT. Subsequent analysis of the sorted populations by flow cytometry revealed that the cells sorted on the basis of low HLA expression were >90% pure (b), while the cells sorted on the basis of high HLA expression were >95% pure (c). Cytospin preparations of these cells were subsequently stained for the expression of CMV IE and late antigens (gB), and examined visually. Approximate percentages of cells positive for these antigens in the sorted populations are shown alongside the figures.

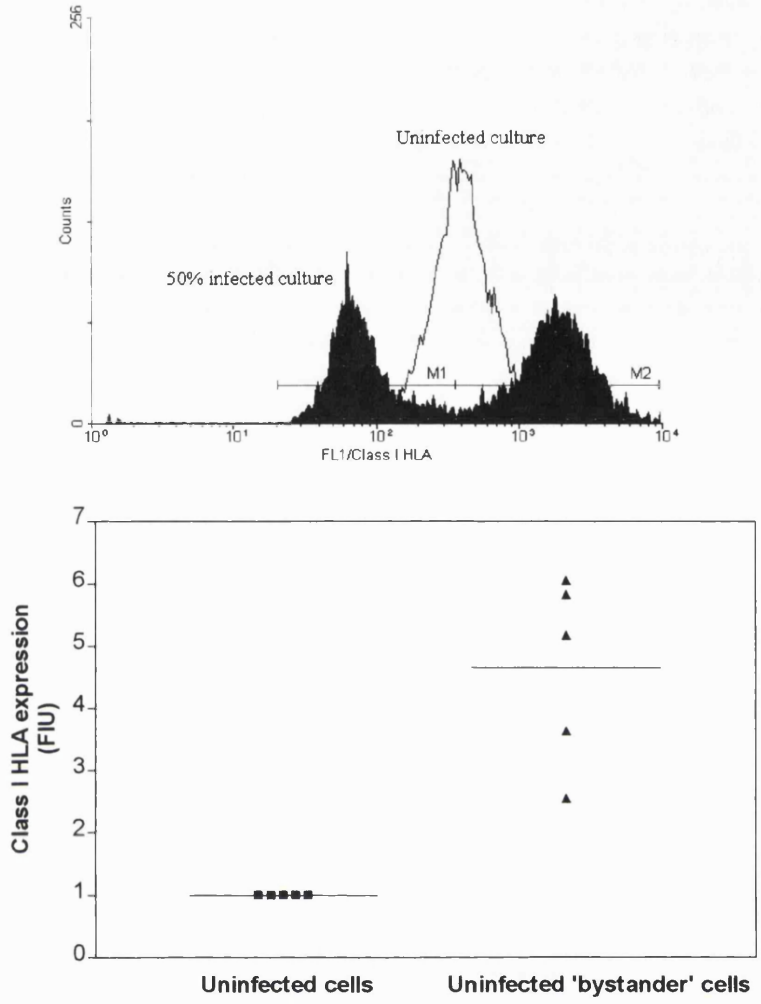


Figure 3.4. The intensity of surface expression of class I HLA on uninfected bystander cells.

Cultures were generated which contained approximately 50% infected fibroblasts. At 2 days post infection the cells from infected cultures, and cells from uninfected control cultures, were removed by trypsinisation and stained for the surface expression of class I HLA. A marker was placed around the cells from the mixed cultures (shown in black) expressing high class I HLA (M1), and the fluorescence intensity was determined. This was compared to the intensity of expression on cells from uninfected cultures (shown in white). A profile from a representative experiment is shown (top panel). The bottom panel shows the combined results from 5 separate experiments, in which the data are expressed as fluorescence intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0.

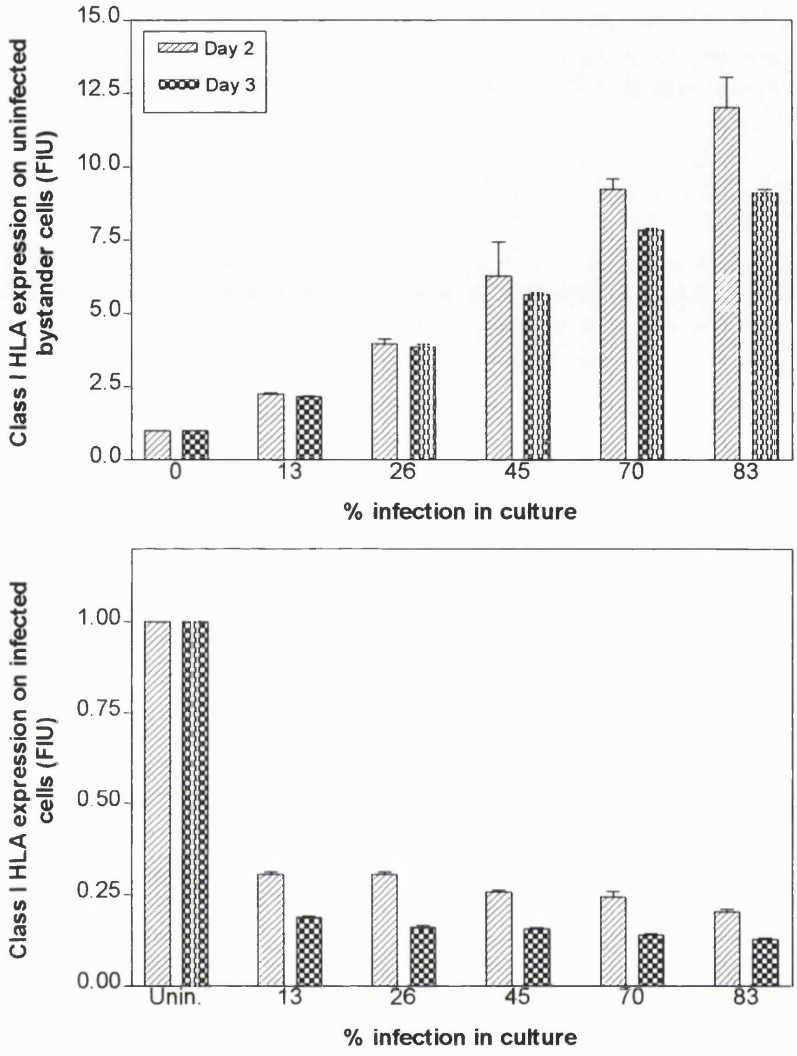


Figure 3.5. The effect of virus dose on the intensity of class I HLA expression on uninfected 'bystander' cells and infected cells.

Fibroblasts were infected with two-fold serial dilutions of viral inoculum ranging from neat to 1/64. The percentage of infection shown on the x axis was determined by staining replicate cultures for the expression of CMV IE antigen at 1 day post infection. The remaining cultures were stained for the surface expression of class I HLA at day 2 (top panel) and day 3 (bottom panel) post infection. Dead cells and debris were excluded by gating, and markers were placed on the low and high peaks of class I HLA expression, representing infected and uninfected 'bystander' cells respectively, as shown in Figure 3.4. The fluorescence intensity of the high and low HLA peaks from triplicate wells of each culture are expressed as fluorescence intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0.

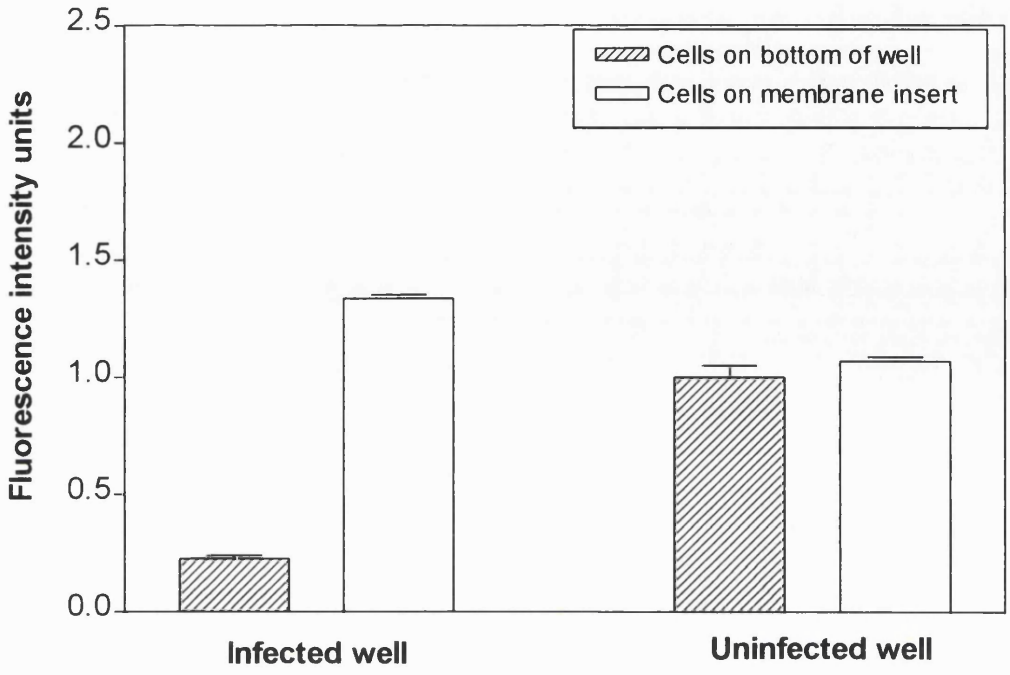


Figure 3.6. Class I HLA expression of uninfected cells exposed to soluble factors released from CMV infected fibroblasts across a semi-permeable membrane.

A dual chamber system was used in which cells were separated by a semi-permeable membrane. Cells grown on the base of the well were infected or uninfected as indicated, whilst those growing on the membrane insert were uninfected. In the case of wells containing CMV infected cells, the membrane inserts were added to the wells following the 1 hour virus adsorption period. Cells were removed by trypsinisation 2 days after infection, and the cell surface expression of class I HLA measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells grown on the base of the well, which has been assigned the value of 1.0. The data shown is one of four representative experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates.

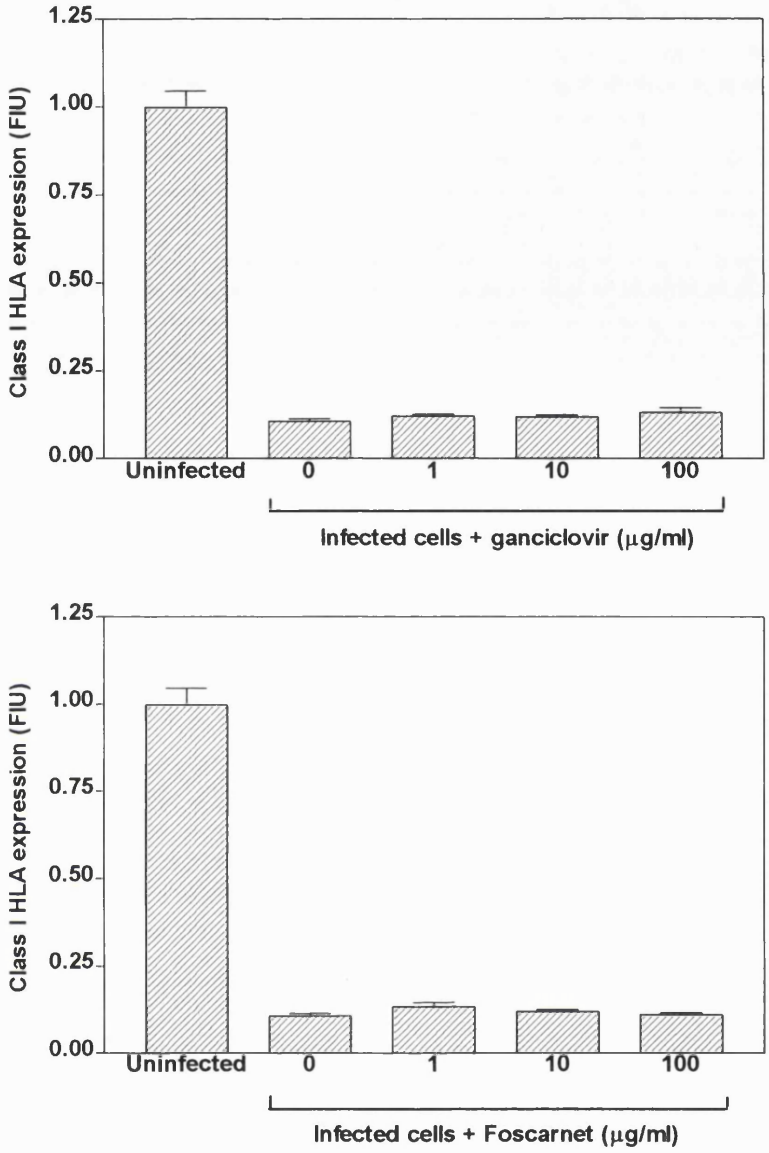


Figure 3.7. The effect of ganciclovir or foscarnet treatment on class I HLA expression on infected fibroblasts.

Fibroblasts were infected with CMV at an MOI of 2-4 pfu/cell, and treated with various concentrations of ganciclovir (top panel) or foscarnet (bottom panel). Cells were removed by trypsinisation 3 days post infection, and cell surface class I HLA expression measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells, which have been assigned the value of 1.0. The data shown is one of three representative experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates.

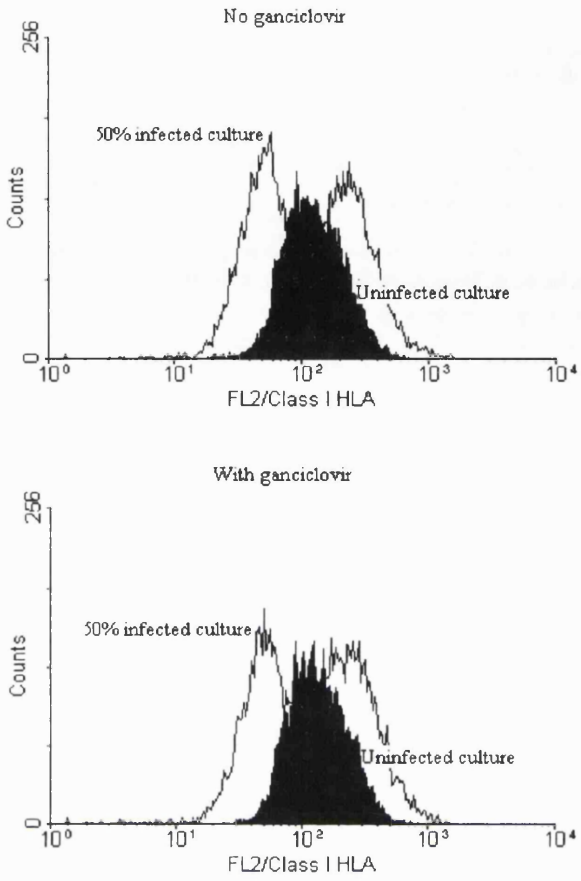


Figure 3.8. The effect of ganciclovir treatment on class I expression on uninfected bystander cells.

Cells were infected with CMV at an MOI of 0.5pfu/cell (unfilled histograms), or remained uninfected (filled histograms), and incubated with medium alone (top panel) or medium containing ganciclovir at 50μg/ml (bottom panel). At 2 days post infection the cells were harvested by trypsinisation and stained for the surface expression of class I HLA. Markers were placed around the high class I HLA peaks as shown in Figure 3.4, and the fluorescence intensity was determined. This was compared to the fluorescence intensity of uninfected fibroblasts. Flow cytometric profiles from one representative experiment from three independent experiments is shown.

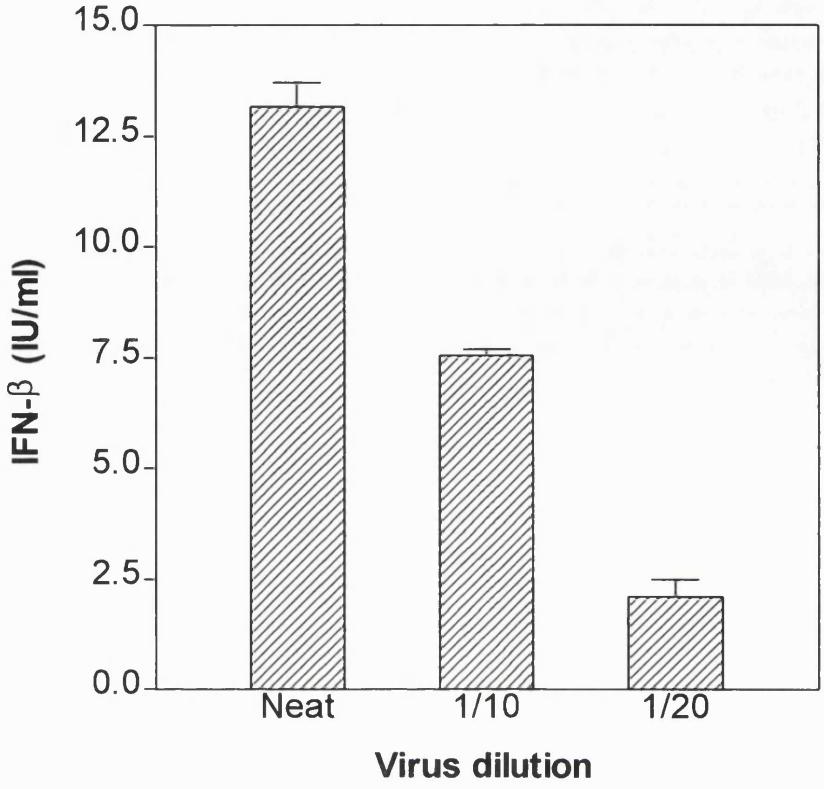


Figure 3.9. Production of IFNβ by cells infected with CMV.

Cells were infected with 3 dilutions of a CMV preparation which had been concentrated by ultracentrifugation and resuspended in medium. At 24 hours post infection the supernatant was removed and assayed for the presence of IFNβ by ELISA. The data shown are mean ± SD from triplicate samples.

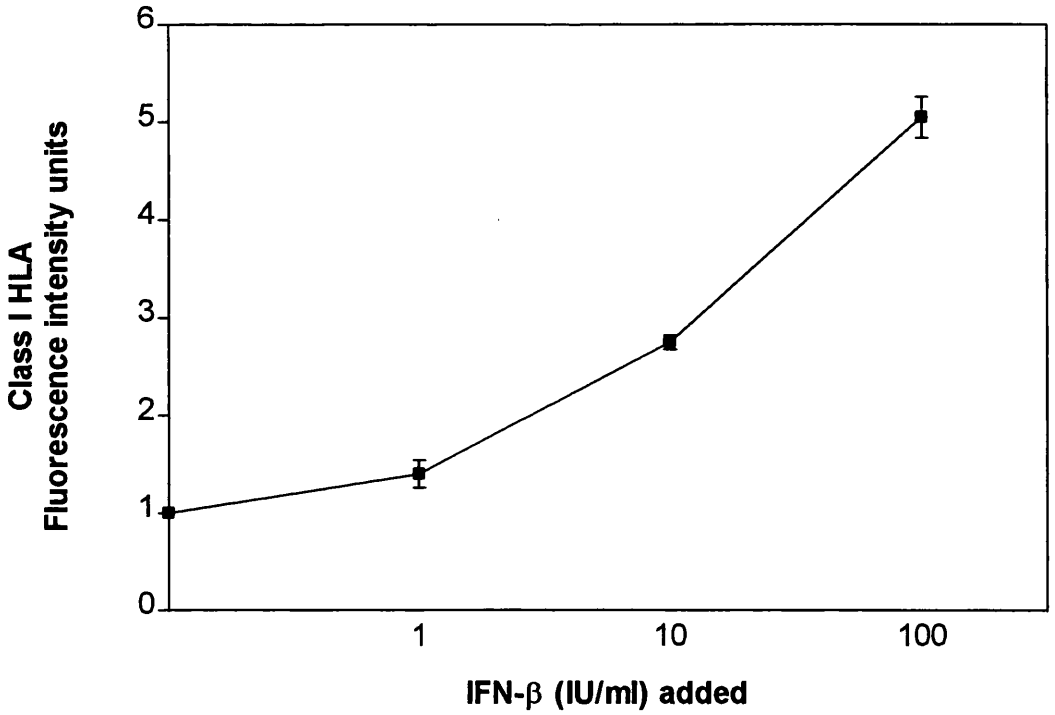


Figure 3.10. Effect of recombinant IFNβ on class I HLA expression.

Following treatment with recombinant IFNβ for 24 hours, fibroblasts were stained for the surface expression of class I HLA. The results are expressed as fluorescent intensity units relative to the mean level of expression on untreated fibroblasts, which has been assigned the value of 1.0. The data shown are the mean ± SD from triplicate samples.

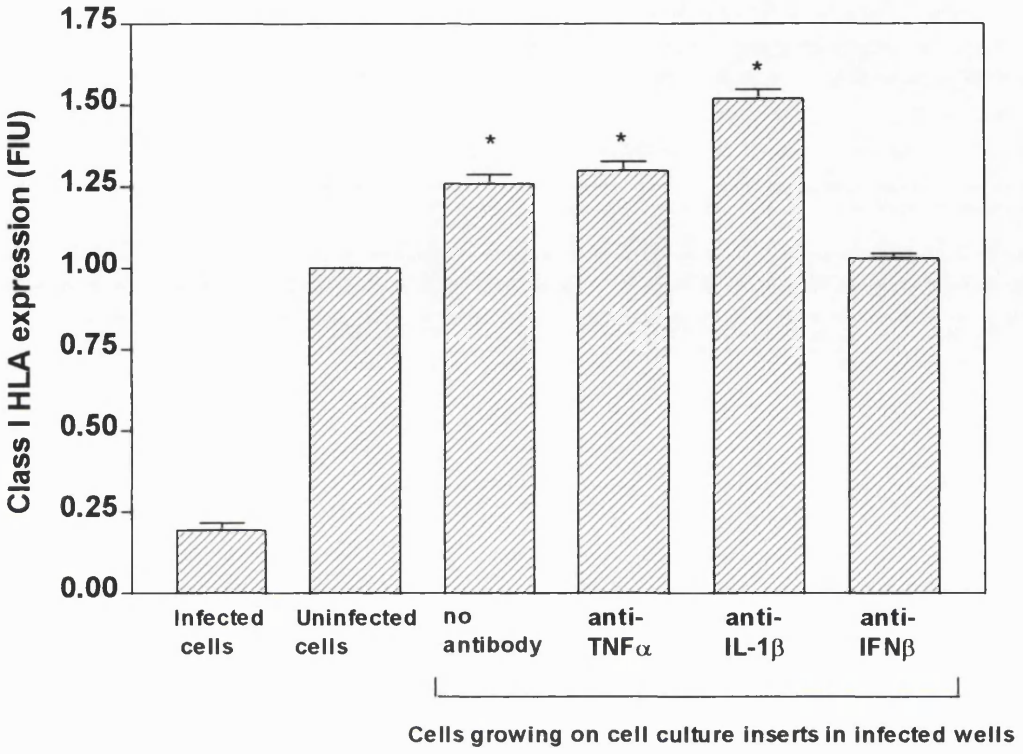


Figure 3.11. The role of IFNβ in the class I HLA increase on uninfected cells exposed to infected cells across semi-permeable membranes.

A dual chamber system identical to that described in Figure 3.6 was used. Immediately following infection, neutralising antibodies specific for IL-1β, TNFα, and IFNβ were added to the infected wells as indicated on the figure, and supplementary antibody (1/10 of the original concentration) was also added the next day. Cells were removed by trypsinisation 2 days after infection, and the cell surface expression of class I HLA was measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells grown on the base of the well, which has been assigned the value of 1.0. The data shown is one of two representative experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates. The asterisks indicate significantly increased ($p<0.05$) levels of expression compared to that on uninfected cells.

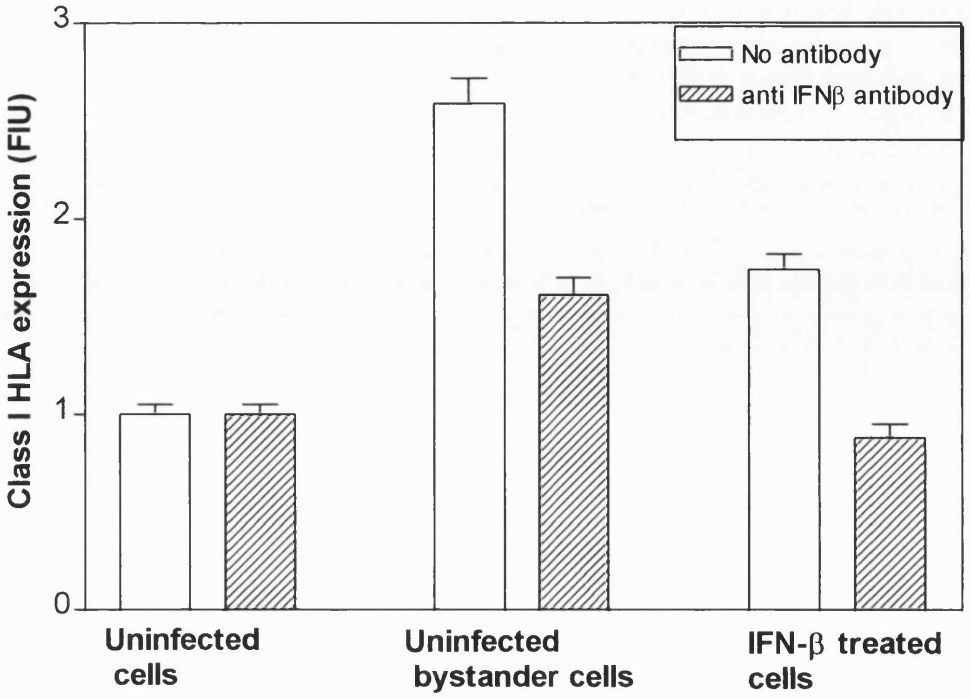


Figure 3.12. The role of IFNβ in the class I HLA increase on uninfected 'bystander' cells from mixed cultures of infected and uninfected cells.

Cultures were generated which contained approximately 50% infected fibroblasts. Neutralising antibodies specific for IFNβ were added to infected cells both during and immediately following infection. Control cells were treated with 10 IU/ml recombinant IFNβ to confirm the neutralising activity of the antibody used. At 2 days post infection or treatment the cells from infected cultures, and cells from control cultures, were removed by trypsinisation and stained for the surface expression of class I HLA. A marker was placed around the cells from the mixed cultures expressing high class I HLA, and the fluorescence intensity was determined. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0. The data shown is one of two representative experiments, each of which was carried out in triplicate. The figure shows the mean ± SD of the three replicates.

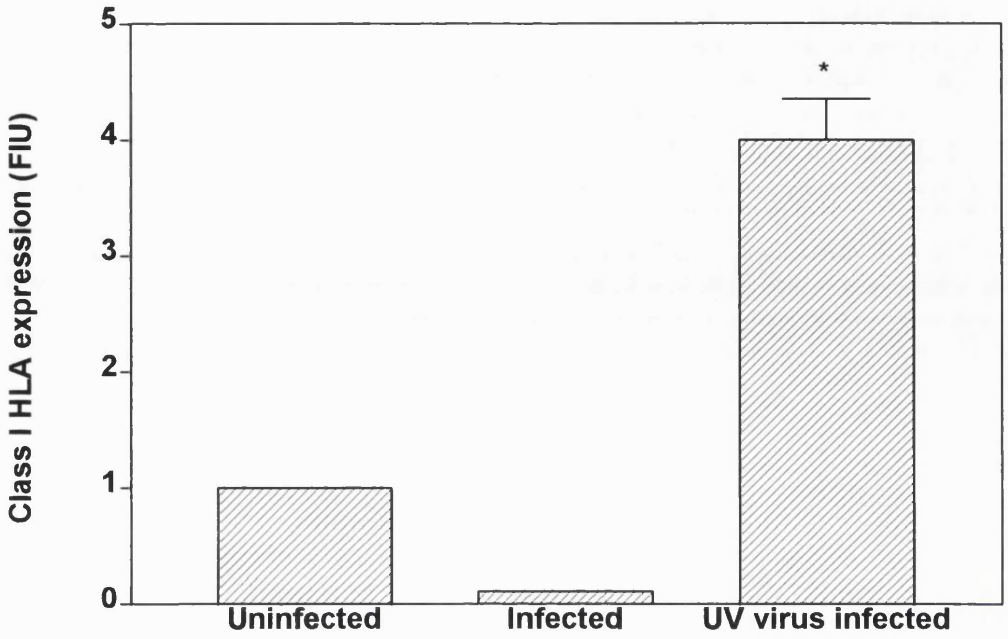


Figure 3.13. The effect of UV inactivated virus on class I HLA expression.

Virus was UV inactivated and used to infect cells alongside infectious virus. Cells were removed by trypsinisation 2 days after infection, and the cell surface expression of class I HLA was measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0. The data shown is one of three representative experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates. The asterisk indicates significantly increased ($p<0.05$) levels of expression compared to that on uninfected cells.

3.3 DISCUSSION

The results presented in this chapter provide evidence that class I HLA surface expression was downregulated specifically on infected fibroblasts following CMV infection, and was upregulated on uninfected cells which were exposed to infected cells. The correlation of the percentage of infection with the percentage of class I HLA_{low} cells provided indirect evidence that the virus infected cells had specifically downregulated their class I HLA. In order to ascertain that this was the case, cells with class I HLA_{low} and class I HLA_{high} expression were physically separated by cell sorting, and double stained for the expression of CMV antigens. Almost all of the cells with low class HLA showed expression of CMV immediate early antigens, and around 25% expressed the CMV late antigen, gB. The class I HLA_{high} population, in contrast, were predominantly negative for CMV antigens, although a small proportion of the cells were expressing CMV immediate early antigen. It is probable that the IE positive cells were present due to inaccurate separation of the two populations by the sorter, or that they had recently become infected and had not yet downregulated their class I HLA, as there was no expression of CMV late antigens in this population. These 'uninfected bystander' fibroblasts had higher expression of class I HLA than uninfected fibroblasts. From this data it was concluded that the downregulation of class I HLA is a direct effect of viral infection, and that increased class I HLA is a secondary effect which occurs on uninfected cells co-cultured with infected cells.

Since the completion of this part of the present study, similar conclusions have been reached by another group (Steinmassl and Hamprecht, 1994) using fibroblasts which were permeabilized with digitonin before double staining for class I HLA and CMV antigens. In the latter study, virtually all cells with low class I HLA expressed CMV antigens, as we have shown in the present study. However, in the Steinmassl and Hamprecht study, the HLA_{high} population also contained a large proportion of cells expressing CMV IE antigen, as opposed to the results presented here, showing a maximum of 5% of CMV IE positive cells in the HLA_{high} population. The digitonin permeabilization of the cells before staining in the latter study, as opposed to surface staining in the current study, may be responsible for these differences. Class I HLA has been shown, by both immunofluorescent staining and immunoprecipitation, to accumulate inside infected cells, concomitant with decreased surface expression (Barnes and Grundy, 1992a; Yamashita *et al.* 1993). It is thus possible that the cells expressing both CMV IE antigen and high cytoplasmic class I HLA also had low surface class I HLA expression. The differences may also have been due to the

time point at which the cells were analysed. In the present study, the experiments were carried out at 2 days post infection, before virus had been released from the cells, in order to avoid secondary infection. In the Steinmassl and Hamprecht study, cells were analysed at 3 days post infection, when virus could already have been released from infected cells. The cells expressing high levels of class I HLA alongside CMV IE antigen may therefore have been recently infected but had not yet downregulated their class I HLA.

Several investigators have concluded that class I HLA was increased following CMV infection (Grundy *et al.* 1988a; van-Dorp *et al.* 1989; van Dorp *et al.* 1993b; Ustinov *et al.* 1991; Tuder *et al.* 1991; Tuder *et al.* 1994). However, in these studies, the levels of class I HLA expression were measured by techniques which provided an average reading of the intensity of expression on a population of cells. In retrospect it seems obvious that the readings were flawed by the contamination of the cultures with a large proportion of uninfected cells which skewed the readings and gave an overall impression of an increase in class I HLA, due to the high levels on uninfected bystander cells. From the experiments presented here, examining the effect of different dilutions of virus inoculum on class I HLA expression, it was clear that two different populations of cells, with high and low levels of class I HLA, were present in fibroblast populations infected at a low MOI, and that it was essential to use either flow cytometry or fluorescent microscopy to determine the class I HLA expression on each population separately.

In the present study we have shown that IFNβ was released by infected cells, and was responsible for at least some of the upregulation of class I HLA on 'uninfected bystander' cells. The addition of anti-IFNβ antibody to dual chamber experiments abrogated class I HLA upregulation on uninfected cells exposed to soluble factors released from infected cells. However, in mixed populations of infected and uninfected fibroblasts, the addition of anti-IFNβ antibody, both in the viral inoculum and during the subsequent culture period, did not completely reduce the class I HLA expression on uninfected bystander cells to uninfected cell levels. It was unlikely that insufficient antibody was the cause of the incomplete abrogation of the effect, as the antibody had been tested against recombinant IFNβ. Furthermore, the successful abrogation of class I upregulation on cells from dual chamber experiments, using an identical concentration of anti-IFNβ antibody, suggested that the amount of antibody used was in excess. The incomplete abrogation of class I HLA upregulation on uninfected bystander cells therefore supports the idea that the fraction of the

class I HLA upregulation not neutralised by anti-IFNβ antibodies was caused by another constituent of the viral inoculum.

A similar type of experiment performed by another group (Steinmassl and Hamprecht, 1994), also showed incomplete abrogation of class I HLA upregulation on uninfected bystander cells following incubation with a neutralising antibody specific for IFNβ. Thus it is possible that other cytokines capable of upregulating class I HLA are released by infected cells, or that another component of the viral inoculum is responsible. Candidates for this activity are defective or non infectious virus particles. Others have shown a discrepancy between the magnitude of the class I HLA upregulation on uninfected bystander cells in mixed cultures, and the upregulation seen using supernatant transfer, and suggested that this was due to the presence of incomplete or defective viral particles, which are known to be a major component of the viral inoculum (Kobayashi *et al.* 1995). However, these authors did not formally demonstrate the role of either IFNβ or incomplete viral particles in the process. The increase in class I HLA expression in response to UV inactivated virions in the present study further supports the idea that defective virus particles may be capable of inducing the class I HLA upregulation. This may occur via the induction of IFNβ mediated by UV inactivated or non infectious viral particles, or by the action of a virion structural protein.

The current influx of reports of downregulation of class I HLA by different viruses suggests that the process is instrumental to the development of persistent viral infections (Rinaldo, 1994). Resistance to CTL lysis by CMV infected fibroblasts has been shown *in vitro*, using HLA A2.1-restricted CD8+ve CTL clones and MRC-5 fibroblasts (which are HLA A2.1 positive) exogenously loaded with influenza virus matrix protein peptides. Infection of the fibroblasts resulted in a decrease in specific lysis, which was evident from 20 hours post infection, and persisted throughout the infection. This effect was also found in the presence of phosphonoformate (foscarnet), demonstrating that the class I downregulation was mediated either by immediate early or early proteins, but did not occur following treatment with UV-inactivated viral inoculum (Warren *et al.* 1994). These findings correlate with the findings from the present study, as class I HLA was downregulated in the presence of ganciclovir and foscarnet, and was not downregulated by UV-inactivated virions. Presentation of constitutively expressed endogenous peptides and recognition by alloreactive CTL clones, was also decreased by CMV infection, although alloreactivity was sustained for longer than peptide specific reactivity (Hengel *et al.* 1995). That

such a severe decrease in specific lysis occurs following CMV infection is an indication of the potential immune evasion by CMV infected cells, as it is known that only a few hundred class I HLA molecules per cell are required to render a target cell susceptible to T cell killing. The ability of the infected cell to process structural proteins of the incoming virions and present them via the class I pathway appears to be an adaptation of the host to the efficient downregulation of the class I HLA peptide presentation pathway which occurs at early time points following infection (Riddell *et al.* 1991).

It has been shown that pre-treatment of fibroblasts with the cytokines IFNγ, TNF-α, and type I interferons (IFNα and β) can compensate for the negative effect of CMV infection on class I HLA expression, but cannot restore defective antigen presentation in cells which are already infected (Hengel *et al.* 1995). Although the mode of action of these cytokines is not clear, it appears that they enhance the production of class I HLA molecules, and maintain the stability of the mature complexes. The abrogation of the downregulation of class I HLA by cytokines may explain the paradoxical findings of the important role for CMV specific CTLs for protection from CMV disease *in vivo*, alongside resistance to CTL lysis by CMV infected fibroblasts *in vitro.* In normal individuals the immune system may compensate for the actions of CMV in decreasing downregulation of class I HLA, by producing the appropriate cytokines, and thus prevent severe disease. The production of IFNβ by infected fibroblasts would be expected to affect class I HLA downregulation in cells undergoing secondary infection from neighbouring infected cells. Pre-treatment of fibroblasts with supernatants from infected fibroblasts prior to infection was reported to have a protective effect on downregulation of class I HLA (Hengel *et al.* 1995). However in our experience, in cultures with less than 100% infection, the uninfected cells in the culture gradually decrease their class I HLA expression over a period of days (data not shown). It is possible that the release of IFNβ from the infected cells in such cultures does not precede the release of infectious virus by a sufficient time period, but such a mechanism may have a protective role *in vivo.*

The increased expression of class I HLA on neighbouring uninfected cells may be important in allograft rejection *in vivo*, as in infected organs only a small proportion of the cells are infected. That this occurs *in vitro* in the presence of ganciclovir may be important for the understanding of *in vivo* observations. Increased expression of class I HLA by uninfected cells in allografts may lead to recognition of the allograft as foreign by the host immune system. It was shown in the previously mentioned study (Hengel *et al.* 1995) that lower concentrations of cytokines restored the reactivity of alloreactive T cell clones

than that required by peptide-specific clones, suggesting that alloreactive cells have different activation requirements than peptide specific clones. In the following chapter changes in adhesion molecule expression have been investigated following CMV infection, as adhesive interactions between lymphocytes and target cells are important in the recognition of infected cells by T cells.

CHAPTER 4

ADHESION MOLECULE UPREGULATION FOLLOWING CMV INFECTION: CYTOKINE MEDIATED OF DIRECT VIRAL EFFECT?

Chapter 4: Adhesion molecule upregulation following cytomegalovirus infection: cytokine mediated or direct viral effect?

4.1 INTRODUCTION

The cellular immune system plays an important role in the host response to viral infection generally, and to CMV infection specifically. Adhesion molecules are important in the migration of leukocytes into tissues, and for the activation of T cells which specifically recognise a foreign peptide presented on a class I HLA molecule. It is well known that the use of antibodies or soluble ligands to inhibit interactions between adhesion molecules on leukocytes and target cells has a profound effect on lymphocyte function. In recent years, there has been considerable interest in the alteration of the constitutive expression of adhesion molecules following CMV infection. Since the initial report from this laboratory demonstrating the upregulation of ICAM-1 and LFA-3 on the surface of CMV infected fibroblasts (Grundy and Downes, 1993), other reports of increased adhesion molecule expression in a range of cell types infected with CMV have been published, but the mechanisms involved have not yet been identified (Sedmak *et al.* 1994b; Ito *et al.* 1995a). Adhesion molecule upregulation at sites of inflammation *in vivo* is generally believed to occur as a result of the secretion of inflammatory cytokine by T lymphocytes and monocytes. The upregulation of adhesion molecules following CMV infection *in vitro* takes place in the absence of lymphocyte derived cytokines, and could result from: 1) cellular activation initiated by virus binding to the cell membrane, which also occurs in response to non-infectious virions (Boldogh *et al.* 1990; Albrecht *et al.* 1992); 2) the action of viral proteins produced during the replication cycle; or 3) the effects of cytokines either present in the viral inoculum or produced by infected cells (Span *et al.* 1991).

In this chapter we have investigated whether the upregulation of ICAM-1 and LFA-3 expression is a direct effect of virus infection, whether infectious virus is required, and if there is a role for cytokines which may be induced following CMV infection. An effect mediated directly by the virus would be restricted to infected cells only, whereas increased adhesion molecule expression regulated by cytokines may lead to a widespread induction on surrounding cells, with potentially important consequences for the cell surface signals encountered by leukocytes in CMV infected tissues. The differentiation between the two was important, as *in vivo* only a small proportion of the cells in tissues are infected. Ganciclovir and foscarnet are currently used for the treatment/prophylaxis of

CMV infection in transplant recipients and other immunocompromised patients. They are specific inhibitors of CMV late protein synthesis, and have minimal effects on host cell proteins. The effect of these agents on adhesion molecule upregulation may have implications for the design of strategies for the treatment and prevention of CMV disease, and in particular on whether effective antiviral therapy would be likely to affect pro-inflammatory activities of the virus. The effect of these agents on the CMV induced upregulation of adhesion molecules has therefore been investigated.

4.2 RESULTS

4.2.1 Characteristics of cell surface expression of LFA-3 and ICAM-1 following CMV infection of fibroblasts.

To confirm that LFA-3 and ICAM-1 were upregulated following CMV infection, infected and uninfected fibroblasts were analysed for the expression of these two adhesion molecules. Cells were infected at an MOI of 2-4 pfu/cell for this and all subsequent experiments except where a different viral dose is stated in the text. This virus dose resulted in 100% infection of the fibroblast population when tested by staining for CMV immediate early antigen. Levels of adhesion molecules on infected cells throughout this chapter are expressed in fluorescence intensity units (FIU), relative to the levels of each molecule on uninfected control cells at the same time point.

Cells were stained at 3 days post infection, as this was previously shown to be the time when adhesion molecule expression peaked following CMV infection (Grundy and Downes, 1993). Figure 4.1 shows the characteristic forward and side light scatter profiles of uninfected and CMV infected fibroblasts at 3 days post infection. The cell population was gated to exclude dead cells and debris from the subsequent analysis.

LFA-3 expression on the population of uninfected fibroblasts was represented by a single peak that was slightly brighter than the background autofluorescence of the cells, or cells stained with an irrelevant control antibody (Figure 4.1). Infection of the fibroblasts resulted in an increase in the intensity of staining for LFA-3, exemplified by the shifting of the peak to the right. The resulting profile showed a tighter distribution of LFA-3 expression among the population of infected cells compared to the uninfected cells (Figure 4.1).

ICAM-1 expression was present at higher background levels on uninfected cells than LFA-3 expression. The uninfected cells showed a wide distribution of levels of ICAM-1 among the population, as shown by a broad flow cytometric profile (Figure 4.1). At 3 days post CMV infection, the profile of ICAM-1 expression was represented by a much tighter peak, at a higher intensity than that found on uninfected cells (Figure 4.1).

4.2.2 Visualisation of increased ICAM-1 and LFA-3 expression on infected and uninfected fibroblasts.

When using flow cytometry, it is always important to examine the stained cells using a fluorescence microscope, in order to ensure that the values for intensity of expression of a molecule generated by the flow cytometer are accompanied by the correct staining pattern on the cells. In the case of surface staining, it is of paramount importance that the antibodies have not permeated the cells, and that the results obtained are in fact a measure of surface intensity. As a characteristic of CMV infection is the generation of 'cytomegalic' or enlarged cells, it was particularly important to examine the adhesion molecule expression microscopically. It was possible that the increased fluorescence detected by the flow cytometer did not represent an increase in the density of the molecules on the cell surface, but was merely a function of the increase in cell size or autofluorescence. In order to visualise the density of expression of each molecule on the cell surface, samples of the cells stained for flow cytometric analysis were also examined by confocal scanning laser microscopy.

Microscopic examination confirmed that the cells were stained on the surface only. ICAM-1 expression could be seen on uninfected cells, and reflected the wide range of intensities represented by the broad flow cytometric profiles. The intensity of fluorescence on infected cells stained for ICAM-1 appeared to be more uniform among this cell population, and was considerably brighter, than that on uninfected cells, confirming the flow cytometric results (Figure 4.2). In the case of LFA-3, the level of staining on uninfected cells could not be distinguished from unstained cells by microscopic examination. However, infected cells stained for LFA-3 displayed brighter fluorescence as a 'ring' around the cell surface (Figure 4.2). The fact that both molecules appear to be present at a greater density on the cell surface despite the obvious increase in cell size and change in cell shape, and hence surface area, confirms that the increase detected by flow cytometry reflects a true increase in the density of expression of LFA-3 and ICAM-1 on the cell surface.

4.2.3 The effect of recombinant cytokines on ICAM-1 expression.

IL-1β and TNFα are known to upregulate ICAM-1 expression. In order to compare the ICAM-1 levels on CMV infected fibroblasts with those induced by recombinant cytokines, cells were treated with recombinant IL-1β or TNFα at concentrations between 0.0001 ng/ml and 10 ng/ml, and their ICAM-1 levels

measured by flow cytometry at 24 hours post infection. A representative experiment is shown in Figure 4.3. ICAM-1 expression in response to IL-1β was maximal at 1ng/ml (2.2 ±0.9 FIU; mean ± SD). In contrast, ICAM-1 expression in response to TNFα continued to increase up to the highest dose tested, 10ng/ml (3.6 ± 0.5 FIU). At levels of IL-1β and TNFα in the picogram range (0.0001-0.01 ng/ml), ICAM-1 levels were not significantly increased above control levels ($p<0.05$). Significantly higher levels of ICAM-1 expression were achieved by treatment with TNFα than IL-1β at all concentrations above 0.01ng/ml ($p>0.05$). Cells from the above experiments were also stained for cell surface LFA-3, but this was not increased by treatment with any of these cytokines (data not shown).

Over a number of experiments, the average fold increase in LFA-3 expression on infected cells relative to uninfected levels was found to be 4.17 ± 0.254 (mean ± SEM, n=12, range=2.88-5.62). The average increase in ICAM-1 expression on infected cells was 3.15 fold ± 0.45 (n=10, range=1.9-6.26) (Figure 4.4). That the CMV induction of ICAM-1 was of similar magnitude to the induction by inflammatory cytokines suggested that CMV is indeed a very potent stimulator of ICAM-1.

IFNβ has been reported by some groups to upregulate ICAM-1 expression in human embryonic lung fibroblasts (Shen *et al.* 1995), whereas data from others is not in agreement with this (Dustin *et al.* 1986b). To test the effect of IFNβ on ICAM-1 expression, cells were treated with recombinant IFNβ at concentrations of 1, 10 and 100 IU/ml, and their ICAM-1 levels measured by flow cytometry at days 1 to 4 post treatment. There was no significant increase in the surface expression of ICAM-1 from uninfected control cells under any of these conditions (data not shown).

4.2.4 The relative roles of virions and cytokines from CMV inoculum on adhesion molecule upregulation.

CMV inoculum is harvested from infected cells, and thus contains cytokines and other factors released from infected cells, in addition to viral particles. To investigate the role in the adhesion molecule upregulation of the virus and of the other factors from the inoculum, virions were removed from the inoculum by ultracentrifugation prior to 'mock infection' of fibroblasts, and the virus free

inoculum and the 'purified' virions were tested separately for their effect on adhesion molecule expression.

Fibroblasts were treated with virus free inoculum, 'purified' virions, or unprocessed viral inoculum for 1 hour, or remained uninfected. Cells were analysed by flow cytometry for the expression of CMV IE antigens, and for surface expression of ICAM-1 and LFA-3 at 3 days post infection. The cells infected with unprocessed infectious viral inoculum or 'purified' virus showed >95% infection. Cells infected with unprocessed viral inoculum showed upregulation of both ICAM-1 and LFA-3 to 2.2 and 4.9 fold the uninfected levels, respectively, in the experiment shown. Levels of expression of these molecules induced by treatment with purified virions were not significantly different from these values ($p>0.05$) (Figure 4.5). In contrast, cells treated with virus free inoculum showed no expression of CMV IE antigen, and no upregulation of ICAM-1 and LFA-3 surface expression in comparison to levels seen on uninfected cells. These results suggested that virions alone were sufficient to induce the upregulation of ICAM-1 and LFA-3, and that cytokines in the inoculum were not responsible for the effect.

4.2.5 UV inactivation of CMV inoculum.

UV inactivation damages the viral DNA by the formation of thymidine dimers, thus preventing viral replication. However, the virions are still able to bind to cells as their proteins are relatively unaltered by such treatment. Treatment of cells with UV inactivated virions was therefore used to test whether viral infectivity was required for the induction of adhesion molecule expression, or whether early activation events resulting from virus binding to the cell were sufficient.

Cells treated with UV irradiated inoculum for the usual 1 hour infection period were stained for the expression of CMV IE proteins, and adhesion molecule expression, at 3 days post infection. Cells treated with UV inactivated inoculum showed no expression of CMV IE antigens, confirming effective inactivation of viral infectivity. They also showed no increase in adhesion molecule expression above that found on uninfected cells (Table 4.1). This result suggested that some degree of viral protein expression was required for adhesion molecule upregulation to take place.

Cytokines present in the viral inoculum would not be expected to be affected by UV treatment. To confirm that this was the case under the conditions used here, cells were treated with IL-1β or IFNβ which had been subjected to identical UV irradiation as the viral inoculum. Cells treated with these cytokines manifested the appropriate biological effects of these cytokines, namely the induction of ICAM-1 (for IL-1β) or class I HLA induction (for IFNβ), whether the cytokines were irradiated or not (Table 4.1). This suggested that other cytokines in the inoculum would probably also not be affected by UV irradiation, and thus agreed with the previous result showing that cytokines present in the inoculum were not responsible for adhesion molecule upregulation.

4.2.6 The effect of virus dose on adhesion molecule upregulation.

Having determined that viral infectivity was required for adhesion molecule upregulation, the effect of multiplicity of infection was investigated. Infection of fibroblasts at a low MOI, resulting in a mixture of infected and uninfected cells, was useful for determining whether infection of a small proportion of cells resulted in a general upregulation of adhesion molecules throughout the cell population.

Cells were infected with CMV inoculum at a range of dilutions that resulted in 96% infection at the highest dilution (MOI= 5), and 12% infection at the lowest dilution (MOI= 0.078). The percentage of cells infected was measured by staining for CMV IE antigen using the monoclonal antibody E13, and flow cytometric analysis. LFA-3 and ICAM-1 cell surface expression were measured at 3 days post infection by flow cytometry.

The average level of LFA-3 expression of the cell population was dependent on the levels of the input virus. A population of cells with increased LFA-3 expression became evident at the lowest virus dose, resulting in the appearance of a bimodal peak, presumably representing basal expression in the uninfected cells, and higher expression in the infected cells. The proportion of cells with high LFA-3 expression increased with increasing virus dose, until at the highest dose all cells showed increased LFA-3 expression (Figure 4.6). CMV infection of fibroblasts therefore resulted in the uniform expression of high levels of LFA-3 which were greater than the levels in uninfected cells.

The change in ICAM-1 expression in response to increased virus dose showed a different pattern, characterised by the loss of cells from the lower end of the

range of constitutive ICAM-1 expression, and their accumulation in the higher end of the range. Thus the resulting population at the highest virus dilution was composed of cells with ICAM-1 expression equivalent to the highest level of ICAM-1 expression in the uninfected cell range (Figure 4.6). It appeared that the population of cells in the uninfected population expressing high ICAM-1 levels did not increase their ICAM-1 expression further upon infection.

4.2.7 The association of ICAM-1 and LFA-3 upregulation with low class I HLA expression.

In the previous chapter, class I HLA expression on the cell surface was shown to correlate with the percentage of infection. To determine whether the upregulation of adhesion molecules was specifically associated with CMV infected cells, or whether there was a more general effect throughout the infected cultures, class I HLA downregulation was used as an indirect marker of infection to distinguish infected from uninfected cells in a mixed population of infected and uninfected cells.

At 3 days post infection, fibroblasts from 100% infected, 50% infected, and uninfected populations were double stained for the expression of class I HLA and either LFA-3 or ICAM-1. Double staining was carried out using pairs of primary antibodies of different isotypes i.e. LFA-3 (Clone TS2/9, IgG2a) with class I HLA (Clone PA2.6, IgG1), or ICAM-1 (Clone BBIG I1, IgG1) with class I HLA (Clone W6/32, IgG2a). The primary antibodies were then detected using isotype specific secondary antibodies, and the cells analysed by flow cytometry. The flow cytometry data was visualised as contour plots to show the expression of the two markers in relation to one another. Flow cytometric profiles from a representative experiment are shown in Figure 4.7.

It was evident that in the 50% infected populations there were two distinct populations of cells, HLA_{high} and HLA_{low}. For cells stained for LFA-3, it could also be seen that on HLA_{low} ('infected') cells LFA-3 expression was high, in both infected and 50% infected populations. On HLA_{high} ('uninfected bystander') cells from partially infected cultures, LFA-3 expression did not differ from the levels on cells from uninfected cultures. Thus it was concluded that LFA-3 expression was upregulated specifically on infected cells in the culture.

In the case of ICAM-1 the data was more difficult to interpret. Although a similar pattern was seen, showing high levels of ICAM-1 expression on HLA_{low} cells,

the levels on the HLA_{high} cells in 50% infected populations had increased slightly from the levels on uninfected cultures (fold increase= 1.63 ± 0.25 (mean ± SEM), p=0.02, n=4 experiments). However, the increase in ICAM-1 expression on the latter HLA_{high} (uninfected bystander) population was much less than the increase on the HLA_{low} (infected) cells (fold increase from uninfected levels= 4.82 ± 0.78, p=0.02, n=4). In 50% infected populations the increased ICAM-1 expression was therefore predominantly associated with the class I HLA_{low} population. It was possible that cytokines released from infected cells had caused the small degree of ICAM-1 upregulation on the class I HLA_{high} uninfected bystander cells, and this possibility is investigated in a separate section. An alternative explanation is that the increase in ICAM-1 expression on uninfected bystander cells is due to a very recent infection of these cells by the virus produced by the infected cells.

4.2.8 Cytokine production by CMV infected fibroblasts.

Although we had established that the upregulation of LFA-3 and ICAM-1 required infectious virus, it was not clear whether this was a direct viral effect on the adhesion molecule levels, or whether the effect was secondary to CMV-induced cytokine induction in the infected cells. Supernatants from infected fibroblasts were screened for the presence of cytokines known to upregulate the expression of ICAM-1. The cytokines investigated were IL-1β, TNF-α IFNγ, and IL-4. Fibroblasts are not normally associated with the production of these cytokines, but it was possible that CMV infection may have altered the cytokine profile of the fibroblasts. Supernatants were also assayed for the presence of other cytokines known to be produced by fibroblasts, such as IL-6, IL-8, G-CSF and GM-CSF.

Supernatants were harvested from infected and uninfected fibroblasts at 24, 48 and 72 hrs post infection, and tested for the presence of a range of cytokines by immunoassay. No production of IL-4, IFNγ, IL-1β or TNFα was detected from either infected or uninfected fibroblasts at any of the time points tested. Table 4.2 shows the detection level of each cytokine assay, and the results obtained for infected and uninfected supernatants at 48 hours post infection.

Infected fibroblasts produced increased levels of IL-6 and IL-8 relative to the levels produced by uninfected fibroblasts (Table 4.2). The addition of recombinant IL-6 or IL-8 to uninfected fibroblasts did not alter their expression of ICAM-1 or LFA-3 when added in recombinant form at concentrations

between 10 and 1000pg/ml for periods of 1 to 4 days (data not shown). The induction of IL-8 by CMV is investigated in further detail in Chapter 6.

4.2.9 The effect of neutralising antibodies against IL-1β or TNFα on ICAM-1 expression.

Although there have been no reports of IL-1β or TNFα protein production by fibroblasts, and we detected none in the previous experiment, there have been reports of IL-1β and TNFα mRNA expression in CMV infected fibroblasts (St.Jeor *et al.* 1993), and of stimulation of these genes by CMV IE proteins (Iwamoto e*t al.* 1990; Geist *et al.* 1994). To ensure that undetectable levels, or locally acting IL-1β or TNFα were not responsible for the ICAM-1 upregulation following CMV infection, neutralising antibodies to IL-1β and TNFα were added to the culture medium both during and after virus adsorption. Cell surface expression of ICAM-1 on infected cells was not altered by the addition of neutralising antibodies against either IL-1β or TNFα, whilst these antibodies were shown to be capable of significantly reducing the increase in ICAM-1 expression following treatment with 0.5ng/ml of their respective cytokines (Figure 4.8). We thus concluded from these experiments that these two cytokines commonly responsible for ICAM-1 upregulation were not responsible for the ICAM-1 upregulation that occurs following CMV infection.

4.2.10 Treatment of fibroblasts with virus-free supernatants from infected cells.

To examine the potential effect of soluble factors released from infected cells, it was necessary to expose uninfected cells to such factors without the latter actually becoming infected. Firstly, this was achieved by treating uninfected fibroblasts with virus free supernatants from infected fibroblasts. Supernatants were harvested from infected or uninfected fibroblasts at 1 and 2 days post infection. Although only a small amount of virus, if any, would be expected to be present at such an early time point after infection, the supernatants were ultracentrifuged to ensure that no virus was present. Fibroblasts were incubated with the virus free supernatants (diluted 1:1 in fresh MEM/4% FCS) for 2 days, and analysed for changes in adhesion molecule expression. No difference was found in the expression of ICAM-1 or LFA-3 on the cells treated with the virus free supernatant from infected fibroblasts, as compared to supernatant from uninfected fibroblasts (Figure 4.9). In Chapter 3, it was shown

that class I HLA was increased by IFNβ released from infected cells. Thus, as a positive control, fibroblasts treated with virus free supernatants were also analysed for the expression of class I HLA, the levels of which were increased by the transfer of such supernatants (Figure 4.9). This data suggested that under these conditions, the supernatants from infected fibroblasts did not contain factors capable of altering the surface expression of ICAM-1 and LFA-3.

4.2.11 Continuous exposure to infected fibroblasts in a dual chamber system.

The above experimental design required that supernatants were harvested at specific time points and stored prior to ultracentrifugation, raising the possibility that their biological activity may have been altered during the experiment. It was also possible that cytokines were released at earlier time points than those used for supernatant harvests, and thus had already bound back to cellular receptors, and were no longer present in the supernatant. For this reason a second approach was used, in which infected and uninfected cells were grown on either side of a semi-permeable membrane, so that the uninfected cells could be directly and continuously exposed to any soluble factors as they were produced by the infected cells.

Uninfected cells growing on a semi-permeable membrane in a cell culture insert were added to wells of infected or uninfected fibroblasts immediately after the 1 hour virus adsorption period. Uninfected cells co-cultured with infected fibroblasts, and control cells co-cultured with uninfected fibroblasts, were stained for the expression of CMV IE antigen and adhesion molecule expression at 3 days post infection. No viral infection was passed to uninfected cells growing on the membrane, as the time point investigated was 3 days post infection, when very little virus had been released by the infected cells. Neither ICAM-1 nor LFA-3 expression was increased in uninfected cells continuously exposed in this way to the soluble factors released from the CMV infected cells (Figure 4.10). This data agreed with the previous findings that no soluble factors capable of increasing levels of ICAM-1 or LFA-3 were released by CMV infected fibroblasts.

4.2.12 The effect of ganciclovir or foscarnet treatment on CMV induced adhesion molecule expression.

Having determined that adhesion molecule upregulation was a direct viral effect, ganciclovir and foscarnet were used to determine whether CMV late proteins were involved in this process. Infected and control uninfected fibroblasts were treated with ganciclovir or foscarnet at concentrations of 1, 10 and 100μg/ml, or left untreated. Preliminary experiments were carried out to determine the concentrations required to abrogate late protein synthesis. These experiments are described in section 2.7. Cell surface levels of ICAM-1 and LFA-3 were analysed at 3 days post infection. Treatment of infected cells with ganciclovir or foscarnet did not prevent the upregulation of either ICAM-1 or LFA-3 following CMV infection (Figure 4.11). This suggested that CMV late proteins were not required for the upregulation of either of these adhesion molecules, as late protein expression is minimal under these conditions.

Furthermore, treatment of infected cells with all concentrations of ganciclovir or foscarnet used (1-100μg/ml) resulted in significant increases in LFA-3 above the levels found on untreated infected cells. In the case of ICAM-1, the expression on infected cells was significantly increased at 1μg/ml of ganciclovir in the experiment shown, but treatment with ganciclovir or foscarnet did not reproducibly increase levels of ICAM-1 expression compared to CMV infection alone.

4.2.13 The effect of ganciclovir treatment on the kinetics of adhesion molecule upregulation following CMV infection.

As LFA-3 and ICAM-1 upregulation occurred in the presence of antiviral agents, we investigated whether such treatment also affected the kinetics of adhesion molecule induction by CMV. LFA-3 and ICAM-1 levels were analysed at daily time points up to 7 days post infection in the presence or absence of 50μg/ml ganciclovir (Figure 4.12).

Ganciclovir treatment of infected cells resulted in a continued increase in LFA-3 expression at day 4 post infection, when levels on untreated infected cells had already begun to decline. At day 7, LFA-3 expression remained at a higher level on ganciclovir-treated infected cells compared to untreated infected controls. In the case of ICAM-1, there was no significant difference between ganciclovir-treated and untreated infected cells at days 2 and 4 post infection,

but at day 7, ganciclovir-treated infected cells showed significantly increased levels of ICAM-1 compared to untreated infected controls. The ganciclovir-treated cells thus retained higher levels of both adhesion molecules in the presence of effective antiviral treatment, and in the absence of the release of infectious virus. The antiviral treatment thus appeared to prolong the length of time for which adhesion molecule expression was enhanced.

4.2.14 Adhesion of CD2+ve lymphocytes to ganciclovir-treated infected fibroblasts.

In the following chapter we have investigated the effect of increased adhesion molecule expression on the adhesion of CD2+ve lymphocytes to CMV infected fibroblasts. Since ganciclovir-treated infected cells expressed equally high levels of adhesion molecules as control infected cells, the adhesion of CD2+ve lymphocytes to these cells was investigated. A quantitative adhesion assay was carried out at day 3 post infection, using resting CD2+ve lymphocytes purified by negative depletion and labelled with tritiated leucine. The development of the adhesion assay is detailed in Chapter 2 and Chapter 5. Lymphocytes were added to confluent monolayers of either infected, infected with ganciclovir treatment, or uninfected fibroblasts, and the percentage of cells binding was calculated. Three separate experiments were carried out. In 2 experiments, the percentage of lymphocyte adhesion to ganciclovir-treated fibroblasts was significantly higher than adhesion to uninfected fibroblasts, while in the third experiment (Donor 2) there was a slight, but not significant increase (Figure 4.13). The levels of adhesion to ganciclovir-treated infected fibroblasts were always significantly lower than adhesion to untreated infected fibroblasts. As the levels of ICAM-1 and LFA-3 were similar in ganciclovir-treated or untreated infected fibroblasts, the reason for reduced levels of lymphocyte adhesion to the ganciclovir-treated cells is not clear.

TABLE 4.1 The effect of 'infection' with UV inactivated CMV on the cell surface expression of LFA-3 and ICAM-1 of fibroblasts.

	Fluorescence intensity units[ab]		
	LFA-3	**ICAM-1**	**Class I HLA**
Uninfected	1.00 (0.09)	1.00 (0.09)	ND
Infected	4.21 (0.18)	3.75 (0.22)	ND
UV-virus infected	0.86 (0.07)	0.85 (0.08)	ND
IL-1β	ND	2.86	ND
IL-1β + UV	ND	3.01	ND
IFNβ	ND	ND	1.37
IFNβ + UV	ND	ND	1.39

[a]Numbers represent the fluorescence intensity of class I HLA, ICAM-1 and LFA-3 in relation to the expression of each molecule on uninfected cells, which have been assigned a value of 1.0. Means of triplicates are shown with standard deviations in brackets. Cytokine data represent the means of values from duplicate wells.

[b]Cells were analysed at 3 days post infection or cytokine treatment.

TABLE 4.2. Cytokine production by CMV infected fibroblasts.[a]

Cytokine	Detection level of assay	Uninfected	Infected
IL-1β	0.3 pg/ml	-ve	-ve
TNFα	4.8 pg/ml	-ve	-ve
IL-4	3.0 pg/ml	-ve	-ve
IFNγ	0.03 IU/ml	-ve	-ve
IL-8	4.7 pg/ml	125pg/ml	400 pg/ml
IL-6	0.35 pg/ml	80pg/ml	300 pg/ml
GCSF	7.2 pg/ml	-ve	-ve
GM-CSF	1.5 pg/ml	10 pg/ml	7 pg/ml

[a] Supernatants were harvested from infected or uninfected fibroblasts at 2 days post infection and cytokine measurements were performed by immunoassay. The values shown represent the means of triplicate samples.

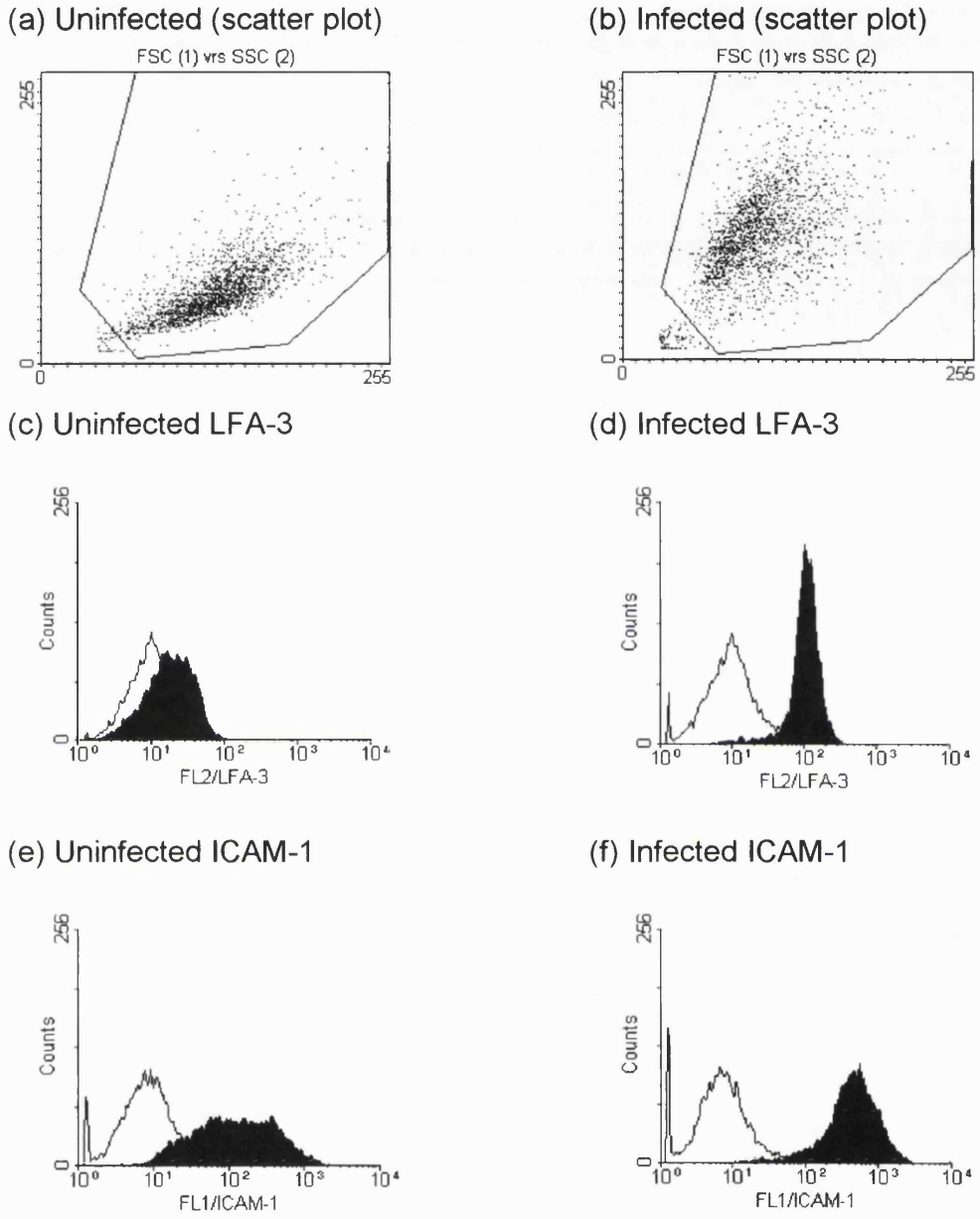


Figure 4.1. Flow cytometric analysis of LFA-3 and ICAM-1 expression on CMV infected and uninfected fibroblasts.

Infected or uninfected fibroblasts were stained for the cell surface expression of LFA-3 or ICAM-1 at 3 days post infection. Scatter profiles (Forward scatter vs side scatter) for uninfected (a) and infected (b) cells were gated to exclude cell debris and dead cells, as shown. Fluorescence histograms show the expression of LFA-3 on uninfected (c) and infected (d) fibroblasts, and ICAM-1 on uninfected (e) and infected (f) fibroblasts. The black filled histograms represent the adhesion molecule expression, while the unfilled histograms represent fibroblasts stained with an irrelevant control antibody. A shift to the right indicates an increase in fluorescence intensity. The number of cells with a particular fluorescence intensity is indicated on the y axis.

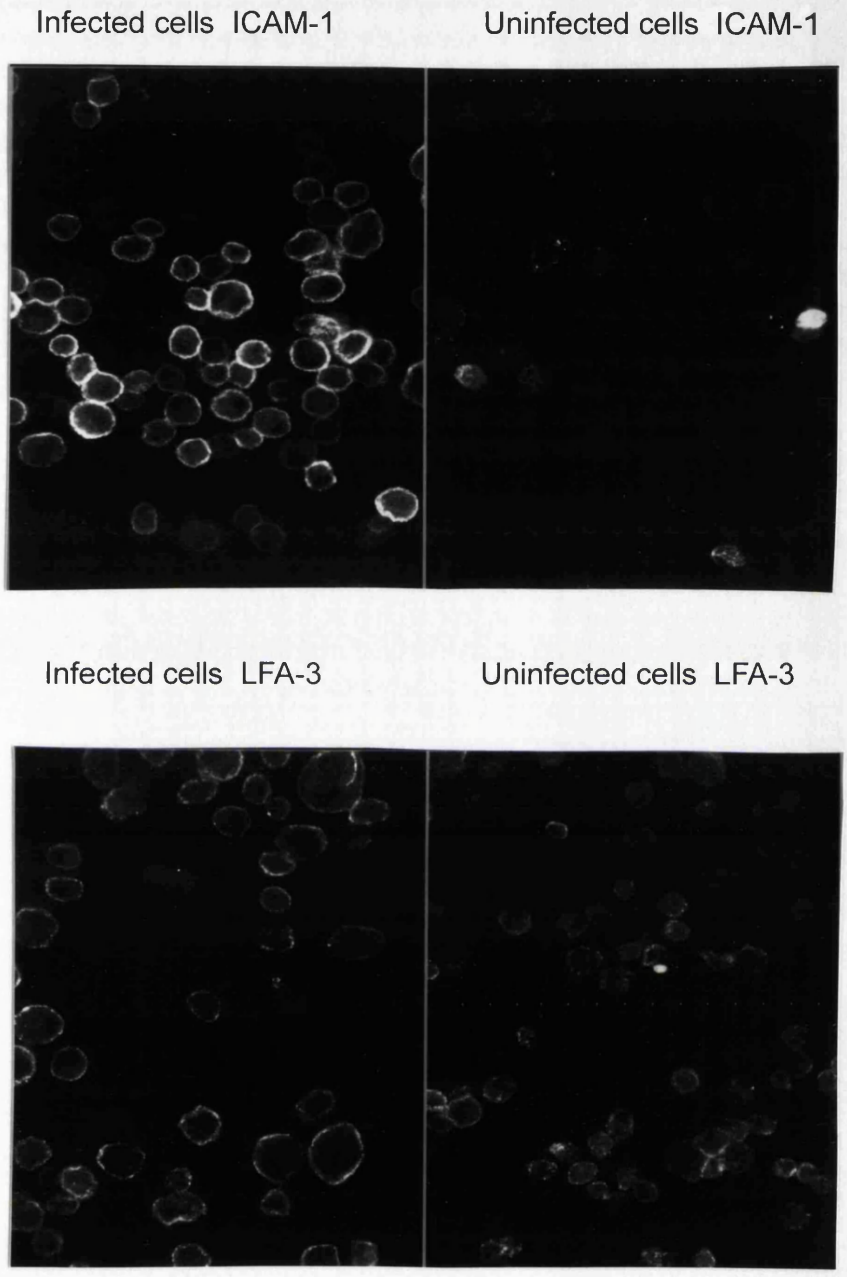


Figure 4.2. Visualisation of the cell surface expression of ICAM-1 and LFA-3 expression on CMV infected and uninfected fibroblasts.

Cells which had been surface stained at 3 days post infection for the expression of ICAM-1 and LFA-3 by flow cytometry were applied to poly-L-lysine coated slides and examined by confocal scanning laser microscopy. Magnification x 400.

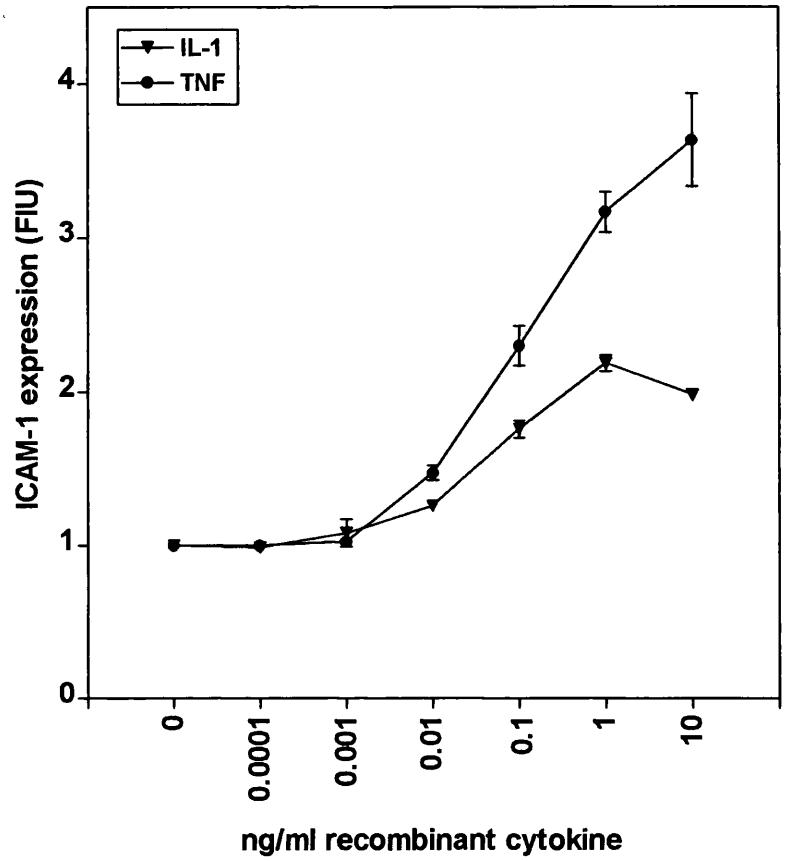


Figure 4.3 The effect of recombinant cytokines on the cell surface expression of ICAM-1 on fibroblasts.

Fibroblasts were treated with a range of concentrations of recombinant IL-1β or TNF-α, from 0.0001ng/ml to 10ng/ml. ICAM-1 levels were measured at 24 hours post infection by flow cytometry, and are expressed as fluorescence intensity units relative to the mean levels on untreated cells which have been assigned a value of 1. The results shown are representative of 3 experiments carried out in triplicate.

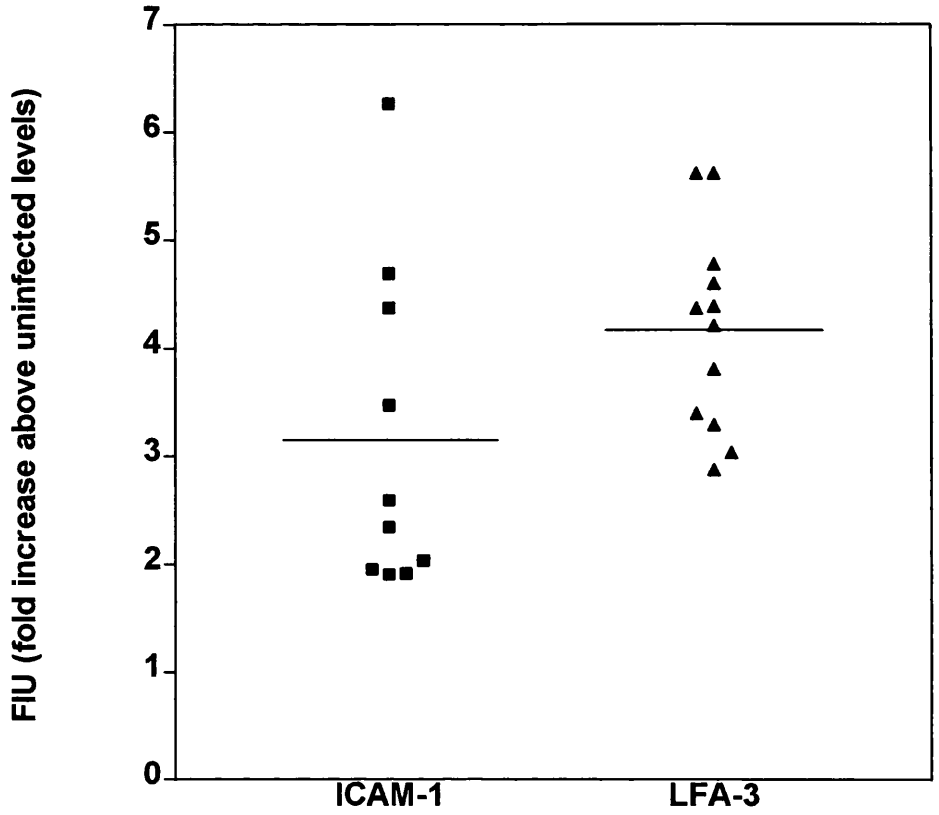


Figure 4.4. The extent of the increase in the cell surface expression of ICAM-1 and LFA-3 following CMV infection.

The fold increases of ICAM-1 or LFA-3 expression on infected cells relative to the levels on uninfected cells were calculated, from a number of separate experiments (ICAM-1, n=10; LFA-3, n=12). Cells were infected with CMV at a MOI of 2-4 pfu/cell and then analysed for the cell surface expression of each molecule at 3 days post infection. The levels of ICAM-1 or LFA-3 on uninfected cells were assigned a value of 1. Each data point represents the fold increase in ICAM-1 or LFA-3 in an individual experiments. The bar represents the mean fold increase for the expression of each molecule.

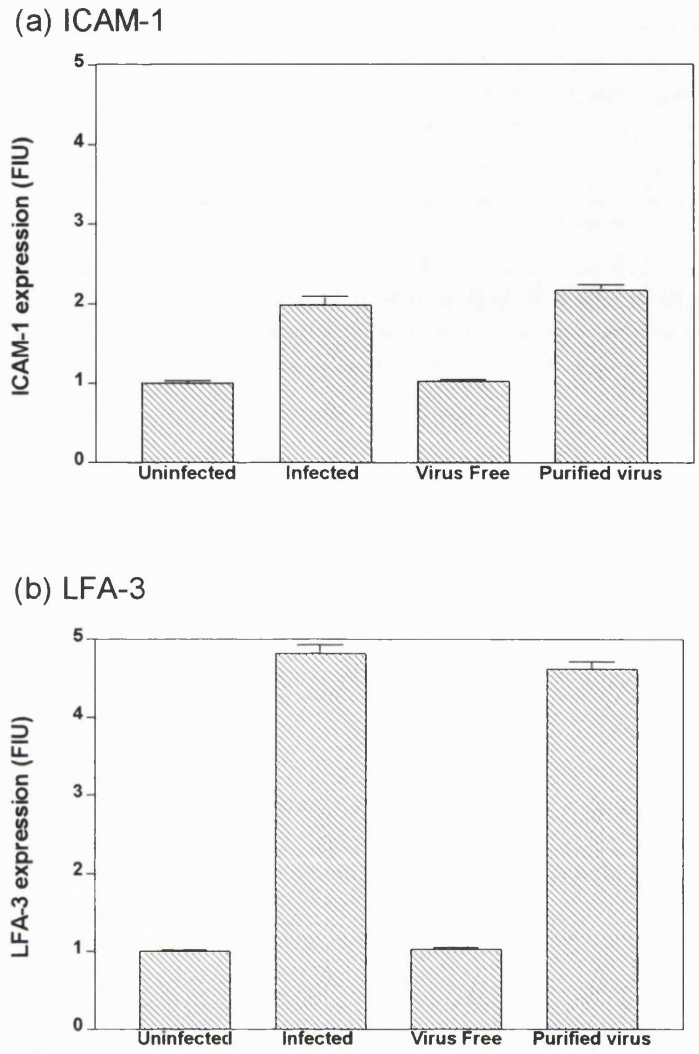


Figure 4.5 The effect of purified virus and virus-free inoculum on the cell surface expression of ICAM-1 and LFA-3.

Fibroblasts were infected with unprocessed CMV inoculum ('Infected'), inoculum which was ultracentrifuged to remove infectious virions ('virus free), or virions removed from the inoculum by ultracentrifugation ('purified' virus). Cells were removed by trypsinisation 3 days after infection, and the cell surface expression of ICAM-1 (a) or LFA-3 (b) measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0. The experiments shown are representative of 3 similar experiments. The data shown represents the mean ± SD for triplicate samples.

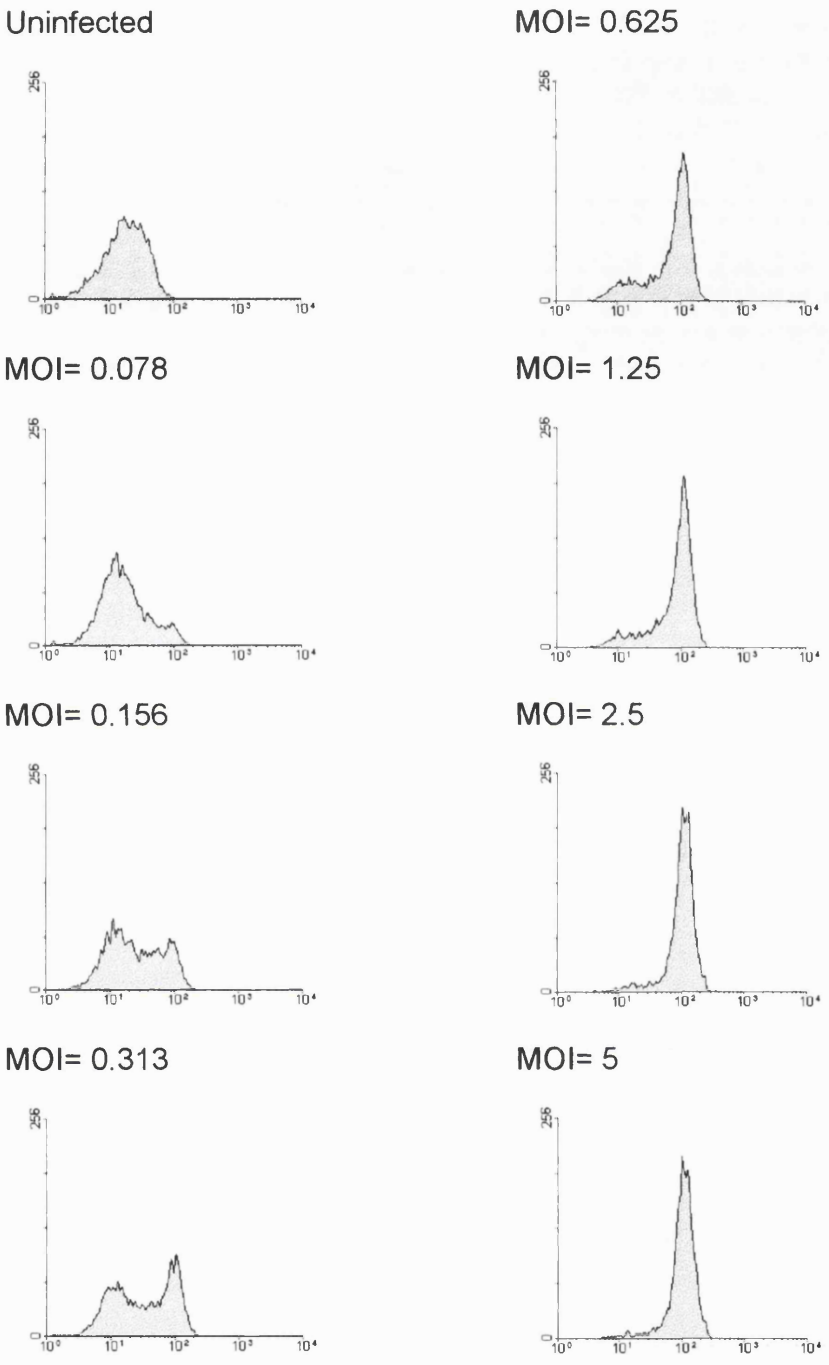


Figure 4.6(a). The effect of virus dose on the cell surface expression of LFA-3.

Fibroblasts were infected with dilutions of virus inoculum which resulted in MOIs ranging from 0.075 to 5 pfu/cell, as labelled on the histograms, or remained uninfected. Cells were stained at day 3 post infection for the expression of LFA-3. Increasing doses of virus resulted in an increase in fluorescence intensity of LFA-3 from the uninfected levels, which is represented by a shift of the cell population to the right.

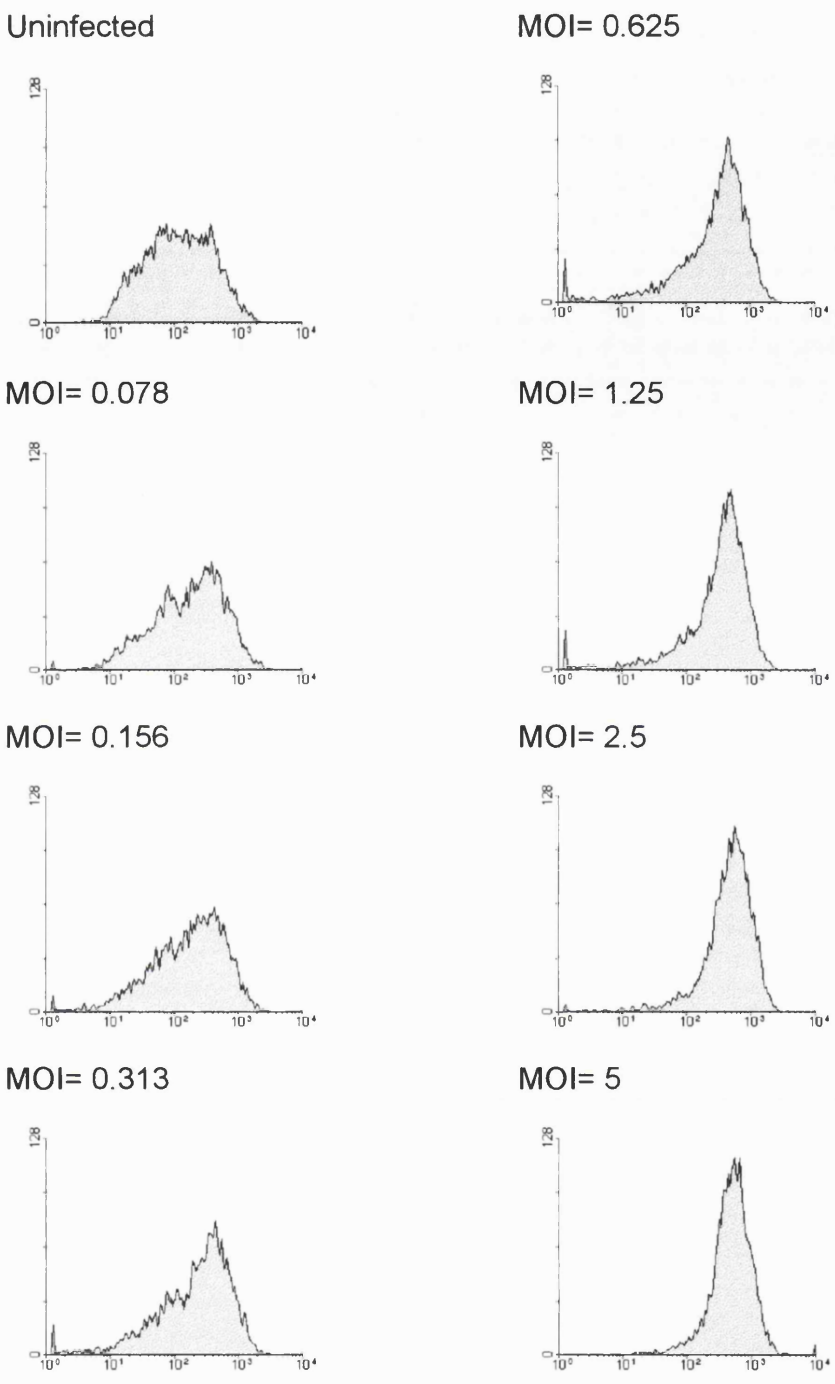


Figure 4.6 (b). The effect of virus dose on the cell surface expression of ICAM-1.

See Figure 4.6 (a) for experimental design.

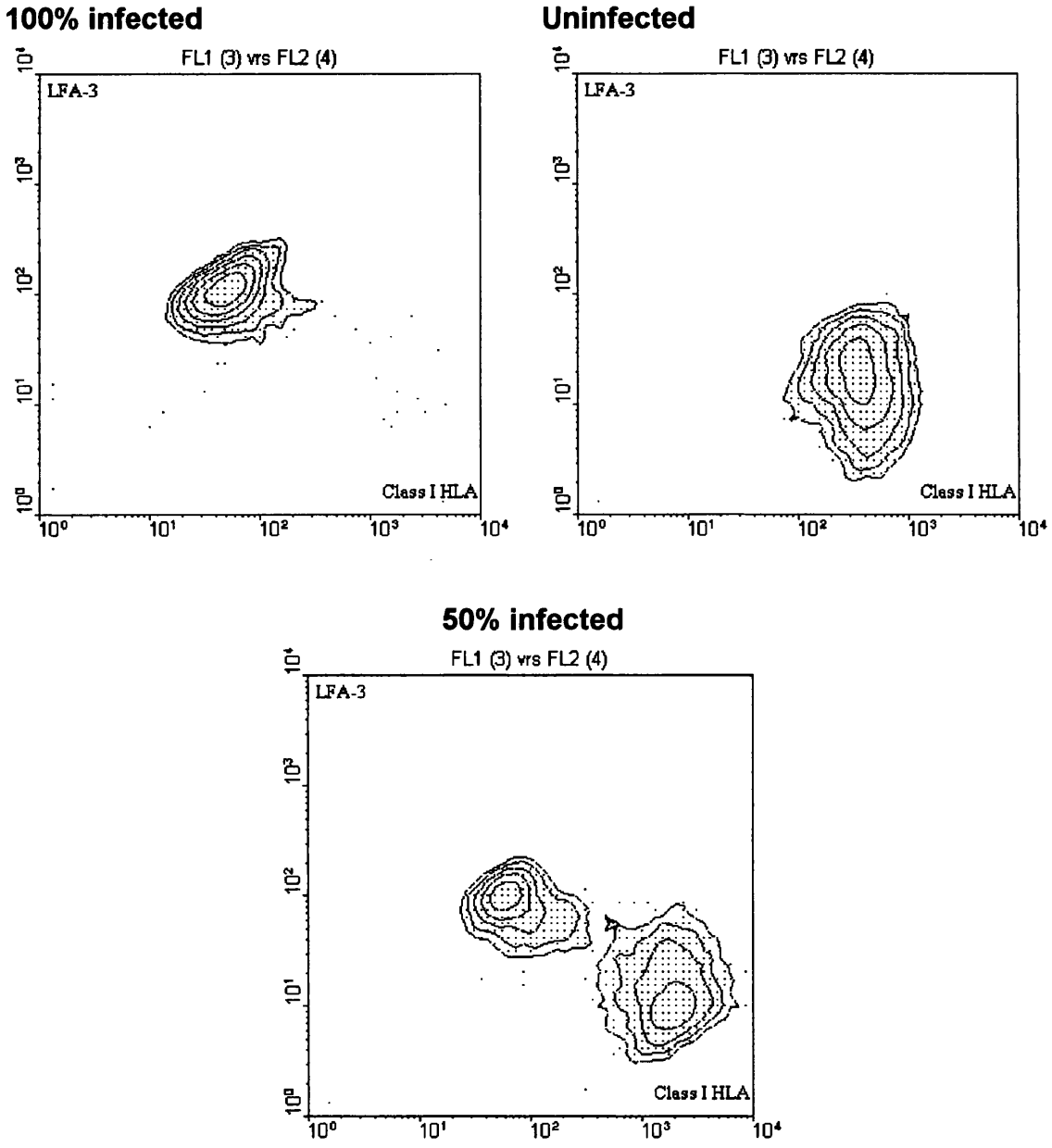


Figure 4.7 (a). The relationship between class I HLA expression and LFA-3 expression following CMV infection.

Fibroblasts were infected with viral inoculum sufficient to infect 100% of cells, 50% of cells, or remained uninfected. Cells were double stained at day 3 post infection for the expression of LFA-3 (Y axis) and class I HLA (X axis). Representative flow cytometric profiles are shown.

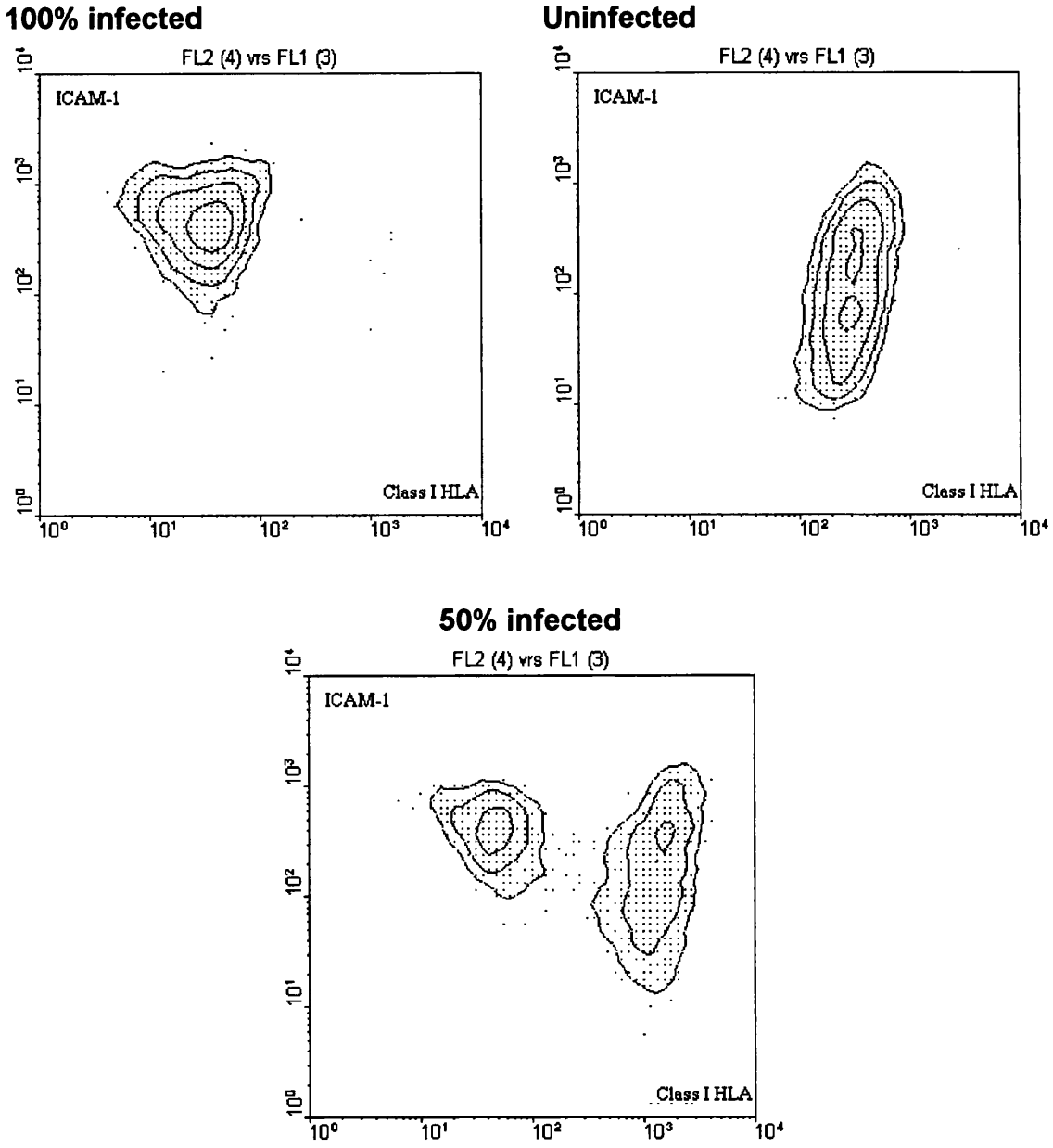


Figure 4.7 (b). The relationship between class i HLA expression and ICAM-1 expression following CMV infection.

Fibroblasts were infected with viral inoculum sufficient to infect 100% of cells, 50% of cells, or remained uninfected. Cells were double stained at day 3 post infection for the expression of ICAM-1 (Y axis) and class I HLA (X axis). Representative flow cytometric profiles are shown.

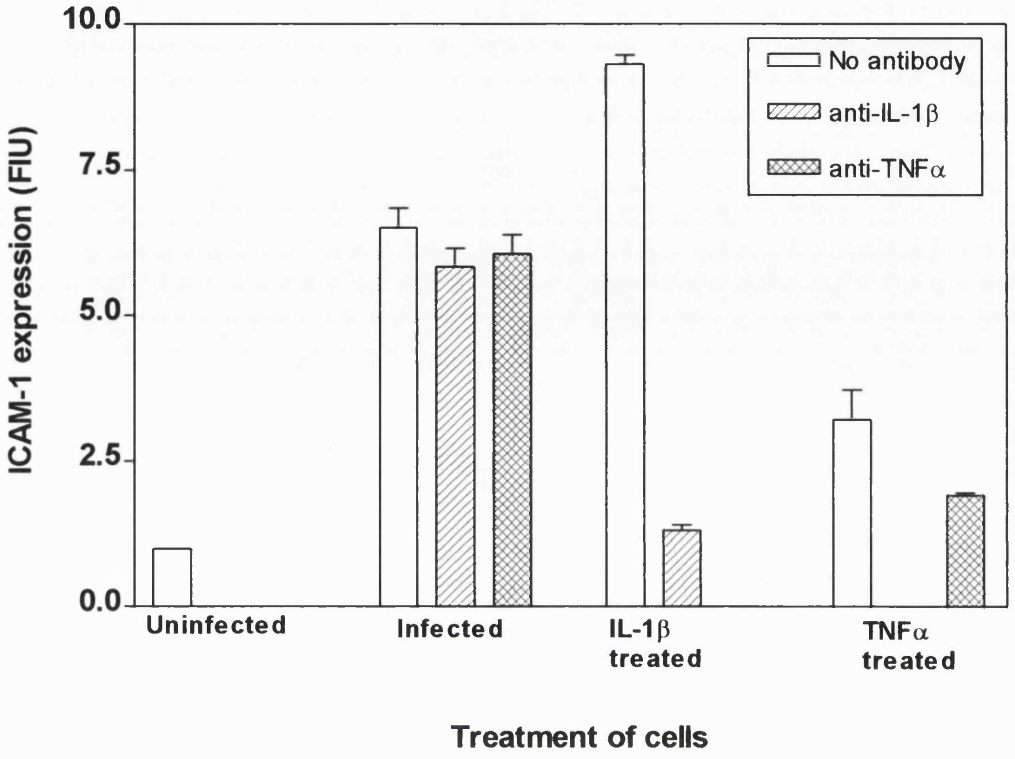


Figure 4.8 The effect of neutralising antibodies specific for IL-1β and TNFα on the expression of ICAM-1 on CMV infected fibroblasts.

The appropriate neutralising antibody was added both during the 1 hour infection and in the medium added to the cells after the infection. Control wells were treated with IL-1β or TNFα at 0.5ng/ml. At 3 days post infection or cytokine treatment cells were stained for the expression of ICAM-1. The results are expressed as fluorescent intensity units relative to the mean level of expression of ICAM-1 on uninfected cells, which has been assigned the value of 1.0. These results are representative of two separate experiments. The figure shows the mean ± SD of 3 replicates.

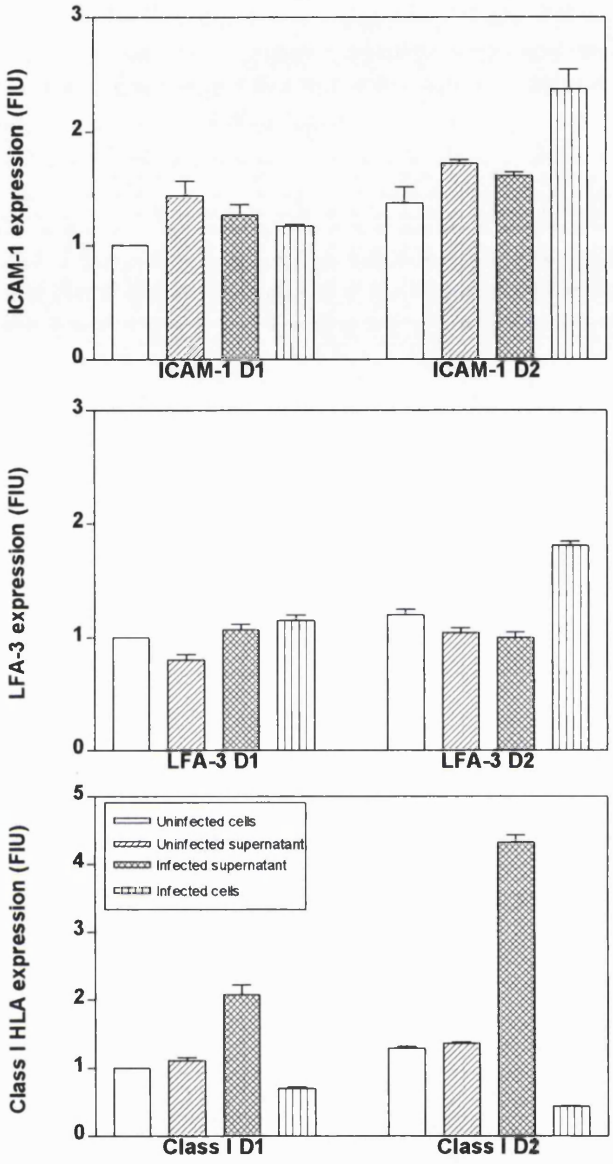


Figure 4.9. The cell surface expression of ICAM-1, LFA-3, and class I HLA on fibroblasts exposed to supernatants transferred from CMV infected or uninfected cells.

Supernatants were removed from infected or uninfected fibroblasts at 1 or 2 days post infection. Any infectious virus in the supernatants was removed by ultracentrifugation. Cells were treated with supernatants from infected or uninfected cells for 2 days, before they were removed by trypsinisation and cell surface adhesion molecule expression measured by flow cytometry. Control infected and uninfected cells were processed in parallel. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells, which has been assigned the value of 1.0. The data shown is representative of three experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates.

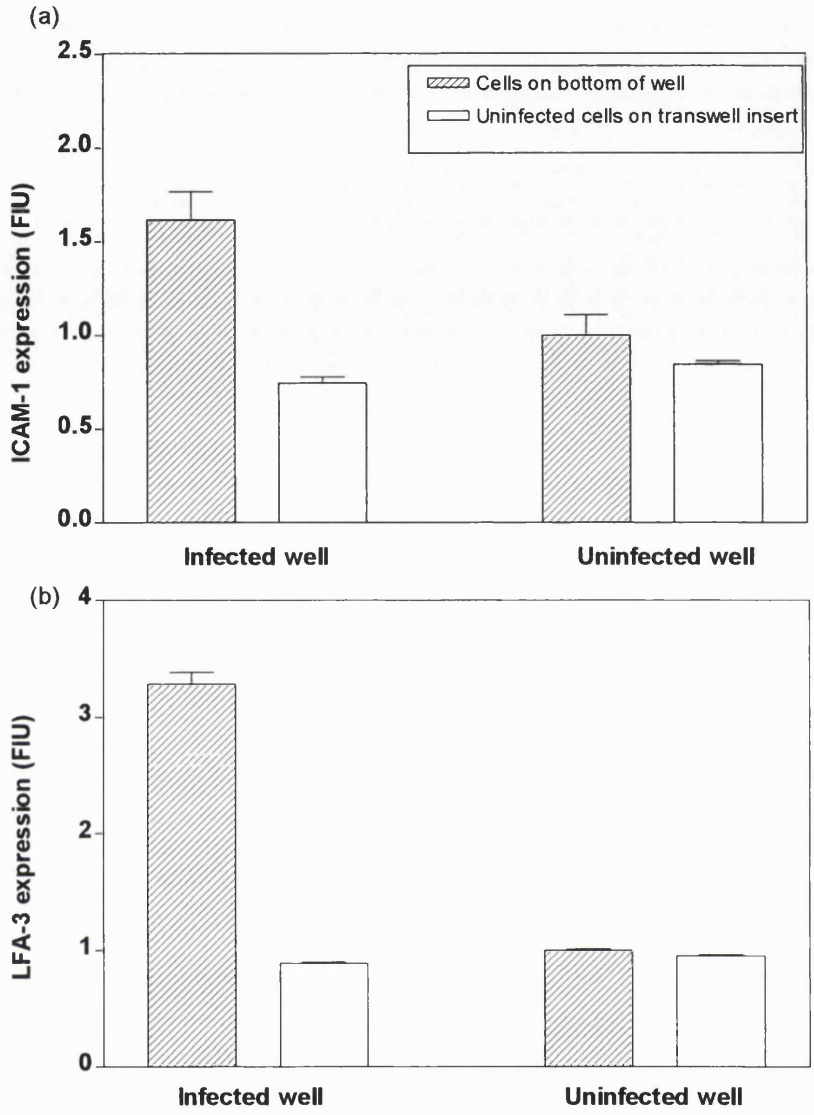


Figure 4.10 The cell surface expression of LFA-3 and ICAM-1 on uninfected cells directly exposed to soluble factors released from CMV infected fibroblasts.

A dual chamber system was used in which cells were separated by a transwell membrane. Cells grown on the base of the well were infected or uninfected as indicated, whilst those growing on the transwell membrane insert were uninfected. In the case of wells containing CMV infected cells, the membrane inserts were added to the wells immediately following the 1 hour virus adsorption period. Cells were removed by trypsinisation 3 days after infection, and the cell surface expression of ICAM-1 (a) or LFA-3 (b) measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells grown on the base of the well, which has been assigned the value of 1.0. The data shown is one of four representative experiments, each of which was carried out in triplicate. The figure shows the mean ± SD of the three replicates.

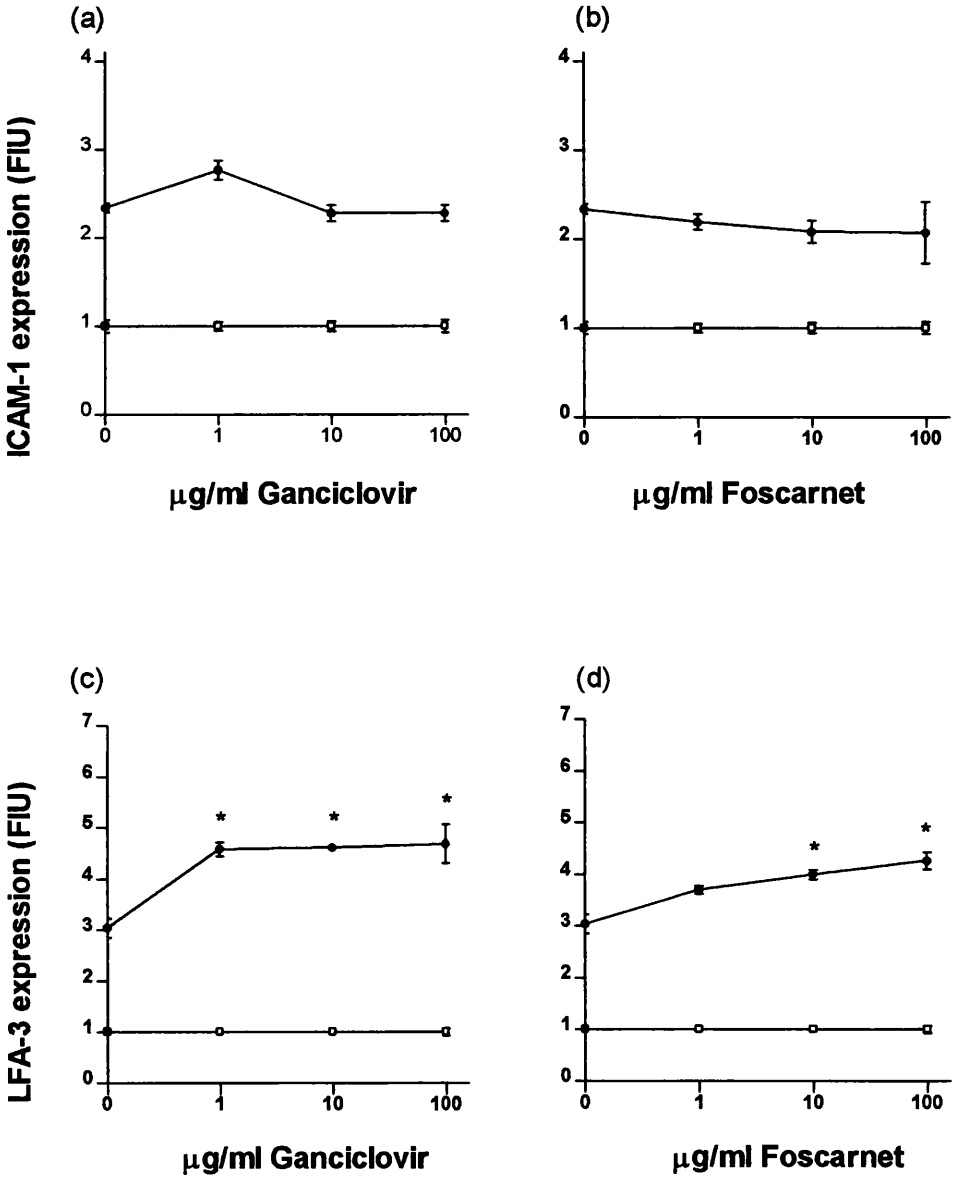


Figure 4.11. The effect of treatment with various concentrations of ganciclovir or foscarnet on levels of expression of ICAM-1 or LFA-3 on CMV infected fibroblasts.

Cells were removed by trypsinisation 3 days post infection, and cell surface adhesion molecule expression measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression of ICAM-1 (a and b) or LFA-3 (c and d) on uninfected cells treated with the relevant drug concentration, which have been assigned the value of 1.0. The data shown is one of three representative experiments, each of which was carried out in triplicate. The figure shows the mean and standard deviation of the three replicates. The asterisks indicate significantly increased ($P<0.05$) levels of expression compared to that on infected cells not treated with the antiviral agents.

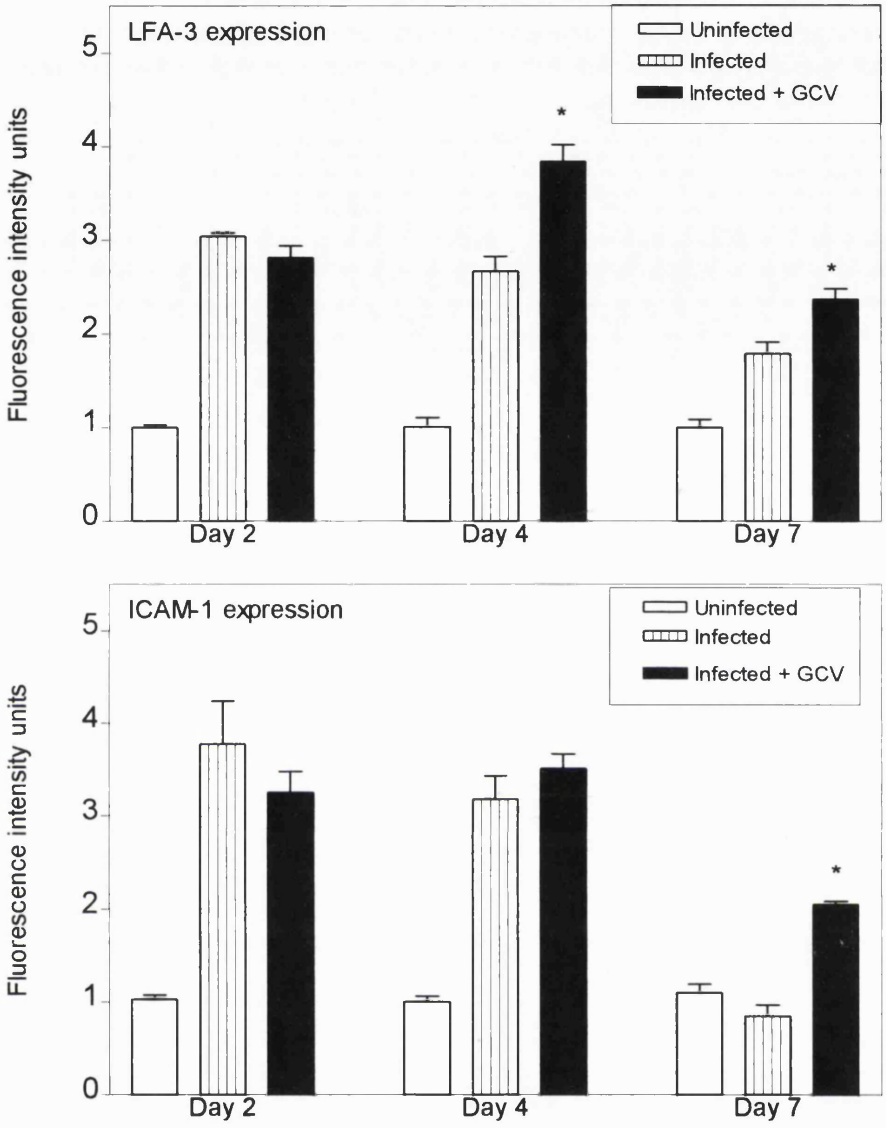


Figure 4.12. The effect of ganciclovir treatment on the expression of ICAM-1 or LFA-3 on CMV infected fibroblasts.

Ganciclovir (50μg/ml) was added following the 1 hour virus adsorption period. Cells were removed by trypsinisation at the time points shown and cell surface expression of ICAM-1 (top) or LFA-3 (bottom) measured by flow cytometry. The results are expressed as fluorescent intensity units relative to the mean level of expression on uninfected cells at that time point, which have been assigned the value of 1.0. The data shown is one of four representative experiments, each of which was carried out in triplicate. The figure shows the mean ± SD of the three replicates. The asterisks indicate significantly increased ($P<0.05$) levels of expression on treated infected cells compared to those on untreated infected controls at the relevant time point.

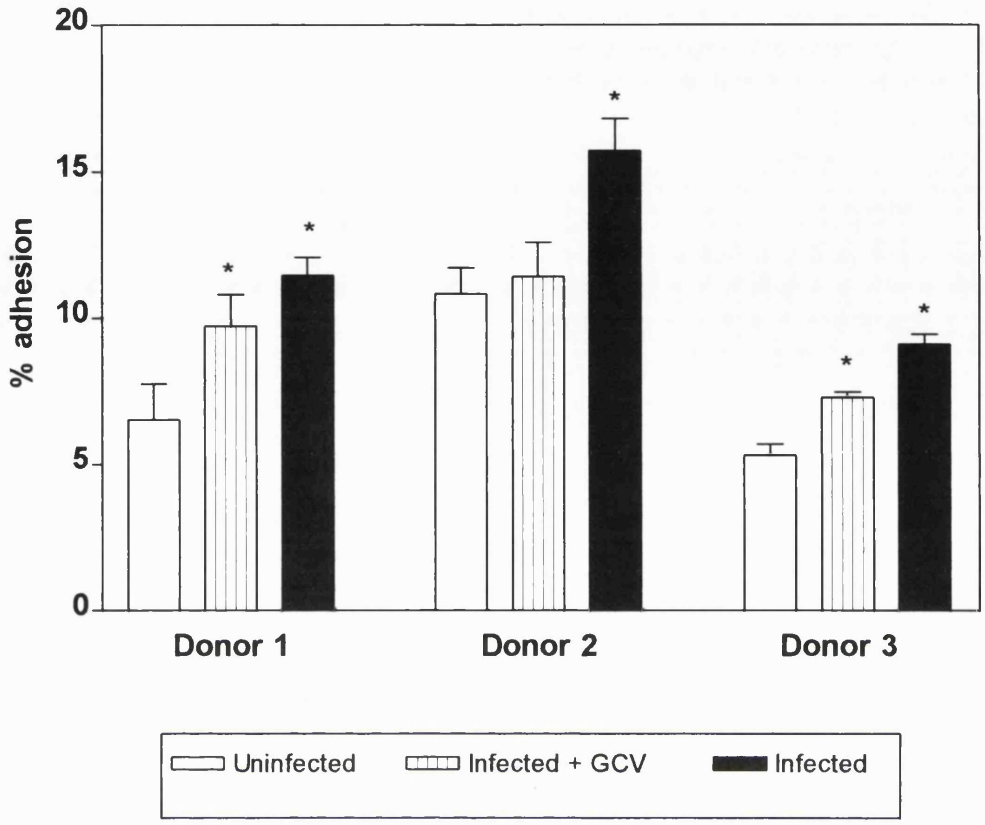


Figure 4.13. The adhesion of T lymphocytes to fibroblast monolayers in a quantitative adhesion assay.

Fibroblasts were seeded at 2 days post infection to form confluent monolayers in a 96 well plate which were used in an adhesion assay the following day. Fibroblasts were either infected, infected and treated with ganciclovir, or uninfected. Purified CD2+ve lymphocytes labelled with tritiated leucine were added to the wells, and unbound T cells were removed by washing. The amount of radioactivity bound was determined by scintillation counting. This value is expressed as a percentage of the radioactivity in the original population of T cells added to the well. The asterisks indicate significantly increased adhesion relative to levels of adhesion to uninfected fibroblasts. Three separate experiments are shown. The figure shows the mean ± SD for triplicate wells.

4.3 DISCUSSION

In the present study, it was confirmed that the cell surface expression of ICAM-1 and LFA-3 is increased following CMV infection. The levels of ICAM-1 expression on infected cells were similar to those achieved by stimulation of uninfected cells with TNFα or IL-1β, which are strong inducers of ICAM-1. Increased LFA-3 expression in response to cytokines on fibroblasts has not been shown by others, and we found no increase in response to recombinant cytokines in the present study. Upregulation of ICAM-1 and LFA-3 expression following infection of cells with purified virus showed that CMV alone was sufficient to cause equal levels of adhesion molecule induction in the absence of extraneous factors normally present in the viral inoculum. Treatment with the virus free portion of the inoculum had no effect on adhesion molecule expression. Likewise, infection of cells with UV inactivated virus inoculum did not result in the upregulation of adhesion molecule expression, showing that the interaction of the virus particles with the cell surface, without the ensuing viral replication, was insufficient to induce expression of ICAM-1 and LFA-3.

The upregulation of ICAM-1 on infected cells was not abrogated by treatment with neutralising antibodies specific for IL-1β or TNF-α either during or following infection, showing that the presence of these cytokines in the inoculum, or induction at low or undetectable levels following infection, was not responsible for the effect. LFA-3 or ICAM-1 expression was not increased on cells treated with virus free supernatant from infected fibroblasts, or on cells continuously exposed to soluble factors released from infected fibroblasts across a semi-permeable membrane. This indicated that adhesion molecule expression is not increased by cytokines released by infected cells, suggesting that *in vivo*, adhesion molecule upregulation on bystander uninfected cells would not take place. In mixed populations of infected and uninfected cells, the upregulation of ICAM-1 or LFA-3 predominantly took place on cells with decreased expression of class I HLA, suggesting that the upregulation of adhesion molecules occurred specifically on infected cells.

It is important to distinguish between effects taking place on infected cells and uninfected cells in the same culture. In this study dual chambers were used to physically separate infected and uninfected cells during the experiment. For other experiments, the two were discriminated between during analysis, by the use of low class I HLA as an indirect marker of infection. In investigating changes which take place in infected cells it is important to ensure that a high level of infection is achieved, so that results are not distorted by the presence of

uninfected cells. Since the beginning of this project, upregulation of ICAM-1 following CMV infection has been confirmed by other groups, both on fibroblasts, and on endothelial cells. However, the discrimination between effects on infected cells and bystander uninfected cells in infected cultures have largely been ignored in other studies. Increased ICAM-1 expression has been found in CMV infected cultures of proximal tubular epithelial cells, alongside increased class I HLA expression (van Dorp *et al.* 1993a). However, in the latter study, only a minority of the cells were infected with CMV, and no attempt was made to differentiate between the ICAM-1 expression on infected and bystander uninfected cells during the analysis, which was carried out using a cell ELISA system. Another group has also reported increased ICAM-1 expression on human embryonic lung fibroblasts, but again only 10-20% infection was achieved by 2 days post infection (Ito *et al.* 1995a), and no attempt was made to identify infected cells separately during analysis of the data. In the latter study, increased LFA-3 expression was not found in infected cultures, which is in conflict with our findings, but this may be explained by the low level of infection achieved. The finding from the present study that ICAM-1 was increased specifically on infected cells was confirmed by a report from another group, who showed by double staining that the expression of CMV early antigen localised with increased ICAM-1 expression (Sedmak *et al.* 1994b). Other results from this laboratory have shown that patterns of expression of ICAM-1 and LFA-3 following infection of endothelial cells are similar to those described here for fibroblasts, and that the mechanism involved also appears to be independent of cytokines (L.P. MacCormac, unpublished observations).

It was shown in the present study that the increase in the expression of ICAM-1 or LFA-3 on the surface of CMV infected fibroblasts was not inhibited by ganciclovir or foscarnet treatment. Indeed such antiviral treatment enhanced the effect of CMV infection on adhesion molecule expression, particularly in the case of LFA-3. In addition the kinetics of alterations in adhesion molecule expression on ganciclovir-treated infected cells was affected, with levels of both ICAM-1 and LFA-3 continuing to increase following the peak of expression in untreated infected cells, and remaining at enhanced levels when those on untreated infected cells had begun to decline. The similarity of the increase in LFA-3 levels following both ganciclovir and foscarnet treatment suggested that this molecule had further increased above normal infected cell levels in response to the effect of the antiviral drugs on the virus replication cycle, rather than to some other function of the drugs on the treated cells. ICAM-1 was not

upregulated further on foscarnet-treated infected cells compared to untreated infected cells, although there was some increase following ganciclovir treatment. However, the data obtained using both these drugs showed that effective antiviral treatment did not prevent the CMV-induced increase in adhesion molecule expression, and thus suggested that the upregulation of ICAM-1 and LFA-3 was due to the effects of CMV immediate early or early genes, rather than to CMV late genes. Despite the high levels of both ICAM-1 and LFA-3 on ganciclovir-treated infected cells at the time of the adhesion assays the levels of resting lymphocyte adhesion were not as high as adhesion to infected fibroblasts which were not ganciclovir-treated. The reasons for this are not clear, but it is possible that differences in the cytoskeleton of ganciclovir-treated infected cells may be responsible for the difference, as the cells exhibit a more rounded appearance than untreated infected cells, which spread out after the initial cell rounding which follows infection. Interactions between adhesion molecules and the cytoskeleton are important in regulating clustering of adhesion molecules and thus their avidity for their ligands. Although lymphocyte adhesion to ganciclovir-treated cells was not as high as to untreated infected cells, it was significantly greater than adhesion to uninfected fibroblasts, suggesting that infected fibroblasts remain a target for CD2+ve lymphocytes when virus replication has been halted by ganciclovir treatment.

The reasons why treatment with ganciclovir results in prolonged upregulation of adhesion molecule expression may be due to the disruptive effect of this agent on the CMV replication cycle. In cells treated with ganciclovir or foscarnet, disruption of the virus replication cycle results in the breakdown of the normal regulatory controls on the expression of the immediate early genes (Hermiston *et al.* 1990; Jenkins *et al.* 1994), some of which are regulated by a feedback inhibition mechanism. One of the gene products of the IE2 gene ($IE2_{338aa}$) is actually a 'late' protein in terms of when it is produced in the replicative cycle (Jenkins *et al.* 1994), and functions in the inhibition of the immediate early gene expression. Blocking late protein synthesis may interrupt the inhibition of IE gene expression, leading to an accumulation of IE proteins. The agents commonly used to block the expression of CMV IE or early gene products (cyclohexamide and actinomycin D) are not specific for CMV genes, but also affect the expression of cellular genes. Thus it was not possible to carry out similar experiments to those carried out using ganciclovir and foscarnet in order to investigate the effect of inhibition of the immediate early and early genes on adhesion molecule expression.

An attempt was made to address the possibility that an accumulation of CMV IE proteins may be responsible for the prolonged adhesion molecule upregulation. Plasmids containing constructs expressing the 72kD and 86kD gene products of CMV IE1 were obtained from Dr. Ray Jupp (Roche) with the intention of transfection of human embryonic lung fibroblasts, so that the characteristics of the resulting transfectants with respect to ICAM-1 and LFA-3 expression could be determined. However, using a variety of different transfection techniques, including Lipofectin™(Gibco), electroporation, and calcium chloride, transfection of human embryonic lung fibroblasts was not achieved, although all these techniques were used successfully in parallel transfections using Cos cells and human kidney epithelial cells. It has been documented that primary cells are more difficult to transfect than cell lines, due to their irregular growth characteristics (Gorman, 1985). It was unfortunate that the intended experiments could not be carried out in the time available. The use of viral mutants with particular viral genes deleted may be enlightening in determining the viral proteins which are important in adhesion molecule upregulation, as has been the case for class I HLA downregulation, but the success of such an approach would depend on whether the proteins of interest were essential for viral replication.

Adhesion molecule expression is upregulated following infection with several other viruses, both *in vitro* and *in vivo*. Increased adhesion molecule expression *in vivo* is generally attributed to the production of inflammatory cytokines by activated T cells recruited to the site of infection (Volpes *et al.* 1992; Marker *et al.* 1995). Cytokines induced in virally infected cells have also been shown to be responsible for adhesion molecule upregulation *in vitro*. A recent study has described enhanced expression of ICAM-1 in pulmonary epithelial cells infected with respiratory syncytial virus (RSV), due to the induction of IL-1 following infection (Patel *et al.* 1995). ICAM-1 expression was also increased in human airway epithelial cells infected with Parainfluenza virus, presumably by a cytokine mediated mechanism. In the latter system, increased ICAM-1 expression was associated with enhanced neutrophil binding and subsequent tissue damage (Tosi *et al.* 1992). Measles virus infection of glial cells was shown to upregulate ICAM-1 expression in an IFNα dependent manner (Kraus *et al.* 1992). This cytokine was shown in a previous study from this laboratory to be absent from the supernatants of CMV infected fibroblasts (J.E.Grundy, unpublished observations). West Nile Virus (WNV), a flavivirus, also induces ICAM-1 expression on human embryonic fibroblasts (Shen *et al.* 1995). This effect was biphasic, with direct viral induction of ICAM-1 at 4 hours post

infection, but induction regulated by virus induced soluble factors occurring at 24 hours post infection. The latter effect was assumed to be due to the release of IFNβ by the infected cells, as this group also showed a small increase in ICAM-1 expression in response to IFNβ (100U/ml), although this was not formally demonstrated. ICAM-1 expression was also increased in a similar manner by the related flavivirus, Kunjin. In the same study, ICAM-1 induction by an interferon-dependent mechanism was seen with Ross river virus, an alphavirus, but no induction of ICAM-1 followed infection with vaccinia, adenovirus 2 and 5 (all DNA viruses), or Semliki forest virus (Shen *et al.* 1995). In the present study, the addition of recombinant IFNβ did not affect the cell surface expression of ICAM-1 on fibroblasts. This is in contrast to the above findings, but in agreement with the findings of Dustin et al (Dustin *et al.* 1986a), who reported no induction of ICAM-1 by IFNβ treatment of human dermal fibroblasts. From the results presented in this chapter, it appears that IFNβ is not responsible for induction of ICAM-1, as class I HLA is increased in an IFNβ dependent manner in supernatant transfer and transwell experiments, but ICAM-1 expression is not altered by exposure to soluble factors released by infected cells by either of these methods.

The direct effect of viral infection on ICAM-1 expression is a novel method of adhesion molecule induction, and one which may occur prior to the recruitment of mononuclear cells. ICAM-1 expression is increased in hepatitis B virus (HBV) infection *in vivo* (Volpes *et al.* 1990), due to the activation of the ICAM-1 gene by the HBV X protein. This protein acts on the ICAM-1 gene in a similar way to IL-1β, which is via NF-κB activation. Class I and II genes are also transactivated by this HBV protein (Hu *et al.* 1992). Both ICAM-1 and LFA-3 are upregulated in human T cells transformed by HTLV-1, due to the action of the *Tax I* protein (Imai *et al.* 1993; Tanaka *et al.* 1995). HTLV-1 possesses the *Tax I* protein in addition to the typical retroviral genes. *Tax I* is a potent inducer of viral transcription and is also capable of transactivating a wide variety of cellular genes (Sugamura and Hinuma, 1993). Both HBV X protein, and Human T cell leukaemia virus type 1 (HTLV-1) *Tax I* increase the expression of ICAM-1, and in the case of *Tax I,* LFA-3, when transfected into cell lines. LFA-3 expression is decreased in EBV positive Burkitt's lymphoma cells, and this is thought to be a mechanism for immune escape by the virus. Burkitt's lymphoma cells with normal levels of ICAM-1, but low levels of LFA-3 are poor targets for virus specific CTLs, stressing the importance of LFA-3 in target cell/CTL conjugate formation (Gregory *et al.* 1988).

Although the LFA-3 gene has been cloned (Seed, 1987), and there is a wealth of information about its structure and function, information about the regulation of its transcription is limited. Cytokine induced LFA-3 upregulation in fibroblasts has not been shown, and reports of cytokine induction in other cell types are rare. Induction of LFA-3 by IL-4 was shown in Burkitt's lymphoma cells (Rousset *et al.* 1989). Induction of LFA-3 was also shown in human kidney epithelial cells in response to a combination of IFNγ and TNFα (Kirby *et al.* 1993). Increased expression of LFA-3 is also found in some disease states. LFA-3 levels were increased in hepatocytes in chronic hepatitis, and the latter was correlated with severity of inflammation, but this was not restricted to viral hepatitis, and the reason for the induction of LFA-3 was not known (Autschbach *et al.* 1991). LFA-3 and ICAM-1 expression were also increased in dermal fibroblasts from patients with systemic sclerosis, and enhanced levels of expression were maintained as the cells were passaged, although again the mechanism for the upregulation of these adhesion molecules was not understood (Shi-Wen *et al.* 1994). Studies in the above areas may elucidate the mechanisms involved in the control of LFA-3 expression.

To summarise the results from this chapter, the *in vitro* finding that ICAM-1 and LFA-3 expression appears to be controlled directly by the virus indicates that a similar mechanism may occur *in vivo*, independently of the induction of pro-inflammatory cytokines. Importantly it was found that the adhesion molecule induction by CMV was accentuated rather than prevented by antiviral therapy with ganciclovir or foscarnet. Thus, despite effective antiviral treatment, CMV infected cells may continue to act as a focus for pro-inflammatory activity, which could contribute to immunopathology and contribute to the pathology of CMV disease. In subsequent chapters we have investigated the functional consequences of adhesion molecule upregulation for leukocyte binding to CMV infected fibroblasts and their activation.

CHAPTER 5

ADHESION OF CD2+ve LYMPHOCYTES TO CMV INFECTED FIBROBLASTS

Chapter 5. Adhesion of CD2+ve lymphocytes to CMV infected fibroblasts.

5.1 INTRODUCTION

Adhesion is the critical first step in a number of cellular immune responses, including antigen-specific and antigen-independent leukocyte activation, cytotoxicity of T cells and NK cells, and migration of leukocytes to sites of infection. Such interactions are regulated both by the levels of adhesion molecule expression, and the affinity or avidity of the molecules for their ligands, in order to prevent inappropriate adhesive interactions taking place (Springer, 1990).

Although T cell activation induces increased levels of CD2 expression, and the expression of a high avidity form of CD2, adhesion of LFA-3 to CD2 on resting T cells also takes place (Hahn *et al.* 1992b). This does not occur at the normal physiological densities of CD2 and LFA-3, but is enhanced by increased expression of either molecule (Dustin and Springer, 1991). In contrast, the LFA-1/ICAM-1 interaction does not take place under resting conditions, as the activation of the LFA-1 molecule is a prerequisite for its function (Dustin and Springer, 1989; Cabanas and Hogg, 1991). It is known that many interactions increase the adhesiveness of LFA-1 for ICAM-1, including classical chemoattractants, lipopolysaccharide, treatment with certain anti-LFA-1 antibodies, and engagement of the TCR/CD3 complex (Rothlein and Springer, 1986; van Kooyk *et al.* 1989). Evidence is emerging that different activation stimuli exert distinct effects on the LFA-1 molecule. Both CD3 and phorbol ester activation increase T cell adhesion by inducing clustering of LFA-1 leading to the acquisition of a high affinity state, while magnesium activation increases the avidity of individual LFA-1 molecules by changing the conformation of the molecule (Stewart *et al.* 1996). Since activated cells are commonly found in the peripheral blood during CMV infection (Yang *et al.* 1989; Reinke *et al.* 1994a; Bertoletti *et al.* 1994a), the effect of T cell activation on lymphocyte adhesion to CMV infected fibroblasts was investigated in the present study.

Other changes following CMV infection involve the expansion of certain subsets of lymphocytes. In particular, the CD57+ve subset, which encompasses subpopulations of both T cells and NK cells, is expanded at 2 to 6 months after infection, and remains elevated in CMV seropositive individuals (Labalette *et al.*

1994; Wang *et al.* 1995). Expansion of the CD28-ve population also occurs during CMV infection and in other viral infections, and appears to constitute the most cytotoxic population of T cells (Labalette *et al.* 1994; Borthwick *et al.* 1994). There is also an inversion of the CD4/CD8 ratio in the peripheral blood during CMV infection, due to an increase in the absolute numbers of CD8+ve lymphocytes (Bukowski *et al.* 1983; Carney *et al.* 1983). High levels of CD8+ve lymphocytes are found in tissues affected by viral infections and graft versus host disease (Janossy *et al.* 1982), while tissues affected by autoimmune disorders are infiltrated by a large proportion of CD4+ve T lymphocytes.

The selective accumulation of memory cells in inflammation is also well known, such as in rheumatoid synovium and pleural and peritoneal infiltrates (Pitzalis *et al.* 1988). Memory cells can be defined by their expression of different isoforms of the leukocyte common antigen, CD45. CD45RO is a marker for memory T cells (Smith *et al.* 1986), while CD45RB is expressed at high levels on naive T cells and is lost as cells are repeatedly activated and differentiate further. $CD45RB_{low}$ thus represents the most differentiated subset of memory cells (Salmon *et al.* 1994). CD45RA is the marker for naive cells, and thus the reciprocal subset of the CD45RO subset. The memory subset of T cells (CD45RO+ve cells) also express increased levels of CD2, LFA-3 and LFA-1 in comparison to the CD45RA+ve subset, and are thus functionally adapted to increased adhesion (Sanders *et al.* 1988). The adherence of the T cell subsets mentioned above to CMV infected and uninfected fibroblasts has been examined.

The aims of this chapter were to assess the functional consequences of the ICAM-1 and LFA-3 upregulation following CMV infection. The adherence characteristics of CD2+ve lymphocytes to CMV infected fibroblasts have been investigated, following activation by three different stimuli: CD3 crosslinking, treatment with phorbol ester, and treatment with magnesium ions in the absence of other divalent cations. An analysis of the resting T cell subsets, or the NK cell subset, which adhered to infected fibroblasts was also carried out. Finally, the effect of CMV infection on the ability of fibroblasts to enhance CD3 mediated activation T cell activation was investigated.

5.2 RESULTS

5.2.1 Development of a quantitative adhesion assay

An important part of preliminary work in this chapter was the development of a quantitative adhesion assay, to be used for the investigation of the important parameters in adhesion of both resting and activated lymphocytes to CMV infected and uninfected fibroblasts. In the initial stages of development of the adhesion assay, it was necessary to establish a reliable method for quantitation of the number of lymphocytes bound to fibroblast monolayers. Several methods for quantitation of lymphocyte adherence were compared, including detection with antibodies, radiolabelling of the T cells with tritiated leucine, or chromium-51, and visual counting of bound cells from photographs of the cells.

A cell ELISA was devised, utilising detection of CD45 on the adherent lymphocytes by a monoclonal antibody, followed by detection of this antibody using a HRP conjugated secondary antibody, and the addition of the substrate, TMB, to produce a measurable colour reaction. This technique resulted in high background levels of colour development, which could not be reduced by further washing without detachment of a large proportion of the cells from the wells (data not shown). It was therefore not possible to quantitate the numbers of adherent cells using this technique.

Labelling of lymphocytes with chromium-51, which enters the cells by diffusion, was assessed as a potential method of quantitation of adherent cells. Chromium-51 uptake was a simple and reliable method of cell labelling, at least for resting lymphocytes. However, due to the nature of the chromium uptake, the label also leaks out of the cells relatively easily. The degree of leakage from resting lymphocytes was assessed over a one hour period, and was found to be minimal. An example of the amount or radioactivity incorporated into the cells and lost through leakage is shown in Table 5.1. However, the leakage may be increased following T cell activation, either due to changes in the lymphocytes following activation, or due to the multiple washing steps required in some of the protocols used in this study.

Visual counting involves counting the number of adherent lymphocytes bound to the fibroblasts. This can be achieved by photographing the cells and counting the number of adherent cells in random fields of view on the

photographs. Visual counting limits the number of wells which can be screened due to the time required for photography and counting of the cells, and the large numbers of photographs generated when photographing replicate fields of view under each condition. Examples of the photographs which were used for visual counting are shown in Figure 5.2(a).

Labelling with tritiated leucine involves the incorporation of the labelled amino acid into proteins synthesised by the cell during the labelling period. To determine the optimal conditions for leucine labelling of lymphocytes, the cells were incubated with either 10 or 50μCi/ml ^{3}H-leucine either for 1 hour or overnight. An example of the amount of labelling achieved is shown in Figure 5.1. Overnight incubation resulted in high levels of leucine incorporation, but a significant decrease in viability of the lymphocytes. During a one hour incubation, 50μCi was shown to result in a greater incorporation of radiolabel than 10μCi for the same time period. These conditions were used for subsequent experiments.

^{3}H leucine incorporation, was the assay chosen for quantitation of lymphocyte adherence to fibroblasts. The incorporation of the amino acid into cellular proteins dispensed with the problem of leakage of the label from the cells during the assay as was the case using chromium-51. β emitters such as tritium have a better safety profile than γ emitters such as chromium-51. In addition, ^{3}H has a half-life of 12 years, and the compound is stable for at least 1 year, in comparison to the half-life of chromium-51 which is 28 days. Tritium is thus a more convenient isotope for use in the laboratory.

5.2.2 Determination of the lymphocyte number resulting in maximal binding of resting CD2+ve lymphocytes

Having shown that tritiated leucine was incorporated into resting lymphocytes at sufficient levels for use in a quantitative adhesion assay, the next stage of development of the adhesion assay was to determine the effect of changes in the number of lymphocytes added to each well of fibroblasts. It was also important to confirm that ^{3}H leucine labelling provided a reliable representation of the levels of lymphocyte adherence which could be seen by visual examination. Experiments were carried out in which ^{3}H leucine labelled lymphocytes were added to wells of infected and uninfected fibroblasts at concentrations resulting in between 1 x 10^{5} and 1 x 10^{6} lymphocytes per well of

fibroblasts. The wells were photographed before solubilization of the contents and measurement of the amount of radioactivity present in each well.

Figure 5.2(a) shows representative random fields of view from wells of infected and uninfected fibroblasts, to which between 10^5 and 10^6 CD2+ve lymphocytes had been added per well. The mean numbers of adherent cells from 3 random fields of view are shown in Figure 5.2(b). Figure 5.2(c) shows the mean number of counts per minute of ^{3}H leucine bound to each well. The results obtained by these two methods show similar patterns, both in the number of cells resulting in maximal binding, and in the difference in T cell binding to infected and uninfected cells. At all cell numbers used there was significantly increased T cell binding to infected cells in comparison to uninfected cells. Figure 5.2(d) shows that a linear relationship exists between the number of cells counted visually and the counts per minute (CPM) bound/well.

Figure 5.2(e) shows the percentage of ^{3}H leucine labelled lymphocytes binding to the fibroblasts in relation to the original aliquot of cells added to the well. A higher percentage of lymphocytes were bound to infected fibroblasts in comparison to uninfected fibroblasts at all cell numbers used. The differential between the percentage of adherent lymphocytes to infected and uninfected fibroblasts was maximal at 5 or 7.5 x 10^5 cells per well. The percentage of adherent cells declined when 1 x 10^6 cells were added per well, suggesting that the binding sites on the infected fibroblasts for resting lymphocytes had been saturated. It was decided to use 5 x 10^5 cells per well in all subsequent experiments, as this provided similar results to using 7.5 x 10^5 cells, but reduced the number of cells required for each experiment.

5.2.3 Characteristics of binding of resting CD2+ve lymphocytes to infected and uninfected fibroblasts.

To define the parameters responsible for the increased adhesion to CMV infected fibroblasts compared to uninfected fibroblasts, adhesion was measured under normal assay conditions (37°C, in RPMI), and either in the absence of divalent cations at 37°C, or in the presence of divalent cations (RPMI) at +4°C. Adhesion between CD2 and LFA-3 is not affected by either of these conditions, whereas the ICAM-1/LFA-1 adhesive interaction is sensitive to temperature and changes in divalent cations, in particular magnesium.

Following ^{3}H leucine labelling, the lymphocytes were divided into three aliquots, one of which was washed and resuspended in divalent cation free buffer. The cells were then added to the fibroblast wells (prewashed in divalent cation free buffer), and the quantitative adhesion assay was carried out. From the remaining two aliquots, one was applied to wells of infected and uninfected fibroblasts as described previously, but following centrifugation was incubated at 4°C for 10 minutes, while the other aliquot of lymphocytes was incubated in the presence of divalent cations (RPMI medium), and at 37°C. Increased adhesion to infected fibroblasts over uninfected fibroblasts was maintained under divalent cation-free conditions, and decreased only slightly when the assay was carried out at +4°C (Figure 5.3). This suggests that the enhanced adhesion of CD2+ve lymphocytes to infected fibroblasts is not due to increased interactions between ICAM-1 and LFA-1, as such interactions would not be favoured under these conditions. As LFA-3 is increased on the surface of CMV infected fibroblasts, and is functional under resting conditions, it is likely to be principally responsible for the increased adhesion.

5.2.4 Binding of activated T cells to infected and uninfected fibroblasts.

To determine the functional effect of the ICAM-1 increase on the fibroblasts following infection, the adherence of activated T cells to infected and uninfected fibroblasts was measured.

Tritiated leucine labelled lymphocytes were either resting or activated by one of three activation stimuli, before use in a quantitative adhesion assay. The percentage of cells adhering to infected or uninfected fibroblasts was calculated in relation to the original population added to the well, as for the above experiments using resting T cells (Figure 5.4).

Resting lymphocytes

Adhesion of resting lymphocytes to infected fibroblasts was 2.18 fold higher than adhesion to uninfected fibroblasts (1.72 ± 0.08 % adhesion to uninfected cells, 3.76 ± 0.48 % adhesion to infected cells, n= 3 experiments).

CD3 activation

CD3 activation of lymphocytes resulted in significantly enhanced the adhesion to infected fibroblasts above resting levels (fold increase 3.76, p=0.004, n=3). Binding of CD3 activated lymphocytes to uninfected fibroblasts was also

increased above resting levels (fold increase 3.32, p=0.06, n=3). Activation of lymphocytes by the CD3 pathway thus resulted in increased adhesion of lymphocytes to both infected and uninfected cells. The adhesion of CD3 activated lymphocytes to infected fibroblasts remained significantly enhanced above adhesion to uninfected fibroblasts (fold increase= 2.5, p=0.013, n=3).

Magnesium activation

Magnesium activation of lymphocytes also resulted in significantly increased adhesion to both infected (3.61 fold, p=0.03, n=3 experiments) and uninfected fibroblasts (2.38 fold, p=0.014, n=3) above adhesion of resting lymphocytes. The differential in adhesion between infected and uninfected fibroblasts was also maintained following this treatment (3.3 fold greater adhesion to infected fibroblasts, p=0.043, n=3).

Phorbol ester activation

Phorbol ester stimulation caused the greatest increase in lymphocyte binding above resting levels to both infected (8.71 fold, p=0.039, n=3) and uninfected fibroblasts (15.12 fold, p=0.002, n=3). From visual inspection, the monolayers of both infected and uninfected fibroblasts appeared to be saturated by lymphocytes (not shown). The adherence of phorbol ester stimulated T cells to infected fibroblasts was increased slightly in comparison to uninfected fibroblasts, but the differential between the two was smaller than that found under other conditions of lymphocyte activation, or using resting lymphocytes (1.26 fold increase, p=0.09, n=3). From the three experiments carried out, the difference between adherence to infected and uninfected fibroblasts was statistically significant in only one experiment.

These results showed that adherence of both activated and resting lymphocytes was increased by CMV infection of fibroblasts above adherence to uninfected fibroblasts. Activated cells generally adhered at higher levels than resting cells suggesting that the activation of integrins had facilitated adhesion of activated lymphocytes to ICAM-1 expressed on fibroblasts and further upregulated on infected fibroblasts.

5.2.5 Blocking of T cell binding using antibodies to ICAM-1 and LFA-3

The contribution of ICAM-1 and LFA-3 to the increased binding of activated lymphocytes to CMV infected fibroblasts was investigated, using blocking

antibodies specific for each of these molecules. One hour before the adhesion assay, the fibroblast monolayers were pre-treated with blocking antibodies specific for ICAM-1, LFA-3, and irrelevant isotype controls, all of which were washed off before the addition of lymphocytes to the wells. The results are shown in Figure 5.5.

CD3 activated T cells

Adherence of CD3 activated lymphocytes to infected fibroblasts was significantly reduced by antibody against ICAM-1. Values were comparable to those seen using uninfected fibroblasts (Infected fibroblasts, 14.1 ± 2.31% binding; infected + anti-ICAM-1, 5.5 ± 1.92 % binding; uninfected fibroblasts, 5.71 ±1.59 % binding; mean ± SEM, n=3 experiments). Treatment of infected fibroblast monolayers with anti-LFA-3 antibody had no inhibitory effect on CD3 activated lymphocyte adhesion. The binding of CD3 activated lymphocytes to uninfected fibroblasts was not significantly altered by treatment of the fibroblasts with antibody against either ICAM-1 or LFA-3.

Magnesium activated T cells.

The adherence of magnesium activated lymphocytes to infected fibroblasts was also significantly reduced by antibody against ICAM-1, to values comparable to the levels binding to uninfected cells (Infected fibroblasts, 13.56 ± 2.27% binding; infected + anti- ICAM-1, 4.11 ± 0.29 % binding; uninfected fibroblasts, 4.10 ± 1.51 % binding; mean ± SEM, n=3 experiments). Treatment of infected fibroblast monolayers with anti-LFA-3 antibody had no inhibitory effect on the adhesion of magnesium activated lymphocytes. Levels of binding of magnesium activated lymphocytes to uninfected fibroblasts were not significantly altered by treatment of the fibroblasts with antibody against either ICAM-1 or LFA-3.

Phorbol ester activated T cells

The adherence of phorbol ester activated lymphocytes to infected fibroblasts was also significantly reduced by antibody against ICAM-1, to values lower than the levels binding to uninfected cells (infected fibroblasts, 32.75 ± 5.38% binding; infected + anti-ICAM-1, 16.49 ± 4.53 % binding; uninfected fibroblasts, 26.0 ± 4.18 % binding; mean ± SEM, n=3 experiments). Treatment of infected fibroblast monolayers with anti-LFA-3 antibody had no inhibitory effect on phorbol ester activated lymphocyte adhesion. Levels of binding of phorbol ester

activated lymphocytes to uninfected fibroblasts was not significantly altered by treatment of the fibroblasts with antibody against either ICAM-1 or LFA-3.

From these experiments it is evident that increased adhesion of activated T cells to infected fibroblasts is related to the increased levels of ICAM-1 which are expressed following CMV infection. The absence of inhibition of adhesion by anti-LFA-3 antibodies suggests that this adhesion pathway is not enhanced by T cell activation.

5.2.6 Phenotypic analysis of the T cell subsets bound to infected and uninfected fibroblasts.

Because increased numbers of CD2+ve lymphocytes adhered to infected fibroblasts, experiments were carried out to determine whether this was due to a general increase in the adherence of all T cell subsets, or whether a specific subset was responsible for the increased adherence. The subsets selected for investigation were of interest due to their increased or decreased presence in the peripheral blood and infected organs of CMV infected patients, or patients infected with other viruses.

Following a large scale adhesion assay using unlabelled lymphocytes, non adherent lymphocytes were removed from the wells, and the adherent lymphocytes were then detached from the fibroblasts. This resulted in five different populations of cells: the original population, the populations adhering to infected, or uninfected fibroblasts, and the non-adherent population from infected or uninfected fibroblasts, all of which were stained for various markers and analysed by flow cytometric analysis. Cells were stained for CD2 expression to enable gating of the CD2+ve population, and for two other markers. The results are expressed as the percentage of cells positive for each antigen, defined by placing a marker at the threshold of fluorescence of the irrelevant antibody control. In the case of CD45RB and LFA-1, cells were defined as expressing high or low levels of each molecule by the placing of a marker between the two populations.

The results of this analysis, which was carried out using lymphocytes from 5 different individuals, are shown in Figure 5.6.

Memory markers

CD45RO+ve cells were present in significantly increased proportions in the adherent populations recovered from both infected and uninfected fibroblasts compared to the proportions in the original population. The same was found for T cells expressing CD45RB at low levels. Although these subsets were present at significantly increased proportions in the adherent cell populations, there was no difference between the proportions bound to infected or uninfected fibroblasts (Figure 5.6(a-c)).

NK markers

Cells expressing NK markers such as CD16, and cells which were CD2+ve/CD3-ve/CD8+ve, did not constitute a higher proportion of the adherent populations than the original population (Figure 5.6(d,e)). Although CD56 expression was investigated in 4 individuals, only two had detectable levels of CD56+ve cells, and the proportions were so low that a conclusion could not be reached regarding the adherence properties of these cells.

CD28 and CD57

CD28-ve cells were present at significantly increased proportions in the populations binding to both infected and uninfected fibroblasts compared with the original population. However, this was not the case for CD57+ve cells which were present at increased levels in the adherent populations in only two out of four individuals, and adhered preferentially to the uninfected fibroblasts (Figure 5.6 (f,g)).

CD4 / CD8 subsets

CD8+ve cells were present in increased proportions in the adherent populations recovered from both infected and uninfected fibroblasts compared to the proportions in the original population, but the increase was not statistically significant. There was a corresponding decrease in the proportion of CD4+ve cells binding to both infected and uninfected fibroblasts, which was statistically significant, but again there was no difference between the proportions binding to infected and uninfected fibroblasts (Figure 5.6 (h,i)).

CD2 expression

CD2 was used as the marker to distinguish lymphocytes from fibroblasts, so was present on all adherent cells analysed. The levels of CD2 expression on adherent cells compared to the original population were examined. It was found

that CD2 expression on lymphocytes adhering to infected fibroblasts was higher than CD2 expression on the original population of lymphocytes for cells from all 5 individuals tested. In two experiments, cells adhering to uninfected fibroblasts also had high CD2 expression, while in the other three experiments, cells adhering to uninfected fibroblasts had CD2 expression equivalent to the level of expression on the original population (Figure 5.7). These results suggest that the increased expression of LFA-3 on the infected fibroblasts preferentially attracts the binding of cells with higher levels of CD2 expression.

LFA-1 expression

LFA-1 is present on all lymphocytes, but two distinct populations of cells exist, expressing either high or low levels. LFA-1 expression was analysed in only two individuals. The cells adhering to either infected or uninfected fibroblasts consisted of a greater proportion of cells expressing high levels of LFA-1 than the original population (data not shown). However, the non-adherent cell population was not devoid of LFA-1_{high} cells, so this marker is not definitive of the adherent population.

The results of the above experiments therefore showed that although certain subsets of lymphocytes preferentially adhere to fibroblasts, the increased adhesion molecule expression on infected cells does not specifically induce the adherence of any of the T cell subsets tested. It appears rather that the increased adhesion results from a greater capacity of the infected fibroblasts to support binding of the population of lymphocytes which have the characteristics which enable them to adhere. Memory T cells, which express higher levels of adhesion molecules, have a greater capacity for binding to fibroblasts than naive T cells.

5.2.7 The effect of CMV infection on lymphocyte proliferation

Due to the role of ICAM-1 and LFA-3 as costimulatory molecules, preliminary experiments were carried out to determine whether CMV infection enhanced the ability of fibroblasts to costimulate CD3 mediated T cell activation. Although fibroblasts are not professional antigen presenting cells, it was hypothesised that the enhanced expression of LFA-3 or ICAM-1 might provide a costimulus to increase anti-CD3 mediated T cell proliferation.

PBMCs were incubated in wells containing one of the following combinations: infected fibroblasts (3 days post infection), infected fibroblasts and anti-CD3, uninfected fibroblasts, uninfected fibroblasts and anti-CD3, anti-CD3 alone, or no stimulus. Proliferation was measured by thymidine incorporation at days 2 to 6 post incubation. The results from day 4 (Figure 5.8), correlated with the peak of proliferation, and are representative of the general pattern of responses at other time points.

Minimal proliferation was observed when PBMCs were incubated with infected fibroblasts in the absence of CD3 activation (resting 269±9.31 CPM; infected fibroblasts 308±27 CPM; mean±SEM, p=0.26). Incubation with uninfected fibroblasts resulted in a small increase in proliferation from resting levels (379±12.7; p=0.001). Addition of anti-CD3 antibody resulted in significantly increased proliferation. Co-incubation of PBMCs with anti-CD3 antibodies and CMV infected fibroblasts resulted in decreased levels of proliferation in 5 out of 6 donors. The average levels decreased from 4873±208 CPM in the presence of anti-CD3 antibodies only, to 3534±693 CPM in the presence of infected fibroblasts (mean±SEM, p=0.06). Incubation with uninfected fibroblasts did not have this negative effect on CD3 stimulated activation. PBMCs from 3 CMV seronegative and 3 CMV seropositive volunteers were used in this experiment, but there was no difference in the responses between the two groups, so the results are presented together. The exception to this was that one CMV +ve donor showed increased proliferation when co-incubated with infected fibroblasts in the presence of anti-CD3 antibodies.

Although proliferation in response to anti-CD3 was decreased by the presence of CMV infected fibroblasts, the nature of the proliferation assay does not provide sufficient information to define whether this was a general effect throughout the population of lymphocytes, or whether some lymphocytes became activated while others died or were unresponsive, leading to an overall decrease in proliferation in the well.

5.2.8 The effect of CMV infection on lymphocyte activation.

During viral infections and in allograft rejection, activation of effector function of both CD4 and CD8 positive cells may take place in the absence of proliferation of resting lymphocytes. To investigate whether there was any evidence of activation of a population of cells without proliferation, the expression of an

activation marker, CD69, was analysed. This marker was selected as it is one of the first indicators of lymphocyte activation, appearing within hours of activation, and continues to be expressed in the presence of the stimulus (Testi *et al.* 1994).

PBMCs from CMV seronegative volunteers were simultaneously activated with anti-CD3 monoclonal antibodies and co-cultured either with infected or uninfected fibroblasts, or in medium only. PBMCs were also incubated with infected or uninfected fibroblasts in the absence of another activation stimuli. PMA stimulation was used as a positive control, and lymphocytes incubated with no stimulus were used as a negative control. Cells were co-cultured for 1 or 3 days, when they were removed from the cultures and stained for the expression of CD4, CD8 and CD69. CD4 and CD8 positive populations were analysed separately for the percentage of cells expressing CD69, by triple colour flow cytometry. A marker was placed around the threshold of CD69 expression in the absence of any stimuli. This was used to determine the percentage of positive cells following activation.

The results from 3 days post incubation for CD4 and CD8 positive cells are shown for 3 separate experiments (Figure 5.9). Due to the variation in levels of CD69 expression in cells from the three donors, mean values have not been calculated. The positive control of PMA stimulation routinely induced CD69 expression on >95% of both CD4 and CD8 positive lymphocytes (data not shown). CD69 was expressed at low levels following incubation with infected or uninfected fibroblasts in the absence of CD3 activation. CD69 expression was increased to between 15 and 27% following CD3 activation. Incubation with infected fibroblasts and anti-CD3 antibodies lead to increased CD69 expression above this level on both CD4 and CD8 positive cells in two individuals, and in CD4 positive cells only in the third individual. Incubation with uninfected fibroblasts had either no effect on anti-CD3 activation, or caused a slight increase in CD69 expression on both CD4 and CD8 positive cells.

These results suggest that a portion of lymphocytes which do not respond to CD3 activation alone are costimulated by CMV infected fibroblasts to express CD69. As CD69 is a very early activation marker it is not possible to ascertain from this experiment whether these cells subsequently become fully activated or alternatively become apoptotic or anergic.

TABLE 5.1 Uptake of chromium-51 by lymphocytes[a].

Activity of ^{51}Cr added to $4x10^7$ lymphocytes	$7x10^6$ CPM, 200μCi
Total activity incorporated by $4x10^7$ lymphocytes	$1.8x10^6$ CPM (25.7% incorporation)
Mean activity in $1x10^6$ lymphocytes	40497 CPM
Leakage of ^{51}Cr from 1 x 10^6 lymphocytes[b] (1 hour at 37°C)	2549 CPM (6.2% of original activity)
Uptake of leaked ^{51}Cr by fibroblasts[c] (1 hour at 37°C)	60.5 CPM (2.3% of leaked ^{51}Cr) (0.002% of original activity)

[a] Chromium-51 was added to lymphocytes and incubated for 1 hour at 37°C. The table shows the amount of chromium-51 incorporated by the lymphocytes.

[b] Aliquots of 1 x 10^6 lymphocytes were incubated for a further 1 hour at 37°C. The lymphocytes were removed by centrifugation and the amount of chromium-51 which had leaked was measured.

[c] The supernatants containing leaked chromium were incubated with fibroblasts for 1 hour at 37°C. The amount of chromium taken up by the fibroblasts was measured.

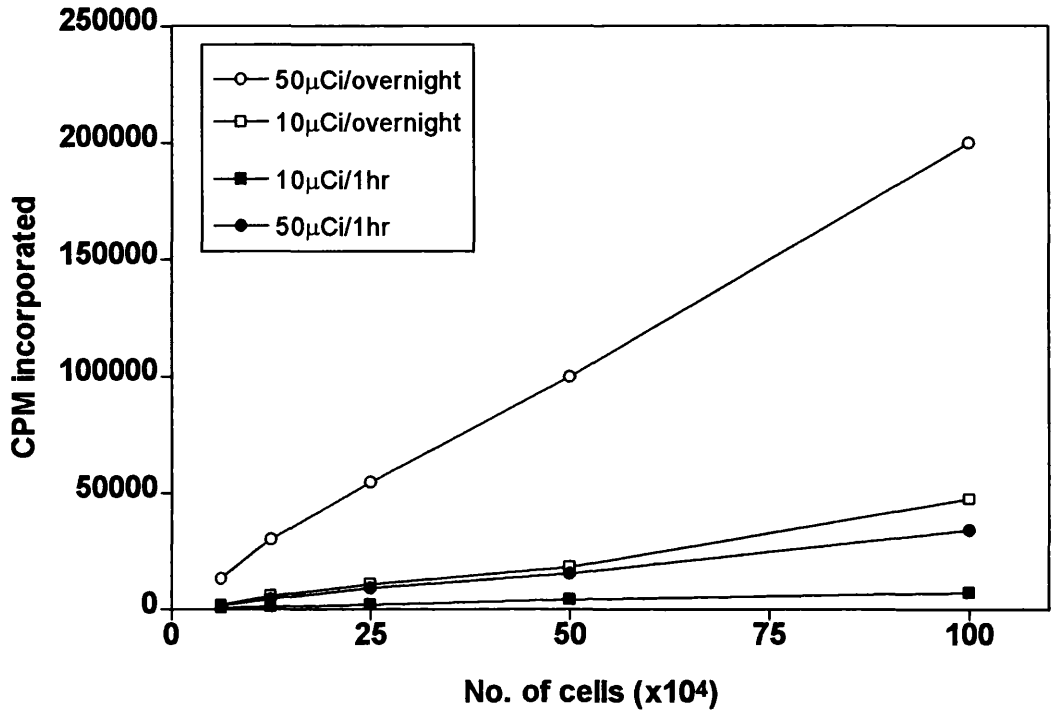


Figure 5.1. Incorporation of tritiated leucine by purified CD2+ve lymphocytes.

Lymphocytes were resuspended at a concentration of 5 x 10^7 cells/ml in leucine-free RPMI. Tritiated leucine was added at a concentration of either 10μCi/ml or 50μCi/ml, and incubated at 37°C for either 1 hour or overnight (16 hours). The cells were then washed twice and resuspended in PBS at 1 x 10^7 cells/ml . Aliquots containing the numbers of cells shown on the *x* axis were applied to a filter mat, and the radioactivity incorporated was measured using a Wallac β plate reader, and expressed as counts per minute (CPM) incorporated in each aliquot.

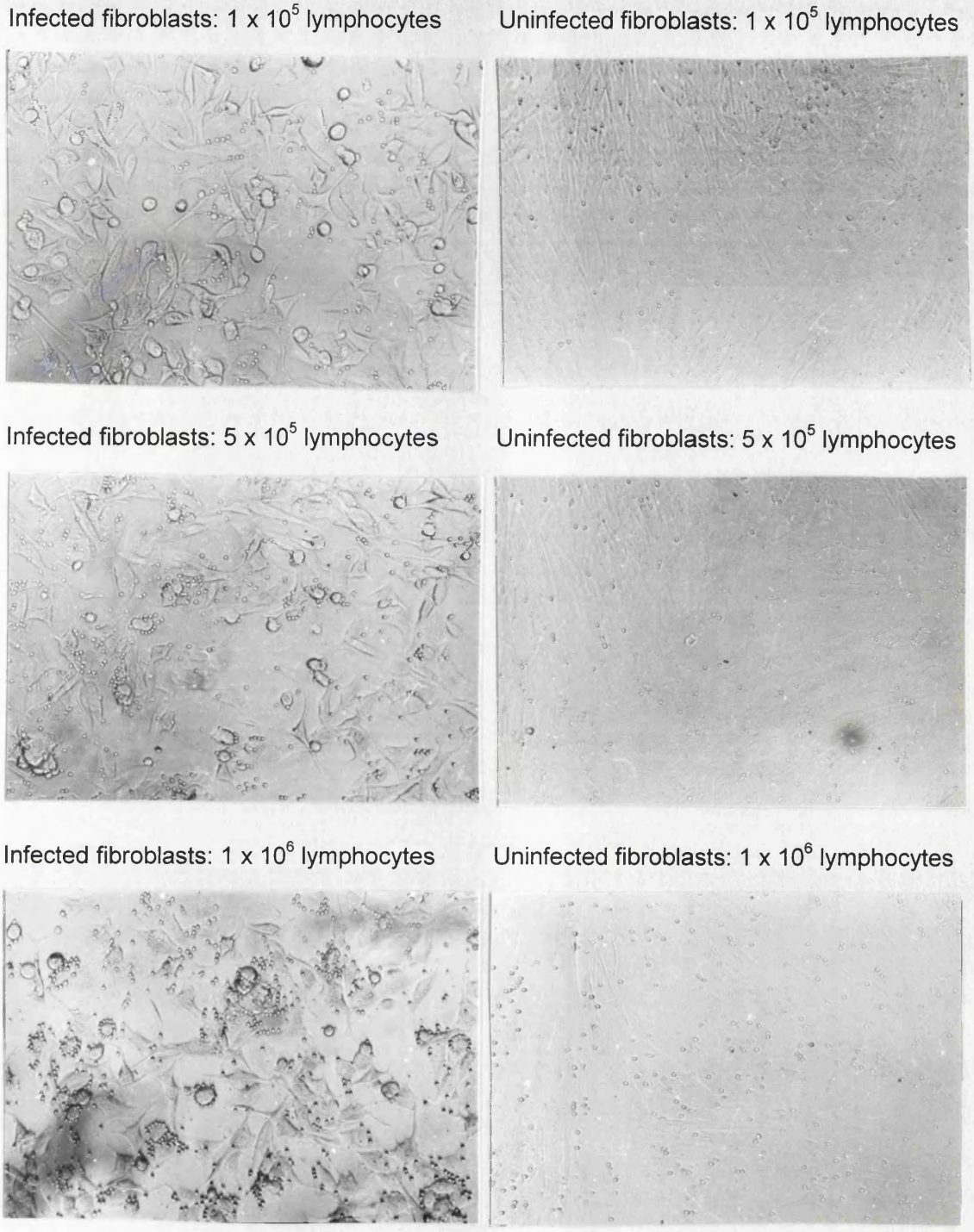


Figure 5.2 (a). Titration of the cell numbers required for optimal T cell adhesion.

Tritiated leucine-labelled resting T cells were added to confluent monolayers of infected (3 days post infection) and uninfected fibroblasts in 96 well plates. T cells were added to each well at the concentrations indicated. The adhesion assay was carried out as described in section 2.8. Photographs of resting T cells adhering to infected (left panel) and uninfected (right panel) fibroblasts are shown (a). The number of T cells added to each well is marked on the figure. *Continued on next page.*

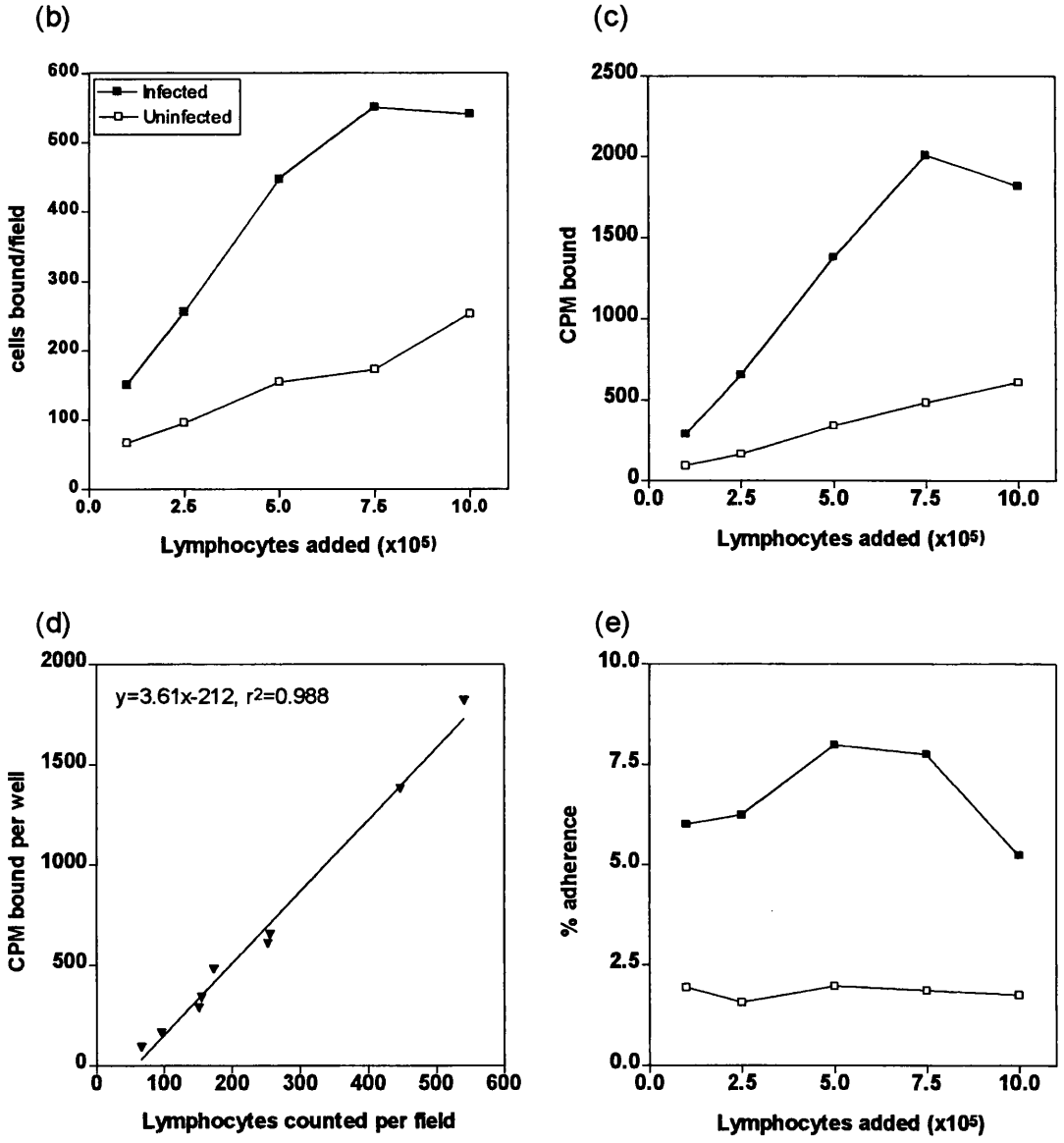


Figure 5.2 (b-e). Titration of the cell numbers required for optimal T cell adhesion.

The mean number of lymphocytes bound to infected or uninfected fibroblasts from 3 random fields of view was plotted against the number of cells added per well (b). Cells were solubilised, the amount of ^{3}H leucine bound per well was measured, and this was plotted against the number of cells added per well (c). The numbers of cells counted visually was plotted against CPM of leucine bound (d). Finally the CPM in the original lymphocyte aliquot added to the well was used to convert the CPM bound to each well to a percentage adherence value (e). The latter method of presentation is used in subsequent experiments. The experiment shown is representative of two separate experiments, each of which was carried out in triplicate.

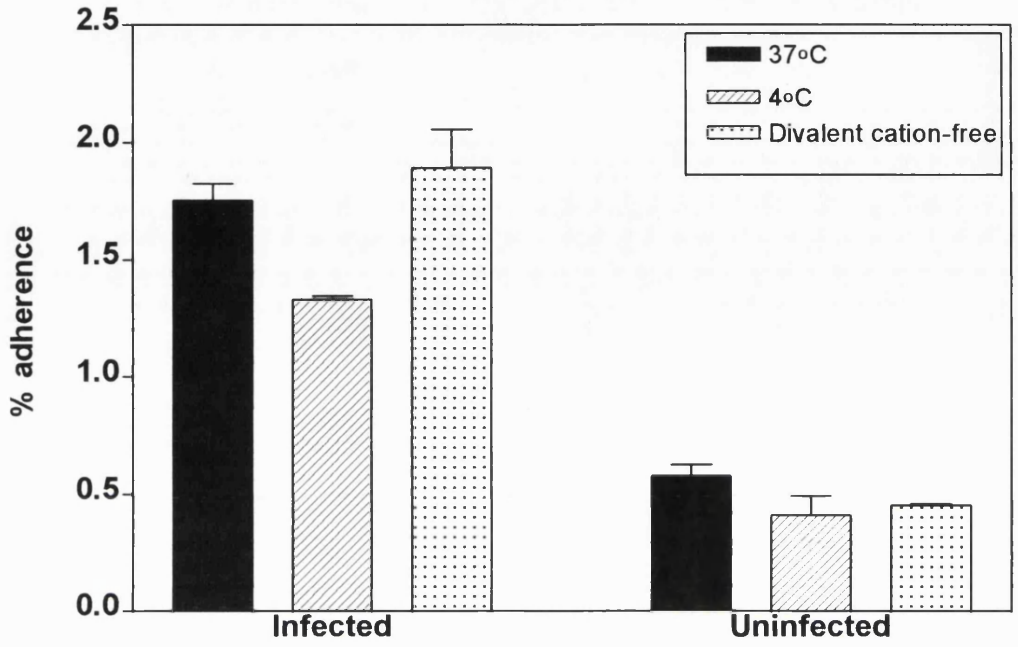


Figure 5.3. The binding of resting CD2+ve lymphocytes to infected and uninfected fibroblasts in the presence of EGTA, and at 4°C.

Resting T cells were labelled with tritiated leucine, resuspended at 5 x 10^6 cells/ml in RPMI, and separated into 3 equal aliquots for use in the adhesion assay. One aliquot was washed in cation-free buffer, containing EGTA, and a second aliquot of cells remained in RPMI, for use in an adhesion assay at 37°C. The third aliquot (in RPMI) was used for an identical assay on a separate plate, which was incubated at 4°C. 100μl was reserved from each aliquot for a total count of the ^{3}H leucine added per well. The results are expressed as the percentage of the original population which bound to the fibroblasts during the assay. The experiment shown is one of two separate experiments. The data shown is the mean ± SD of triplicate values.

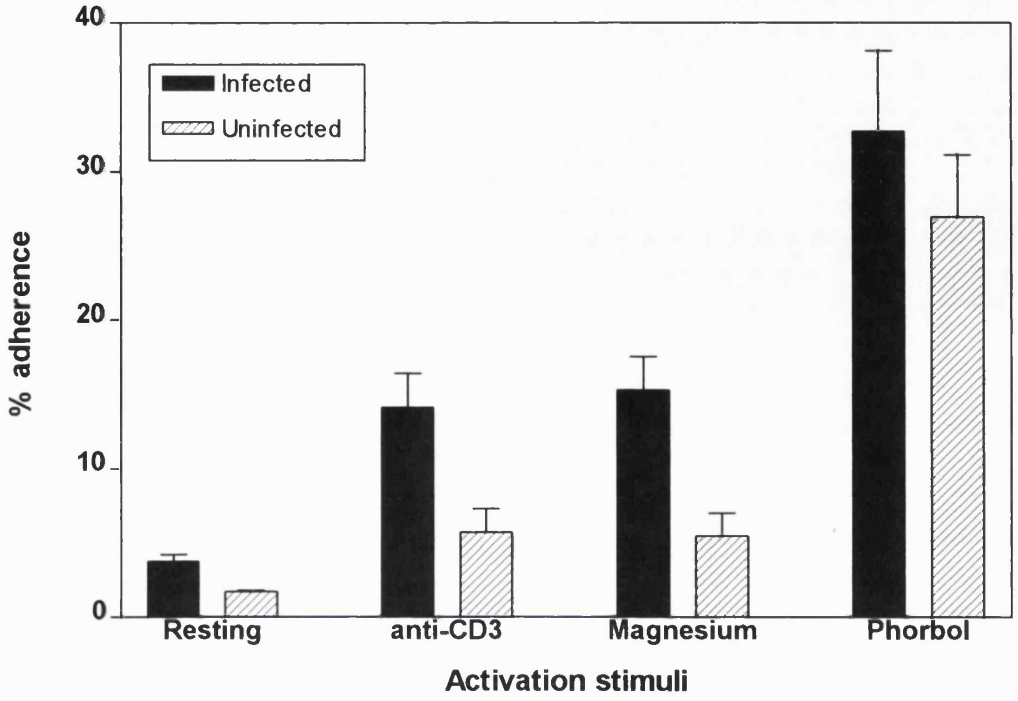


Figure 5.4. Adhesion of activated CD2+ve lymphocytes to CMV infected and uninfected fibroblasts.

Resting CD2+ve lymphocytes were labelled with tritiated leucine and divided into four equal aliquots, which were activated by the different stimuli as indicated, or remained resting. The labels on the X axis refer to lymphocytes either activated by crosslinked anti-CD3 antibodies (anti-CD3), treated with 5mM Mg^{2+} in the presence of 1mM EGTA (magnesium), or treated with 50nM PdBu (phorbol) in the presence of 1mM Ca^{2+} and 0.3mM Mg^{2+}. 5 x 10^5 lymphocytes were added to each well of CMV infected or uninfected fibroblasts in a quantitative adhesion assay. The percentage of cells adhering was calculated in relation to the amount of radioactivity added. The figure shows mean values ± SEM calculated from 3 separate experiments.

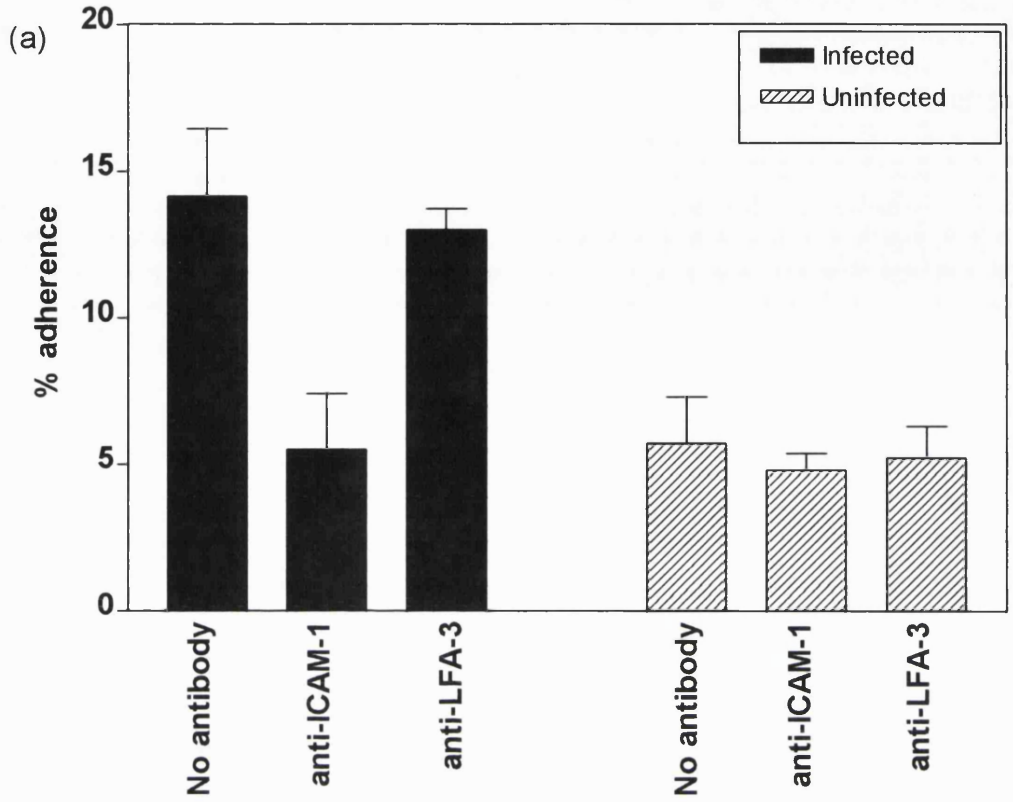


Figure 5.5(a). The effect of blocking antibodies on the adherence of CD3 activated lymphocytes.

Infected and uninfected fibroblast monolayers were treated with blocking antibodies specific for ICAM-1 and LFA-3 prior to the adhesion assay. The results from control wells without antibody pretreatment are shown, but the addition of irrelevant isotype control antibodies produced identical results. All conditions for activation were identical to those in Figure 5.3. The figure shows mean values ± SEM calculated from 3 separate experiments.

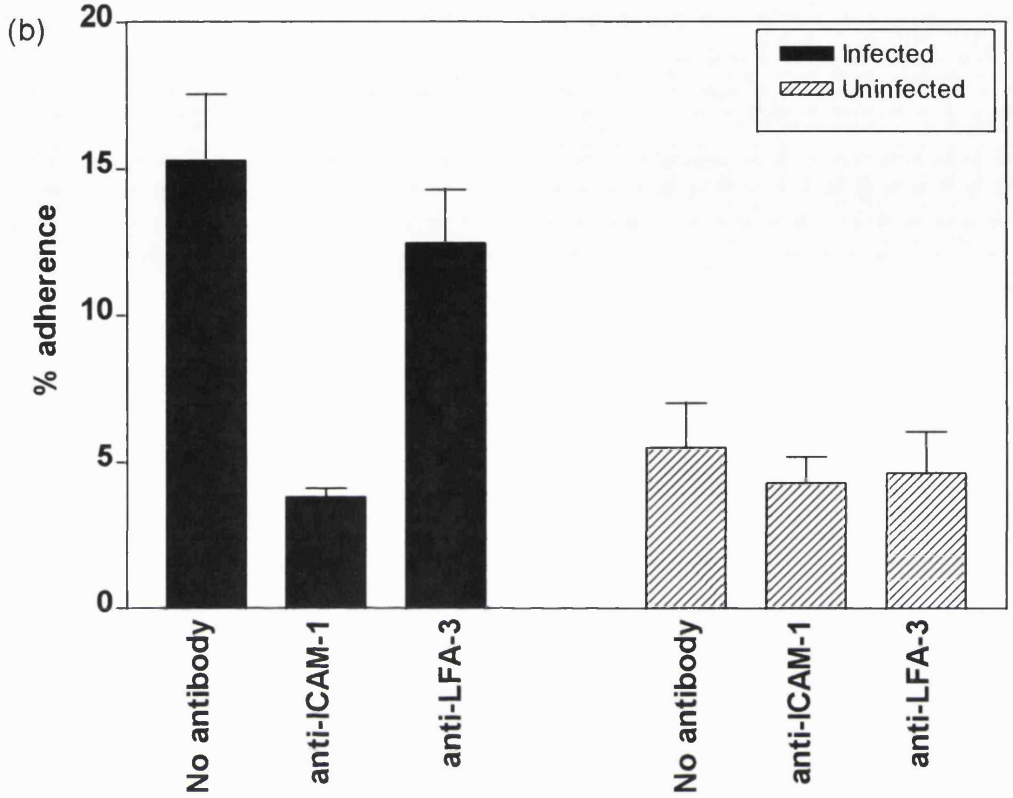


Figure 5.5(b). The effect of blocking antibodies on the adherence of magnesium activated lymphocytes.

Experimental conditions were identical to Figure 5.5(a). The figure shows mean values ± SEM calculated from 3 separate experiments.

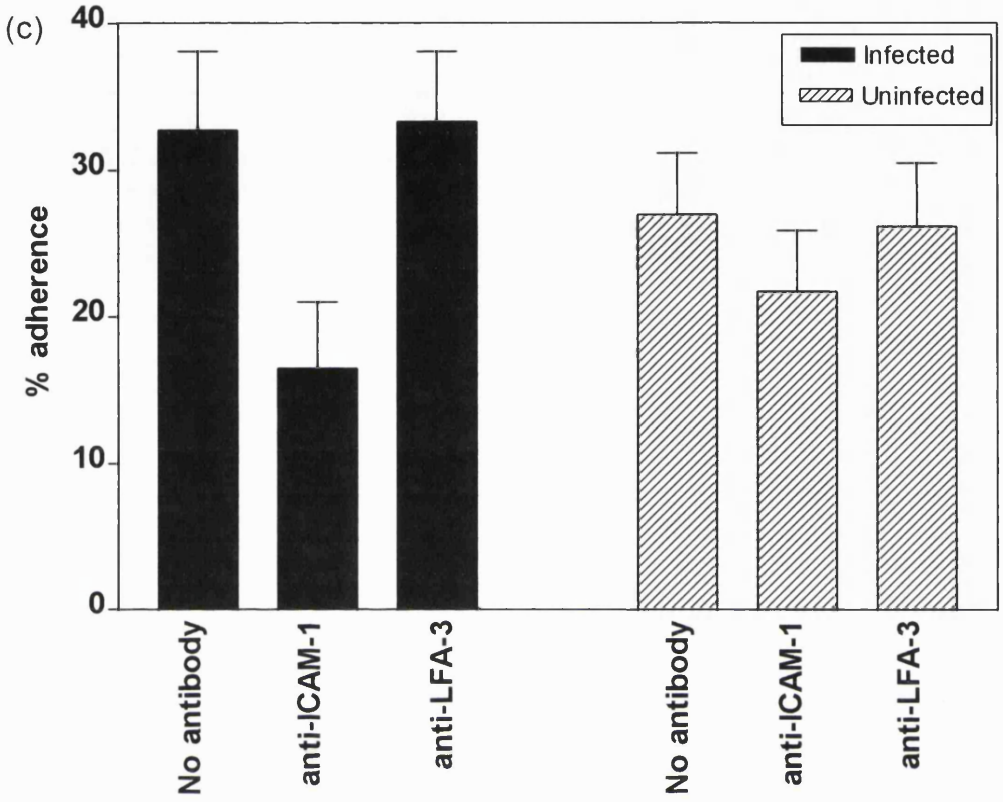


Figure 5.5(c). The effect of blocking antibodies on the adherence of phorbol ester activated lymphocytes.

Experimental conditions were identical to Figure 5.5(a). The figure shows mean values ± SEM calculated from 3 separate experiments.

bigger well
for large
scale
assay?
How done?

RO

more adherive ✓

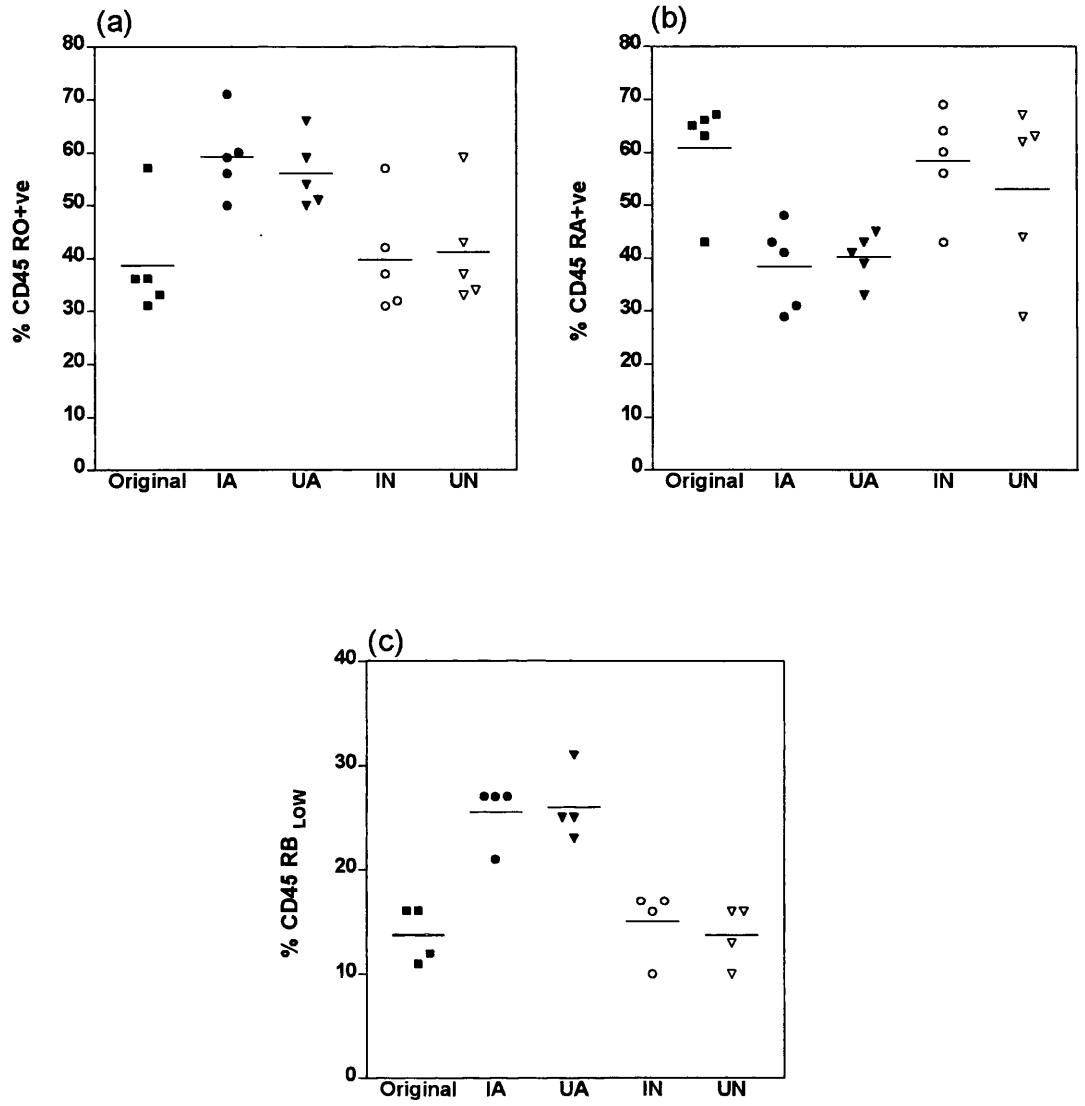


Figure 5.6 (a-c). Expression of CD45 isoforms on lymphocyte populations adherent to infected and uninfected fibroblasts.

A large scale adhesion assay was carried out using unlabelled CD2+ve lymphocytes. 5 x 10^6 lymphocytes were added to each well containing monolayers of either infected or uninfected fibroblasts (3 days post infection), and incubated for 1 hour at 37°C. The non-adherent population was removed, and the adherent cells were then detached from the fibroblasts as described. This resulted in five different populations of cells: the original population, the lymphocytes adherent to infected (IA), or uninfected (UA) fibroblasts, and the non-adherent population from infected (IN) or uninfected (UN) wells. Each was stained for the expression of the surface markers shown on the Y axis of each graph. Each data point represents the mean percentage (from duplicate values) of CD2+ve lymphocytes expressing each marker, from one individual. The expression of most markers was assessed in five individuals. The bars represent the means calculated from the five individual values.

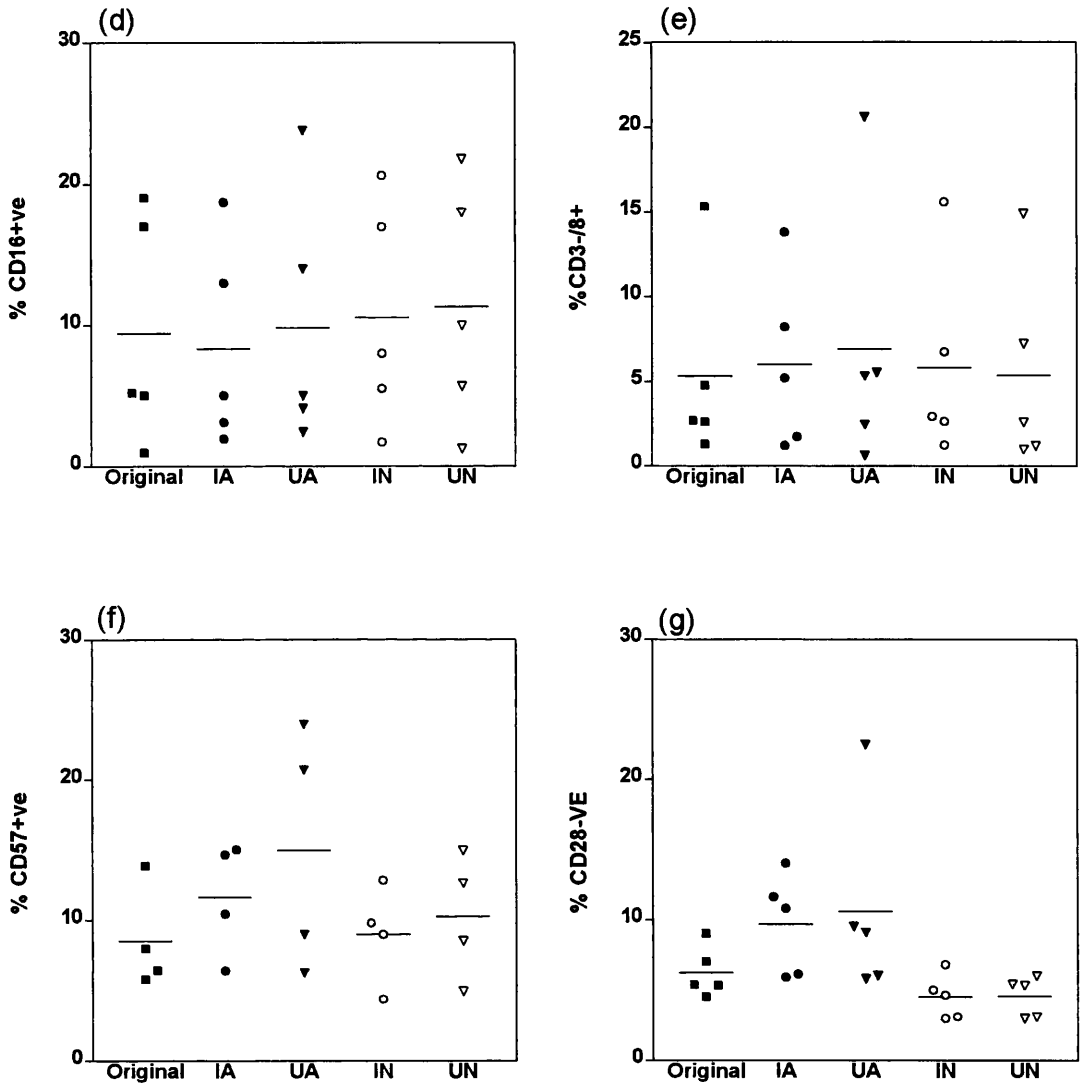


Figure 5.6 (d-g). Expression of NK cell markers, CD28 and CD57 on lymphocyte populations adherent to infected and uninfected fibroblasts.

The experimental design was identical to that described for Figure 5.6 (a-c).

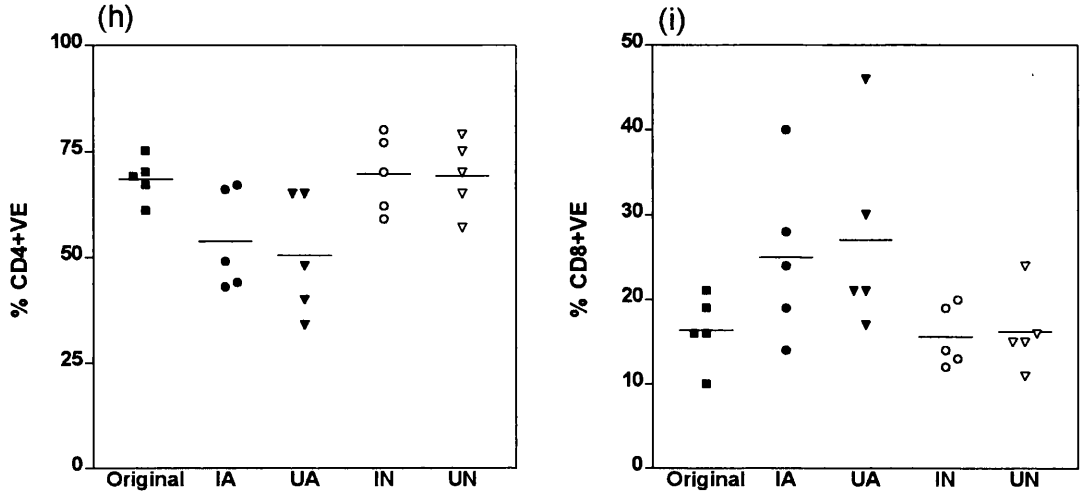


Figure 5.6 (h,i). Expression of CD4 and CD8 on lymphocyte populations adherent to infected and uninfected fibroblasts.

The experimental design was identical to that described for Figure 5.6 (a-c).

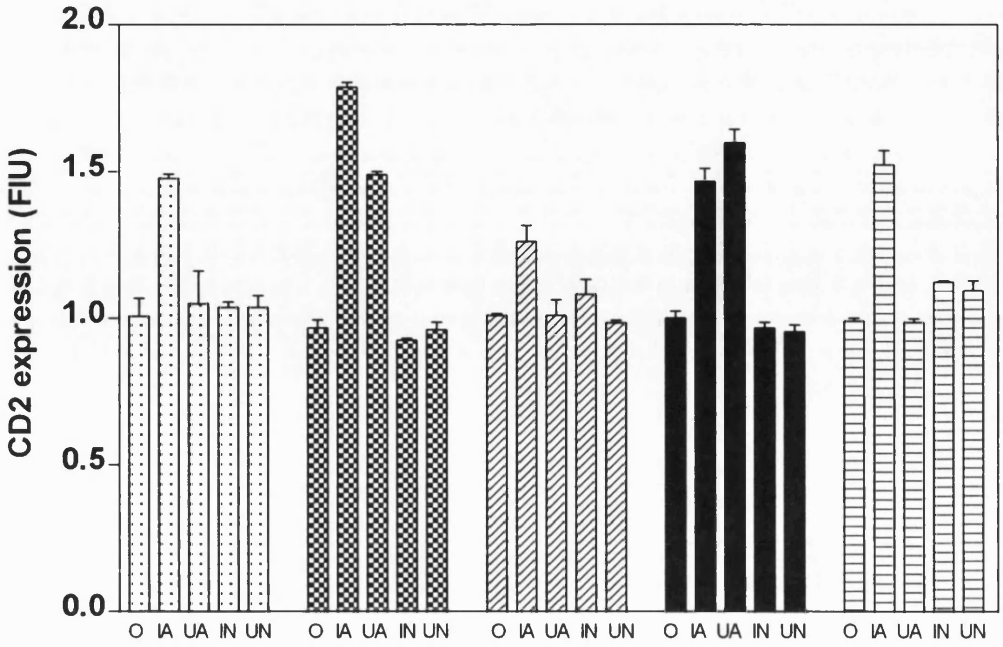


Figure 5.7. CD2 expression on lymphocyte populations adherent to infected or uninfected fibroblasts.

Lymphocytes harvested from the experiments described in Figure 5.5 were stained for the expression of CD2. The latter was used as a marker for lymphocyte gating so was present on all cells analysed. The levels of CD2 expression were calculated in relation to the levels on the original lymphocyte populations from each individual, which were assigned a value of 1. Each set of differently shaded bars represents data from a separate individual. The labels on the X axis correspond to those in Figure 5.6. The bars show mean values of duplicate samples ± SD.

= cpm / 18 hr !!

CD3
bound
or
soluble?

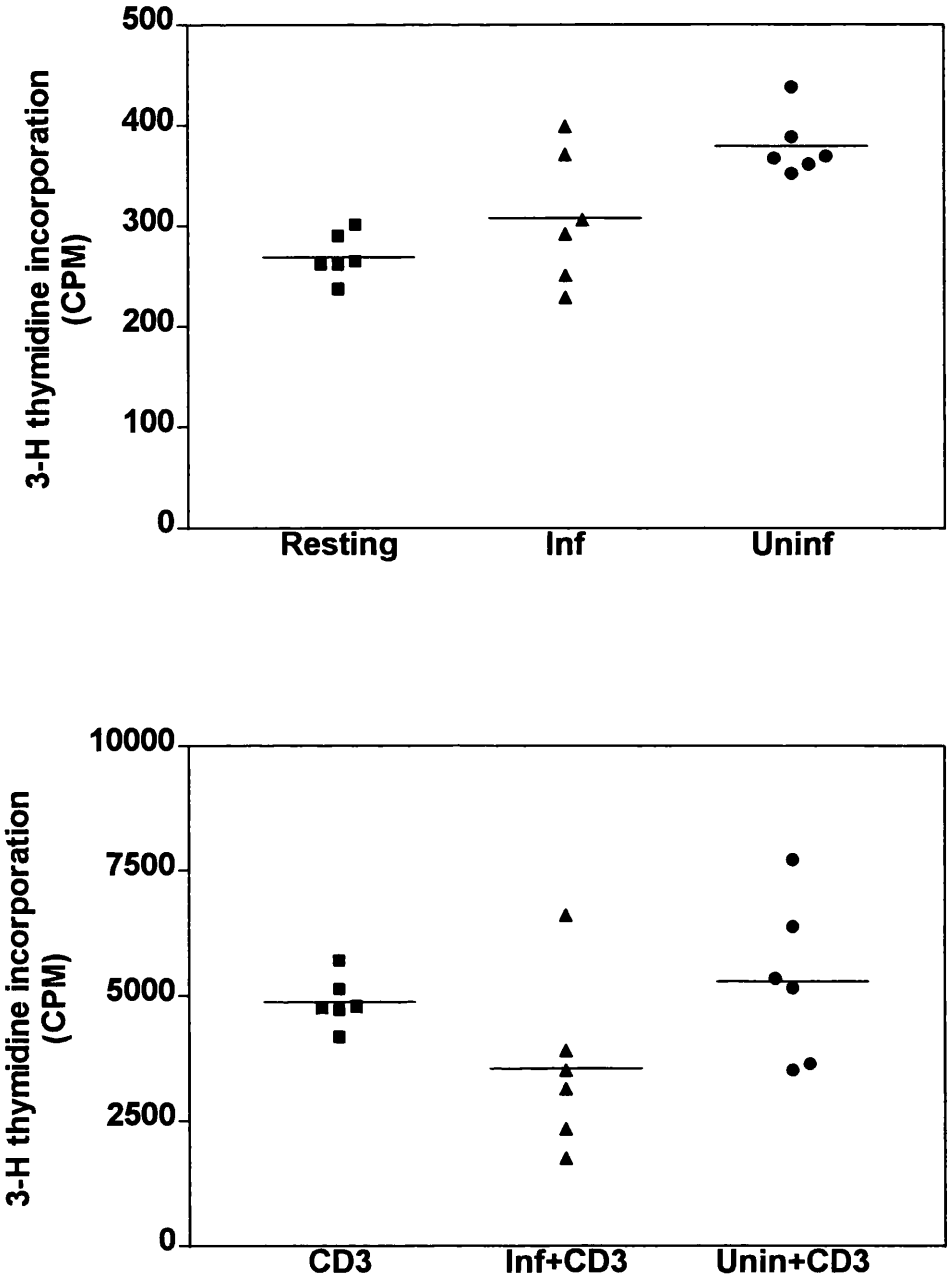


Figure 5.8. The proliferation of PBMCs in response to CMV infected and uninfected fibroblasts.

PBMCs were purified from 6 normal donors and incubated in wells (1 x 10^5/well) containing CMV infected or uninfected fibroblasts (2 x 10^4/well), in the presence or absence of anti-CD3 antibodies. Control wells contained PBMCs without fibroblasts. Fibroblasts were treated with mitomycin C at 3 days post infection for use as stimulator cells. Ganciclovir (1μg/ml) was included in the wells to prevent the production of viral DNA. Tritiated thymidine was added for 18 hour pulses at days 2 to 6 post incubation, after which the amount of incorporated tritiated thymidine was measured. The results from day 4 are shown. The six data points for each condition represent the mean proliferation from triplicate wells for each of six individuals.

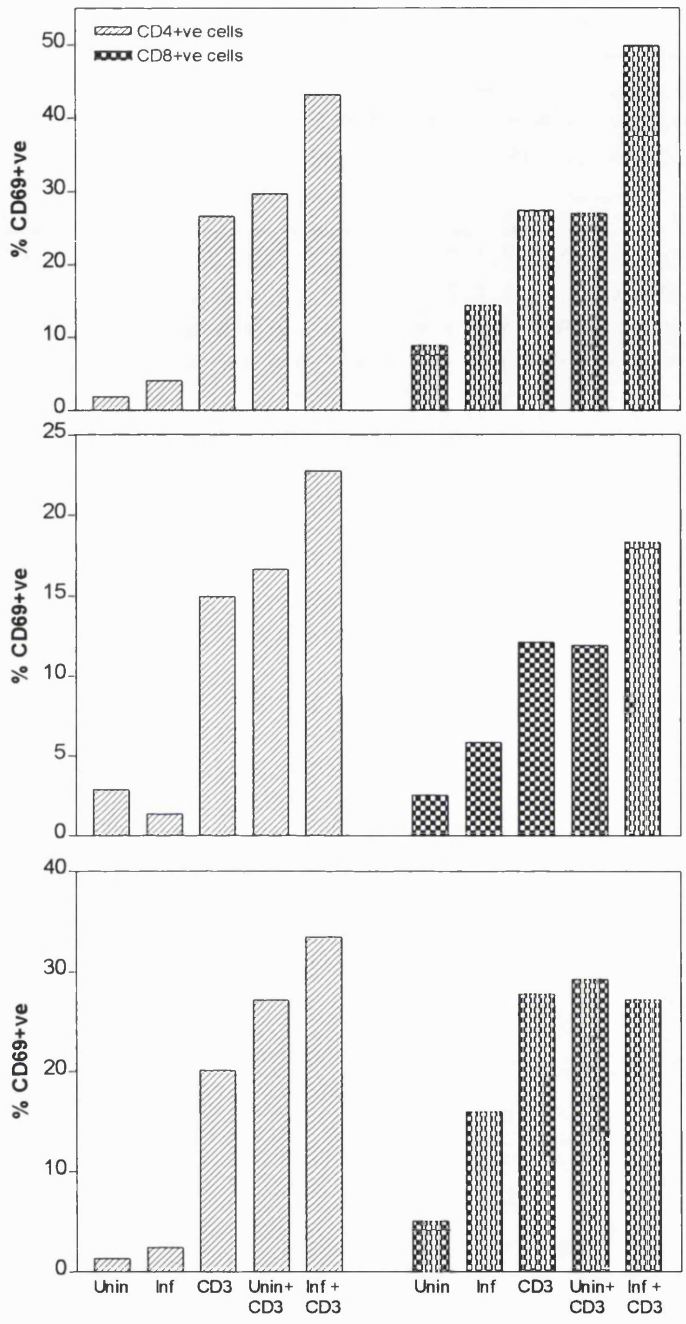


Figure 5.9. CD69 expression following PBMC co-culture with CMV infected or uninfected fibroblasts.

PBMCs were incubated with either CMV infected fibroblasts (immediately following infection), or uninfected fibroblasts, in the presence or absence of anti-CD3 antibodies, or with anti-CD3 antibodies alone. The contents of the wells were harvested at 3 days post infection and the cells triple stained for the expression of CD4, CD8 and CD69. The CD4 and CD8 populations were analysed separately for the expression of CD69. The bars show the mean percentage of lymphocytes expressing CD69 after each stimulus, from duplicate samples for each condition, from 3 separate experiments.

5.3 DISCUSSION

The main aim of this chapter was to determine the differences in T cell binding between uninfected and CMV infected fibroblasts which resulted from the increased levels of cell surface ICAM-1 and LFA-3 following CMV infection. The titration of cell numbers for optimal binding in the adhesion assay showed that at low cell concentrations, binding to infected and uninfected fibroblasts was sparse, and the differential between the two was small. The number of lymphocytes adherent to infected fibroblasts increased concomitant with increases in the number of lymphocytes added per well, until a plateau in the number of adherent lymphocytes was reached at an input lymphocyte number of 7.5×10^5 per well. The binding of lymphocytes to uninfected fibroblasts was less affected by cell concentration, suggesting that the molecules available for adhesion of CD2+ve lymphocytes had been saturated on uninfected cells. The possibility that stearic hindrance or other physical constraints were responsible for the plateau in lymphocyte adherence was ruled out by subsequent observations that activated lymphocytes adhered at substantially higher levels. This pattern of adhesion suggested that only a subset of lymphocytes have the capacity to adhere to fibroblasts, and that the presence of higher levels of adhesion molecules on infected fibroblasts allows a greater proportion of these cells to bind.

The critical parameters for the adhesion of T cells to uninfected fibroblasts have been investigated by others, although the conditions used were slightly different (Abraham *et al.* 1988; Cabanas and Hogg, 1991). Previous studies in dermal fibroblasts fixed with formaldehyde showed that spontaneous binding of resting T cells to fibroblasts was abolished by such fixation (Abraham *et al.* 1988). We have also found this to be true (data not shown). This suggests that movement of the molecules on both the target cell and the lymphocytes is required for efficient adhesion. The molecules involved in the background adherence to uninfected fibroblasts have not been identified, but it is likely that carbohydrate moieties are involved, as the presence of the sulphated fucose polysaccharide, fucoidin, is partially inhibitory to this process (Brandley *et al.* 1987; Abraham *et al.* 1988). Lymphocyte binding to fibronectin and other unidentified extracellular matrix components on fibroblasts also takes place (Abraham *et al.* 1989).

A previous study from this laboratory demonstrated that resting T cell binding to infected fibroblasts utilised the LFA-3/CD2 adhesion pathway, and there was a minimal role for the LFA-1/ICAM-1 interaction (Grundy *et al.* 1993).

Experiments examining the effect of divalent cation-free conditions, and performance of the assay at +4°C were carried out to confirm this finding. The increased binding of CD2+ve lymphocytes to infected fibroblasts above uninfected fibroblasts was maintained under both these conditions. There was a slight decrease in the level of adhesion of CD2+ve lymphocytes to infected fibroblasts at +4°C, but levels of binding remained significantly higher than to uninfected cells. The decrease may be due to a general slowing down of cellular processes at this temperature, as the assay duration was only ten minutes. The maintenance of the increased binding capacity of infected fibroblasts under divalent cation-free conditions confirms the minimal role for integrin mediated interactions in the binding of resting CD2+ve lymphocytes.

The adhesion of activated lymphocytes may be more relevant than that of resting lymphocytes to the interactions which take place between leukocytes and CMV infected cells *in vivo*, as CMV infection, and other viral infections are associated with activation of peripheral blood lymphocytes (Yang *et al.* 1989; van den Berg *et al.* 1992; Reinke *et al.* 1994b), and increased expression of β_2 integrins in particular (Reinke *et al.* 1994c; Borthwick *et al.* 1996). Graft-versus-host disease, which is strongly associated with development of CMV pneumonitis, is characterised by the activation of T cells, NK cells and B cells (Dokhelar *et al.* 1981; Ringden *et al.* 1987; Sullivan and Parkman, 1983). Stimulation with CMV antigen *in vitro* also leads to the generation of cells of an activated phenotype, expressing high levels of adhesion molecules (Nakano *et al.* 1993; Ito *et al.* 1995b). In the present study, activation of T lymphocytes by CD3 crosslinking and magnesium treatment induced significantly increased adherence to infected fibroblasts in comparison to uninfected fibroblasts. Lymphocytes activated by phorbol ester also adhered to infected fibroblasts at high levels, but adherence to uninfected fibroblasts was almost as high. It is possible that the surface area of the infected fibroblasts could not support further lymphocyte binding in line with the higher levels of adhesion molecules due to stearic hindrance by the high levels of binding. Adhesion of all activated lymphocytes to infected fibroblasts could be reduced to low levels by blocking with an anti-ICAM-1 monoclonal antibody, whilst the latter did not affect their adherence to uninfected fibroblasts. From the previous studies of other groups it had been expected that the binding of activated T cells would involve the ICAM-1/LFA-1 adhesion pathway (Dustin and Springer, 1989; Cabanas and Hogg, 1991), and indeed, the LFA-3/CD2 pathway appeared to play a minor role in the binding of CD2+ve lymphocytes following activation in the present study, as treatment with the anti-LFA-3 monoclonal antibody had no significant

effect on levels of adhesion. LFA-1/ICAM-1 interactions may be incompatible with the maintenance of CD2/LFA-3 interactions, as the LFA-1 and ICAM-1 molecules are significantly larger than CD2 and LFA-3. As the ICAM-1 and LFA-1 molecules do not appear to interact in the adhesion of resting CD2+ve lymphocytes, these larger molecules may be excluded from the zone of contact, and thus favour the CD2/LFA-3 interaction under resting conditions (Davis and van der Merwe, 1996).

The analysis of the resting T cell subsets and NK cells which bound to infected and uninfected fibroblasts showed that no subset showed preferential adherence to infected fibroblasts. The identification of memory T cells (CD45RO+ve and $CD45RB_{low}$) as the preferentially adhering population suggests that these cells have evolved to adhere to target cells and hence migrate through tissues and carry out their function when encountering the appropriate antigen. Memory T cells, as defined by the expression of CD45RO, express higher levels of adhesion molecules in order to have this function (Sanders *et al.* 1988; Akbar *et al.* 1991). The cells adhering to infected fibroblasts were found to have increased expression of CD2 and LFA-1 in the present study. A recent observation in a mouse model of viral encephalitis has shown that while antigen specificity does not influence entry of lymphocytes into inflamed tissues at any stage of infection, only antigen specific cells are retained, suggesting that these cells are unable to leave the tissue (Irani and Griffin, 1996) The efficient adhesion of memory T cells defined by expression of CD45RO, and the most differentiated memory cells, $CD45RB_{low}$ may lead to the selective retention of these cells in the tissues. The increased expression of adhesion molecules on CMV infected fibroblasts may act to prolong the adherence of such lymphocytes to infected cells and thus increase the efficiency of antigen recognition.

CD28-ve lymphocytes, which are considered to be the principal CTL effector cells, and contain cytotoxic granules (Borthwick *et al.* 1994; Kern *et al.* 1996), were found to adhere at increased proportions to both infected and uninfected fibroblasts. Cells expressing cytotoxic granules are predominant in bronchoalveolar lavage fluids of lung allograft recipients during CMV pneumonitis (Humbert *et al.* 1994). A population of $LFA\text{-}1_{high}$, CD57+ve, CD8+ve lymphocytes is expanded in patients exhibiting late-acute renal allograft rejection and symptomless CMV infection (Reinke *et al.* 1994c). The co-expression of these markers has not been investigated in the present study, but $LFA\text{-}1_{high}$ cells were shown to preferentially adhere to fibroblasts, while

increased adherence of CD57+ve cells to fibroblasts was not seen. This agrees with the clinical observation that CD57+ve cells were not found in the organs of patients exhibiting increased peripheral blood levels of CD57+ve cells during CMV infection (Reipert *et al.* 1992). The clinical significance of the CD57+ve cell expansion following CMV infection has not been established, but it has been suggested that they are terminally differentiated anergic cells which suppress MHC-restricted CTL function (Wang and Borysiewicz, 1995). It would be interesting to investigate the adhesion properties of lymphocytes from CMV infected individuals, due to the many differences in the lymphocyte populations in comparison to normal individuals.

NK cells accumulate in tissues early after CMV infection (Maher *et al.* 1985; Natuk and Welsh, 1987), and do not require prior activation to migrate or exert their cytotoxic effect. They also migrate more quickly than T cells in *in vitro* assays (Taub *et al.* 1995; Berman *et al.* 1996). NK cells are reconstituted earlier than T cells following BMT, and thus could be important in the control of infection in the absence of the T cell response. In the present study, NK cells, as defined as either CD16+ve, or CD2+veCD3-ve, were not found at increased levels in the adherent lymphocyte populations, either to infected or uninfected fibroblasts. The reason for the poor adherence of NK cells to fibroblasts is not clear.

In addition to the retention of lymphocytes in tissues, adhesion molecules may also function in the co-stimulation of lymphocytes. Both ICAM-1 and LFA-3 have been shown to have a costimulatory function *in vitro* (Tiefenthaler *et al.* 1987; Altmann *et al.* 1989; Hahn and Bierer, 1993). In the present study, incubation of CD3 activated T lymphocytes with infected fibroblasts resulted in increased numbers of activated T cells in comparison to CD3 activation alone, as defined by the early lymphocyte activation marker CD69. However, it can not be assumed that CD69 expression is always followed by full activation of the cell, as many activation stimuli only result in partial activation, and the fate of these cells is not clear, as increased proliferation was not observed in thymidine incorporation experiments. This may be due to the selective activation of a proportion of lymphocytes in the cultures, in conjunction of either death or anergy of the remaining cells. Activation in viral infections is also associated with increased cell death by apoptosis (Akbar *et al.* 1993), but there was no obvious morphological evidence of apoptosis in these cultures. Cell cycle analysis may provide an insight into the numbers of cells which are proliferating, anergic, or apoptotic. Further experiments are therefore required

to determine whether CMV infected fibroblasts are providing a positive or negative signal to lymphocytes. The proportion of cells which became activated may represent effector cells which do not require a signal through CD28 in order to exhibit effector function, in contrast to the proliferation of resting cells which requires a signal from B7 on professional antigen presenting cells (Azuma *et al.* 1992; Azuma *et al.* 1993a). LFA-3 and ICAM-1 are involved in activation of virus specific effector cells (de Waal Malefyt *et al.* 1993), and the upregulation of these molecules following CMV infection may thus enhance the activation of virus effector cells, and provide protection from disease. However increased interaction between allospecific effector cells and CMV infected fibroblasts may lead to increased activation of the latter cells that may contribute to tissue damage and allograft rejection.

CHAPTER 6

ALTERATIONS IN CHEMOKINE PRODUCTION BY CMV INFECTED FIBROBLASTS AND THEIR ROLE IN LEUKOCYTE RECRUITMENT

Chapter 6. Alterations in chemokine production by CMV Infected fibroblasts and their role In leukocyte recruitment.

6.1 INTRODUCTION

Chemokines interact with leukocytes by activating integrins to facilitate strong adhesion to the endothelium and thus migration through interstitial tissues, and additionally provide a concentration gradient for the spatial localisation of migration (Adams and Shaw, 1994). The selectivity of chemokines is achieved by the expression of specific chemokine receptors on leukocyte subsets (Mackay, 1996). Both non-immune cells and migrated leukocytes are sources of chemokines in tissues. The stimuli leading to chemokine production vary among different cell types, and include cellular activation, the action of cytokines, and viral and bacterial infection (Van Damme *et al.* 1989; Okamoto *et al.* 1993). Stimulation of chemokine production by viral infection may be a host mechanism for attracting particular leukocytes to sites of infection.

The accumulation of specific leukocyte subsets within tissue is a fundamental feature of inflammation. Different diseases, such as cystic fibrosis, rheumatoid arthritis, and atherosclerotic disease are associated with particular patterns of leukocyte infiltration that correlate with differential expression of chemokines (Strieter *et al.* 1994). In viral infections, migration of leukocytes to sites of infection is important for control of the infection, but accumulation of leukocytes may also lead to tissue damage. It is well established that T cell activity causes immunopathology in the lung in respiratory syncytial virus infections (Alwan *et al.* 1994; Openshaw, 1995). In certain conditions associated with CMV infection, such as CMV pneumonitis and allograft rejection, activated T cells or cytotoxic effector T cells accumulate in the tissues (Humbert *et al.* 1992; Humbert *et al.* 1994; Ouwehand *et al.* 1994). Altered chemokine production following CMV infection, together with adhesion molecule upregulation, may change the patterns of leukocyte migration through tissues. Furthermore, the expression of the CMV encoded β chemokine receptor may be important in determining the balance of chemokines in infected tissues (Neote *et al.* 1993; Gao and Murphy, 1994).

The aims of this chapter were to establish the profile of chemokines produced by CMV infected fibroblasts, at both the protein and mRNA levels and then to determine whether specific subsets of leukocytes migrated through endothelial

monolayers towards supernatants from CMV infected fibroblasts. The transendothelial migration of neutrophils and different subsets of resting T cells was investigated.

6.2 RESULTS

6.2.1 CMV infection of fibroblasts enhances steady state levels of IL-8 and MCP-1 mRNA.

To investigate the profile of chemokines induced following CMV infection of fibroblasts, total cellular RNA was extracted from infected and uninfected cultures at selected times post infection, and analysed by northern blotting. The blots were probed for the presence of IL-8, MCP-1, RANTES, and MIP-1α mRNA using complementary DNA probes.

At two hours post infection, IL-8 mRNA could not be detected in either uninfected or CMV infected fibroblasts. IL-8 mRNA in infected cells was first detected at 6 hours post infection, and remained above the levels in uninfected cells at all time points measured (up to 48 hours post infection). The levels of IL-8 mRNA in infected cells were lower at 24 hours than at 12 and 48 hours, although still significantly above the levels in uninfected cells. Steady state levels of MCP-1 mRNA were increased following CMV infection, reaching maximal levels at 12 hours post infection after which they declined (Figure 6.1).

There was no detectable expression of RANTES or MIP-1α mRNA at any of the above time points (data not shown). Subsequent experiments therefore focused on the production of IL-8 and MCP-1.

6.2.2 CMV infection enhances production of IL-8 by human embryonic lung fibroblasts.

To characterise the effect of CMV infection on IL-8 production, culture supernatants from infected fibroblasts were harvested and assayed for the presence of IL-8 by immunoassay at 6, 12 and 24 hours post infection. Higher levels of IL-8 were consistently found in infected cell supernatants compared to those from uninfected cells. While constitutive levels of IL-8 produced by uninfected fibroblasts were low in all experiments (2.45±0.56ng/10^6 cells, n=4), peak levels in infected cultures were significantly increased, to a mean of 11.08±1.17ng/10^6 cells (n=4, p=0.001), representing a mean fold increase of 4.87±0.53 from uninfected levels at the same time points. Enhanced IL-8 levels were detected from as early as 6 hours post infection. Figure 6.2 shows mean

IL-8 levels produced in 2 identically prepared cultures of fibroblasts established from different donors.

6.2.3 MCP-1 levels in supernatants from human embryonic lung fibroblasts are decreased by CMV infection.

To investigate the effect of CMV infection on MCP-1 levels at the protein level, supernatants from CMV infected, and uninfected control fibroblasts, were harvested at 24, 48, and 72 hours post infection, and assayed for MCP-1 production by ELISA. The uninfected fibroblasts were found to produce MCP-1 constitutively. At 24 hours post infection, MCP-1 levels in supernatants from infected fibroblasts were significantly lower than the levels present in uninfected cell supernatants (mean = 63% of uninfected level, range 49%-79%). By 48 hours, the levels measured in the infected supernatant had decreased dramatically, to 4% of uninfected levels (range 1.6%-5.4%), as was the case for supernatants harvested at 72 hours post infection (Figure 6.3). Cells treated with virus free inoculum were found to produce similar constitutive levels to the uninfected cells, suggesting that the decreased levels of MCP-1 in the infected cultures were a result of viral infection (data not shown).

Supernatants were also tested for the presence of the β chemokines MIP-1α and RANTES, but these cytokines were not detectable in supernatants from either uninfected or CMV infected fibroblasts (data not shown).

6.2.4 CMV-induced upregulation of IL-8 and MCP-1 mRNA is a direct viral effect.

In addition to virions, CMV inoculum contains other factors released from infected cells. It was therefore possible that the increase in IL-8 and MCP-1 mRNA levels was in response to another factor present in the viral inoculum. To address this issue, cells were infected with a 'purified' virus preparation, and the IL-8 and MCP-1 mRNA levels were measured at 24 hours post infection. This virus preparation induced increases in IL-8 (Figure 6.4) and MCP-1 (Figure 6.5) mRNA levels similar to those achieved by whole CMV inoculum, suggesting that the cytokines in the inoculum were not responsible for this effect.

6.2.5 CMV induced upregulation of IL-8 protein is a direct viral effect.

Although it was shown in Chapter 4 that supernatants from CMV infected fibroblasts contained no detectable IL-1β and TNFα, it was possible that low levels of these cytokines, or other cytokines induced by infection could be responsible for the IL-8 upregulation in CMV infected fibroblasts. IL-8 protein levels in infected supernatants were measured in the presence of neutralising antibodies specific for IL-1β and TNFα, which were added to the cells both during the 1 hour virus adsorption period, and following infection. The concentrations of antibodies used were sufficient to neutralise the activity of 0.5ng/ml of their respective cytokines (Table 2.4). Treatment with neutralising antibodies also had no effect on IL-8 production (Figure 6.6). The effect of virus free inoculum on IL-8 production was also investigated. Fibroblasts were also infected with 'purified' virus, and produced similar levels of IL-8 to cells infected with the usual CMV inoculum. CMV inoculum from which the virus was removed by ultracentrifugation had no effect on IL-8 production by fibroblasts. This further confirmed that the increased production of IL-8 following infection was not a secondary effect of cytokines present in the inoculum.

6.2.6 Sequestration of recombinant MCP-1 by infected fibroblasts.

It was possible that the MCP-1 levels were decreased in infected cultures due to either inhibition of constitutive production by CMV infection, or by sequestration of the MCP-1 by the infected cells as a result of the expression of the CMV encoded β chemokine receptor. In order to test which of these two possibilities was occurring, recombinant MCP-1 was added to infected and uninfected cells at 1000 and 10,000pg/ml at the time of infection, and the supernatants were harvested at 24 and 48 hours post infection. At 24 hours post infection, the levels of MCP-1 in both infected and uninfected cultures were approximately equal to the sum of the constitutive levels produced by uninfected cells and the amount of recombinant MCP-1 added. In contrast, at 48 hours post infection, infected cell supernatants contained greatly diminished levels of MCP-1 in comparison to uninfected cell supernatants (Figure 6.7).

6.2.7 Increased neutrophil transendothelial migration occurs in response to supernatants from CMV infected fibroblasts.

IL-8 is a powerful neutrophil chemoattractant, and also induces the migration of some T cell subsets and NK cells. The supernatants from CMV infected fibroblasts were first tested for the ability to influence neutrophil migration. Supernatants were harvested from infected and uninfected fibroblasts at 6 hours post infection, and tested for their ability to enhance the transmigration of neutrophils across an endothelial cell monolayer. Labelled neutrophils were placed on the top of a cell culture insert coated with endothelial cells, and migration towards supernatants from infected or uninfected fibroblasts placed in the lower chamber, or IL-1β controls, was measured.

Under resting conditions (i.e. only medium present in the lower chamber), 8.5 ± 3.12% (mean±SE, n=4) of the starting neutrophil populations migrated during the period of the assay. This basal level was designated 100%. Neutrophil migration towards supernatant from infected fibroblasts was significantly higher than that towards supernatant from uninfected fibroblasts (321 ± 32% of basal levels for infected cells, 152 ± 16% for uninfected cells, $p<0.05$, n=4 experiments) (Figure 6.8). Supernatants from uninfected fibroblasts were also found to increase neutrophil migration above the basal level. This observation was consistent with the constitutive low level production of IL-8 by different fibroblast lines as measured by ELISA described above.

6.2.8 Inhibition of CMV induced transendothelial migration by neutralising antibody specific for IL-8.

In order to determine whether the increase in neutrophil transmigration was due to the presence of increased levels of IL-8, supernatants harvested from infected and uninfected cells at 6 hours post infection, as well as positive (the chemotactic peptide fMLP, 10^{-7}M) and negative controls (medium), were preincubated with a neutralising antibody against IL-8, or an IgG control antibody for 30 minutes before the transmigration assay was carried out. In wells containing the fibroblast supernatant, this treatment reduced the percentage of transmigrating cells to the levels of control wells, confirming that the chemotactic activity for neutrophils in both infected and uninfected supernatants was due to the presence of IL-8 (Figure 6.9). In contrast,

migration of neutrophils towards the chemotactic peptide fMLP (10^{-7}M) was not affected by the neutralising antibodies specific for IL-8 (632% of control migration with control antibody, 690% with anti-IL-8). These results showed that the predominant factor increasing neutrophil migration towards supernatants from CMV infected fibroblasts was indeed IL-8.

6.2.9 Increased neutrophil transmigration is independent of alteration of endothelial adhesion molecules.

Neutrophil transmigration occurs as a result of a multistep process, involving both adhesion molecules and chemokines. It was possible that cytokines present in supernatants from CMV infected fibroblasts had caused alterations in endothelial adhesion molecule expression, which may have contributed to the increased migration observed. To test for this possibility, endothelial cells were treated with identical supernatants to those which were used in the migration assays, and their adhesion molecule expression analysed. Cells were treated with supernatants from infected or uninfected cells, IL-1β at 10ng/ml, or fresh medium (all at a final concentration of 10% FCS). The endothelial cells were exposed to the supernatants for 2 hours, which was equivalent to the duration of a neutrophil migration assay. The endothelial cells were subsequently stained for expression of ICAM-1, PECAM, VCAM, and E-Selectin, and analysed by flow cytometry. On cells treated with supernatants from infected and uninfected cells, the expression of these molecules was not altered in comparison to cells treated with fresh medium. In contrast, cells treated with IL-1β showed increased levels of E-Selectin, and a small increase in VCAM and ICAM-1 levels, but no changes in PECAM levels, as would be expected on IL-1β stimulated cells at his early time point (Figure 6.10). Thus we concluded that changes in the expression of these adhesion molecules on endothelial cells did not contribute to the CMV-induced neutrophil migration.

6.2.10 T cell migration in response to supernatants from CMV infected fibroblasts.

IL-8 is a T and NK cell chemoattractant in addition to its effects on neutrophil migration. Supernatants from infected and uninfected fibroblasts had been tested for the presence of the chemokines IL-8, MCP-1, MIP-1α and RANTES by ELISA, but assays for other chemokines which attract T cells were not

available. A functional assay was therefore carried out to determine whether the supernatants from CMV infected and uninfected fibroblasts induced T cell transendothelial migration. Supernatants harvested from fibroblasts at 6, 24, 48 and 72 hours post infection were tested in transendothelial migration assays. T cell migration takes place at a much slower rate than neutrophil migration, so migration assays were allowed to proceed for 18 hours. It was not possible to use chromium-51 in such a long assay due to problems with leakage from the cells. Instead, the numbers of migrated cells were counted using the Cytoron Absolute flow cytometer, which counts the absolute number of cells in a cell suspension of known volume.

Transendothelial migration assays were carried out using T cells purified from different individuals and supernatants generated from infected fibroblasts in 5 independent experiments. The amount of T cell migration towards infected or uninfected supernatants harvested at 24 hours post infection are shown for each of the 5 experiments (Figure 6.11). The increase or decrease in T cell migration observed using supernatants harvested at 24 hours post infection was representative of the activity at other time points. No consistent pattern of increased migration towards infected or uninfected fibroblasts emerged from these experiments. The migration of T cells was increased by infected supernatants in 3 out of 5 experiments, while the reverse was true for the other two experiments. It was therefore not possible to speculate on the presence or absence of T cell chemotactic activity based on these results.

6.2.11 Phenotyping of T cells migrating towards supernatants from CMV infected fibroblasts.

Although the previous experiment did not show any consistent increase in T cell migration towards supernatants from infected fibroblasts, it was possible that certain subsets of T cells were preferentially attracted. Large scale transmigration assays were carried out in order to harvest a sufficient number of migrated lymphocytes for flow cytometric analysis. Both starting and migrated populations were stained for the expression of CD4, CD8, CD45 isoforms, the adhesion molecules LFA-1 and L-selectin, the NK cell marker CD16, and CD28, a marker which is absent on cytotoxic T cells. Cells were stained with CD3 to gate the T cell population during analysis, and with CD2 when investigating CD16 positive cells. This experiment was carried out using PBMCs from three different individuals. The results from a representative

experiment are shown in Figure 6.12. There were no differences in the phenotypes of migrating lymphocyte subsets in response to either infected or uninfected supernatants in comparison to the control wells (containing medium only). However, certain subsets were present at increased levels in the migrated populations in comparison to the original populations.

NK cells

NK cells were distinguished by CD16 expression. These cells were not found in any of the migrated populations (Figure 6.12 (a)).

CD28

CD28 positive cells were present at increased levels in the migrated populations, but there were no differences between control migration, and migration stimulated by infected and uninfected supernatants (Figure 6.12(b)). This was in contrast to the results from Chapter 5, where CD28-ve cells showed increased adherence to fibroblasts.

Adhesion molecules

T cells can be divided into those which express high and low levels of LFA-1. The CD45RO+ve populations contained mainly LFA-1_{high} cells. These cells were present at greatly increased levels in the migrated cell populations. The CD45RA+ve populations consisted of equal proportions of LFA-1_{high} and LFA-1_{low} cells. CD45RA+/LFA-1_{high} cells migrated, whereas migration of CD45RA+/LFA-1_{low} cells was undetectable (Figure 6.12(c,d)). L-selectin (CD62L) is a lymphocyte adhesion molecule which is responsible for the initial interaction between the lymphocyte and the endothelium. There was no selective enrichment for cells either expressing or lacking this molecule in the migrated populations (data not shown).

Memory markers

The expression of CD45 RA, RO and RB was investigated in both CD4+ve and CD8+ve T cell subsets. CD4 positive cells showed preferential migration of the memory subsets, represented by CD45RB_{low} and CD45RO+ve populations, but the proportion was not different between uninfected and infected supernatants, or control wells (Figure 6.12(e,f)). CD45RO+ve/CD8+ve cells also migrated preferentially relative to the starting population (Figure 6.12(g)). The proportion of CD8+ve/CD45RB_{low} cells was too small to analyse, as the CD45RB_{low} phenotype was present predominantly in the CD4 population.

Supernatants from CMV infected fibroblasts thus do not appear to preferentially attract any of the above resting T cell subsets or NK cells. The proportion of NK cells in the migrating populations was particularly low, in comparison to the proportions of NK cells in the original populations.

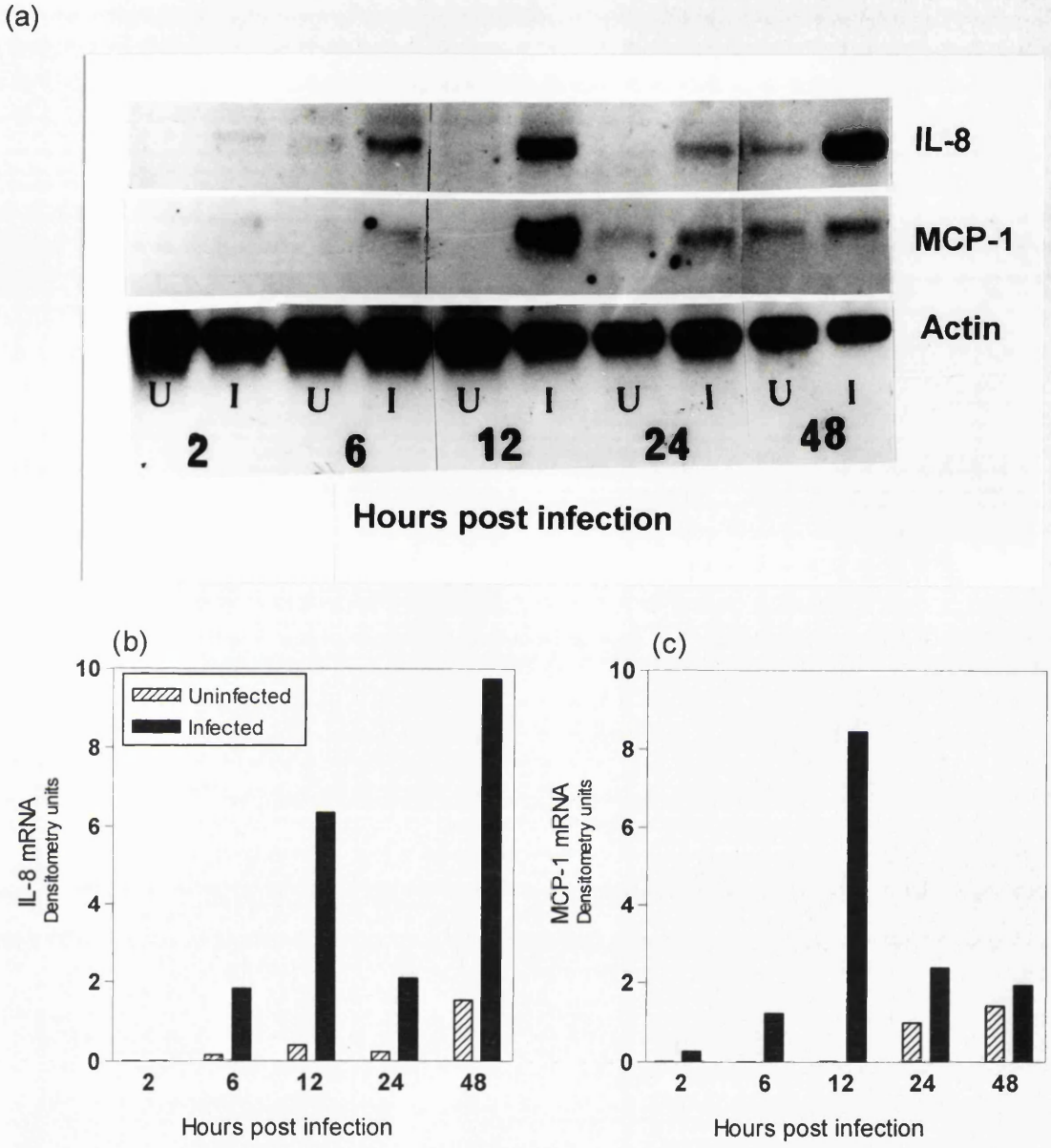


Figure 6.1. IL-8 and MCP-1 mRNA levels in CMV-infected and uninfected fibroblasts.

Fibroblasts were infected with CMV strain AD169 (I), or left uninfected (U). At the times indicated (hours post infection), total cellular RNA was prepared, and IL-8, MCP-1 and actin mRNA were detected by Northern blotting. The bands detected were visualised by chemiluminescence and autoradiography, and the figure shows an autoradiograph from a representative experiment (a). The intensity of the IL-8 or MCP-1 mRNA bands detected in part (a) were quantified by laser densitometry, and the data was converted to arbitrary Densitometry Units (b and c). Adjustments were made for slight differences in loading by densitometric analysis of the actin mRNA levels. The data shown are representative of two similar experiments.

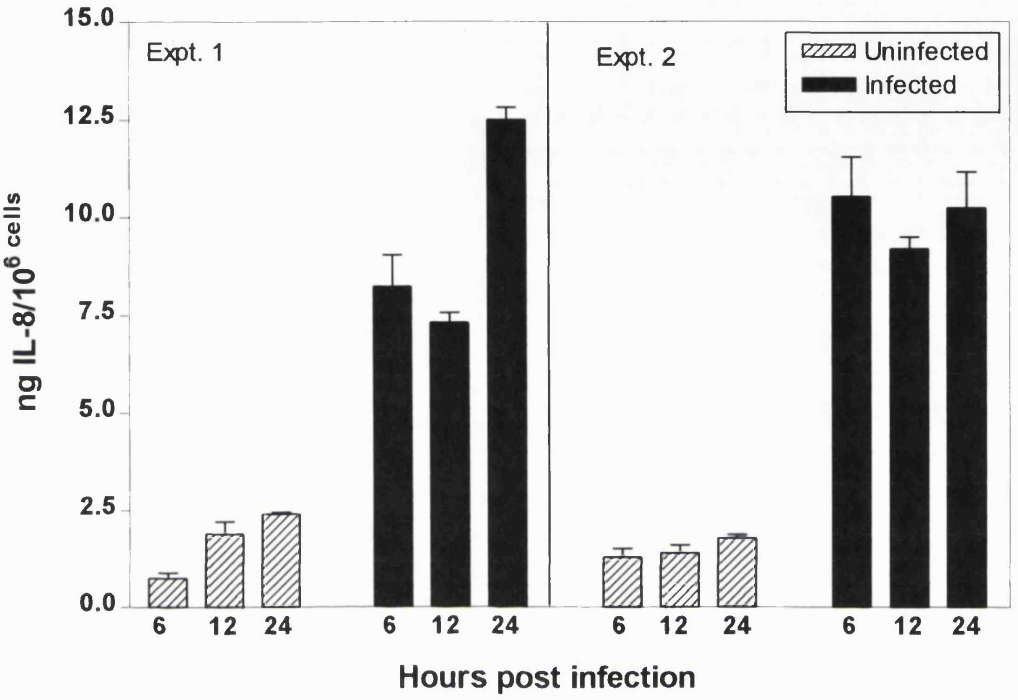


Figure 6.2. IL-8 protein production by CMV infected fibroblasts.

Subconfluent fibroblast monolayers were either infected with CMV strain AD169, or left uninfected, and their supernatants harvested at the times indicated from the time of initial infection. IL-8 protein levels were quantified by ELISA, and are expressed as ng/10^6 fibroblasts. Two representative experiments performed on identically prepared fibroblast cultures derived from different donors are shown. The results shown represent the mean ± SD from triplicate wells in each experiment.

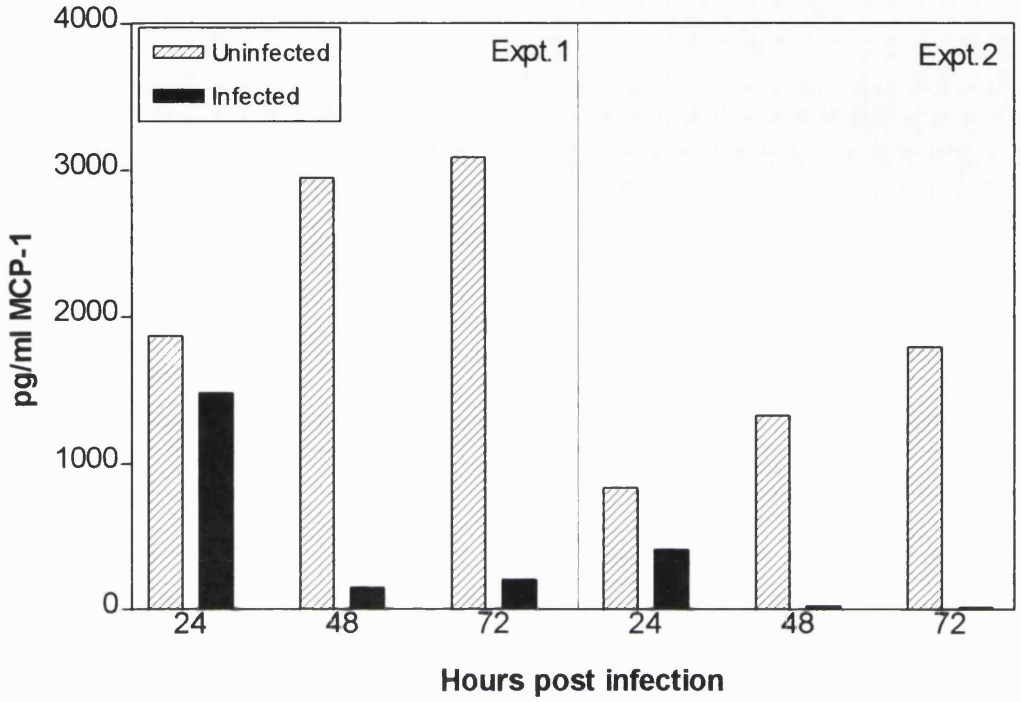
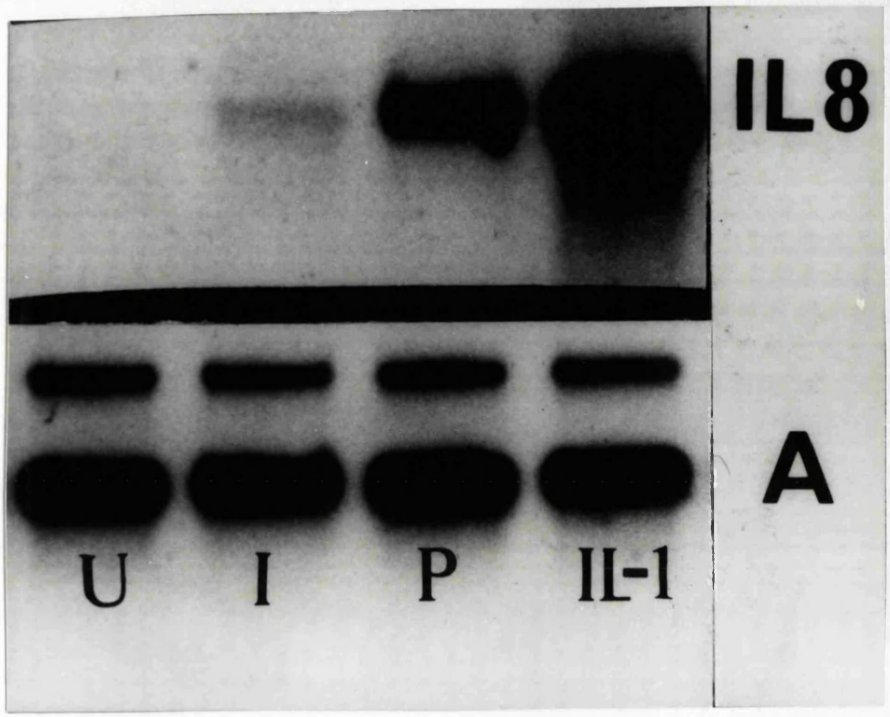


Figure 6.3. Effect of CMV infection on levels of MCP-1 protein.
Fibroblasts were infected with CMV inoculum, or left uninfected. The supernatants were harvested at the times indicated on the graph and assayed for the presence of MCP-1 by ELISA. The results are expressed as pg MCP-1/ml. The data shown represent the mean from duplicate wells. Two representative experiments are shown.



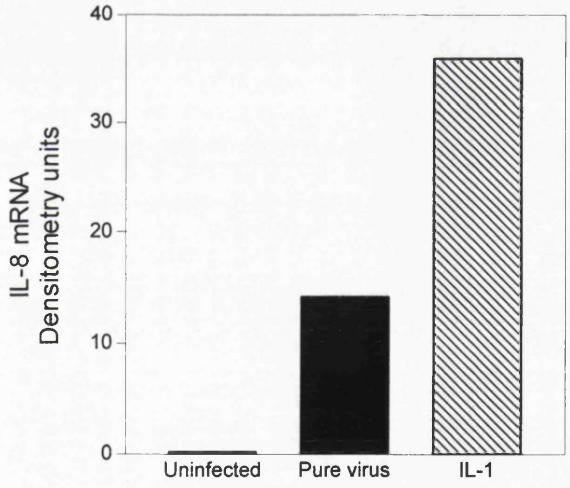
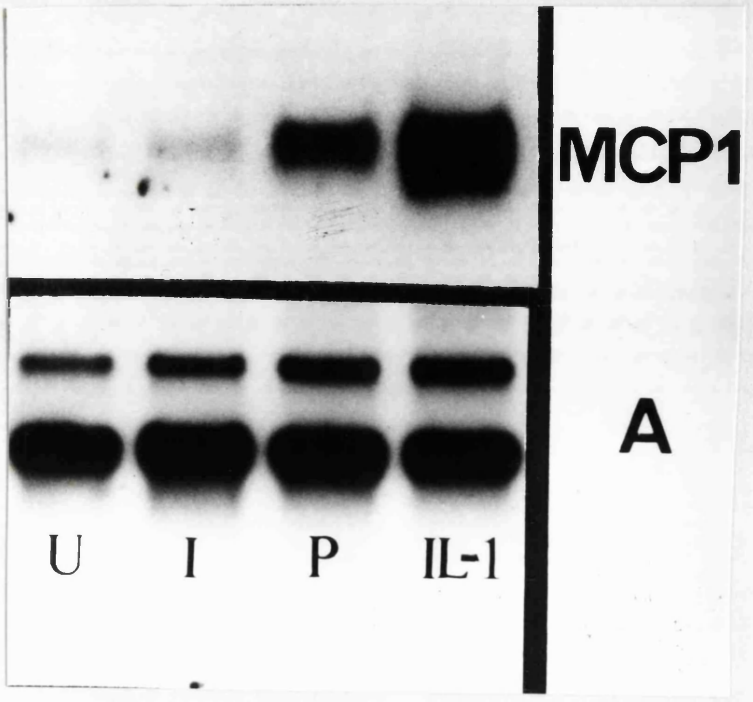


Figure 6.4. The effect of purified virus on IL-8 mRNA expression.
CMV strain AD169 was purified from other extraneous factors in the viral inoculum by two rounds of ultracentrifugation and resuspension in fresh medium ('pure virus'). Total cellular RNA was extracted at 24 hours post infection, from cells infected with this inoculum, uninfected cells, or fibroblasts treated with IL-1β (10ng/ml) and IL-8 and actin (A) mRNA levels were detected by Northern blotting. An autoradiograph of the bands detected by chemiluminescence is shown (top panel). The intensity of the mRNA bands were quantified by laser densitometry and presented as arbitrary densitometry units as described for Figure 6.1 (bottom panel).



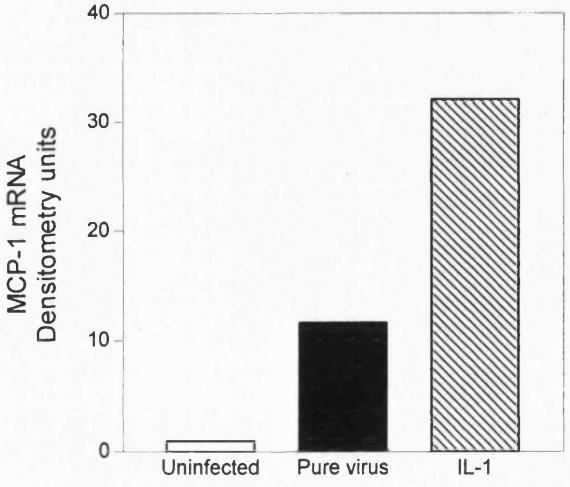


Figure 6.5. The effect of purified virus on MCP-1 mRNA expression.
The expression of mRNA for MCP-1 was investigated by Northern blotting. See figure 6.4 for experimental design.

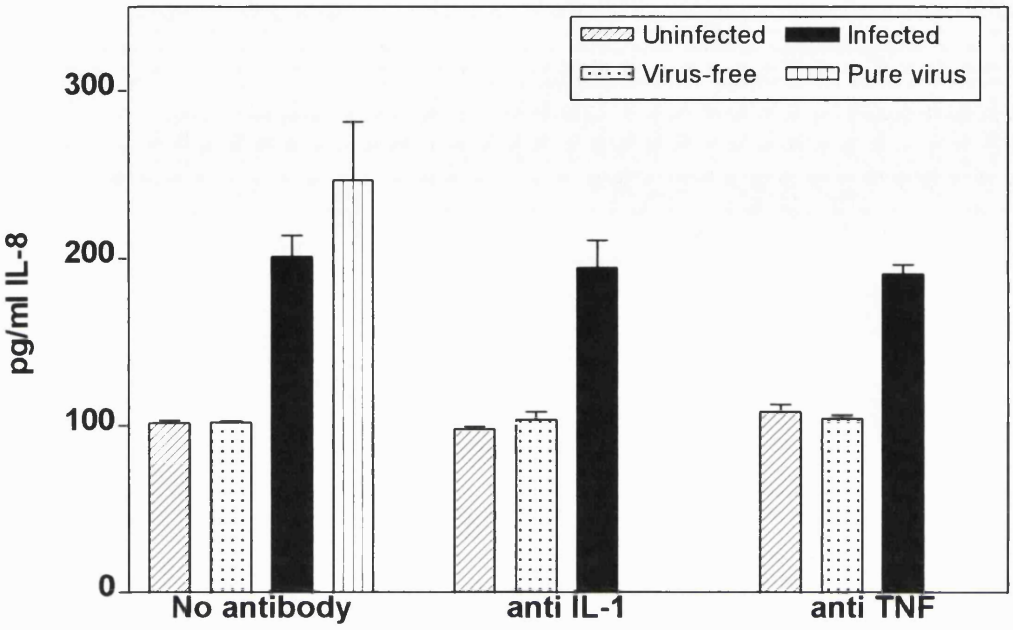


Figure 6.6. Effect of extraneous factors from the viral inoculum on IL-8 protein expression.

Fibroblasts were infected with CMV inoculum, virus free inoculum (virus removed by ultracentrifugation), 'pure virus' (as for Figure 6.4), or left uninfected. Neutralising antibodies specific for IL-1β or TNFα (10μg/ml) were added both during and immediately following infection, to wells treated with CMV inoculum, virus free inoculum and uninfected controls. Levels of IL-8 protein were measured by ELISA at 24 hours post infection, and are expressed as pg IL-8/ml. The data shown represents the mean ± SD from triplicate wells in one representative experiment. Similar results were obtained in a separate experiment.

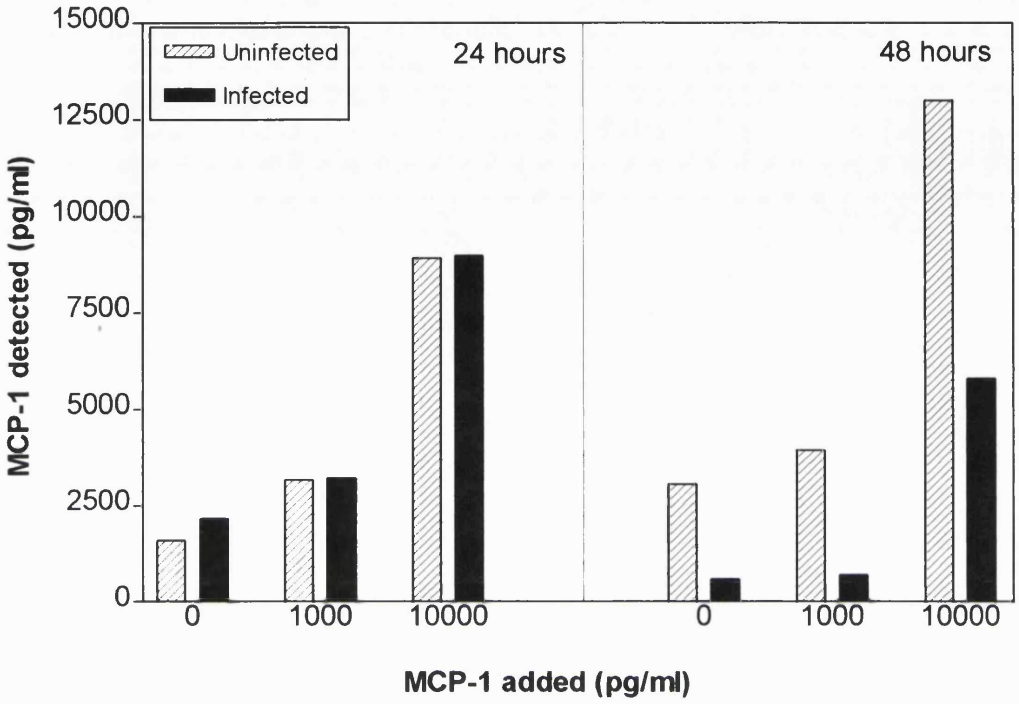


Figure 6.7. The removal of recombinant MCP-1 from supernatants incubated with CMV infected fibroblasts.

Fibroblasts were infected with CMV inoculum at a MOI of 2-4 pfu/cell, or left uninfected. Following the one hour inoculation period, the inoculum was replaced with medium containing 1000 or 10,000 pg/ml recombinant MCP-1, or medium alone. The supernatants were harvested at 24 or 48 hours post infection and their MCP-1 levels measured by ELISA. The data shown represents the mean from duplicate wells. The experiment shown is representative of three similar experiments.

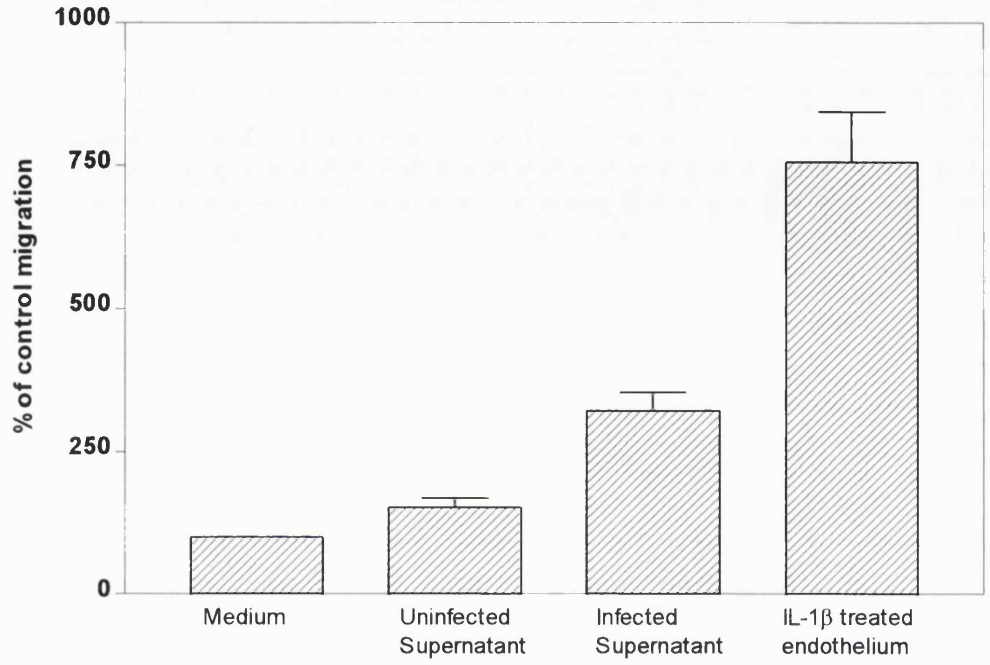


Figure 6.8. Transendothelial migration of neutrophils towards supernatants from infected or uninfected fibroblasts.

Supernatants were harvested from cultures of CMV infected or uninfected fibroblasts at 6 hours post infection. The migration of ^{51}Cr-labelled neutrophils placed in the top of a cell culture insert coated with endothelial monolayers, towards the supernatant, or medium, in the bottom of the chamber, was measured over a 2 hour period. 'IL-1β treated endothelium' used as a positive control was preincubated with 2ng/ml of IL-1β for 3 hours prior to the assay, rinsed and placed in wells with fresh medium. All supernatant samples were tested in duplicate wells. Data from individual experiments were normalised such that % migration of the control (medium only) wells was designated 100%. The data shown represents the mean ± SEM of 4 separate experiments.

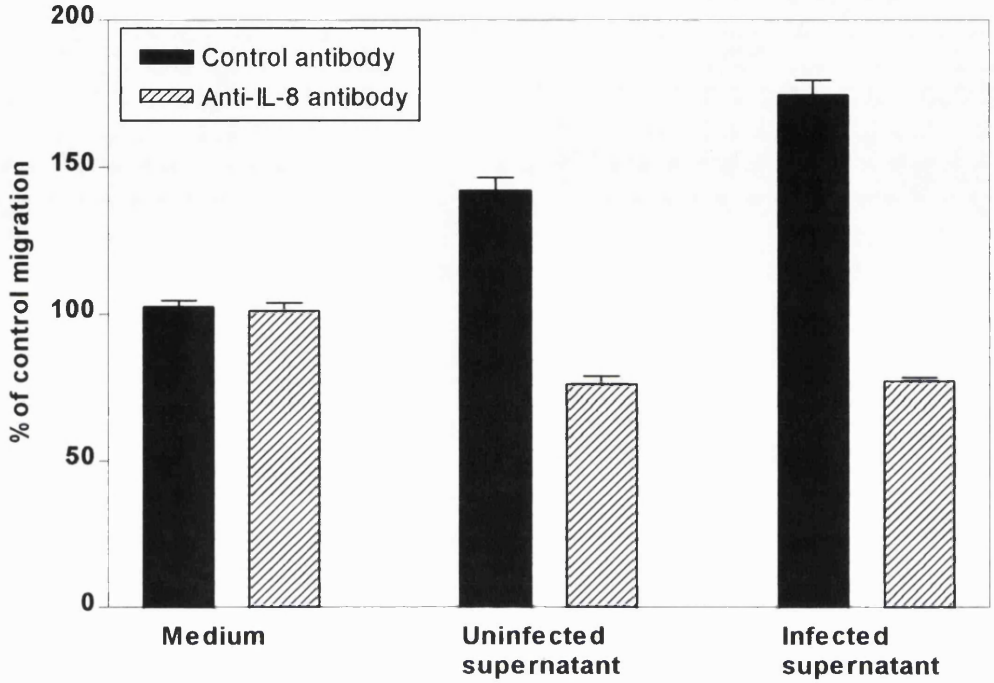


Figure 6.9. The effect of neutralising antibodies specific for IL-8 on neutrophil migration.

Anti-IL-8 antibodies or irrelevant control antibodies were added to supernatants from CMV infected and uninfected fibroblasts at a concentration of 20μg/ml for 30 minutes prior to the transmigration assay. The experimental design was otherwise identical to that described in Figure 6.7. The graph shows % migration relative to the medium only control, for irrelevant antibody wells or wells containing anti-IL-8 antibody. Migration towards the bacterial chemotactic peptide fMLP was not affected by the addition of anti-IL-8 antibody (632% of control migration with control antibody, 690% with anti-IL-8, data not shown). The data points represent the mean of duplicate wells and are representative of four separate experiments.

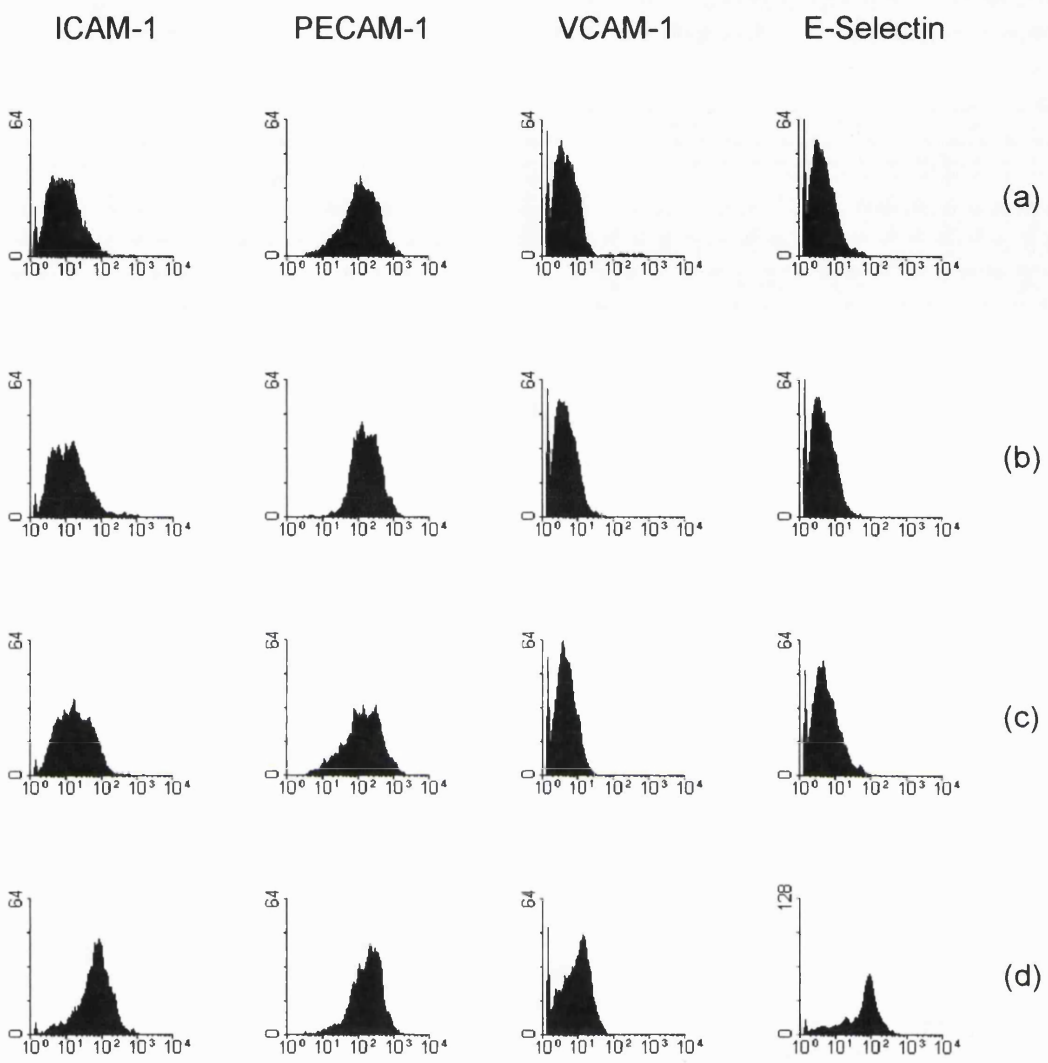


Figure 6.10. Endothelial adhesion molecule expression following treatment with supernatants used for migration assays.
Endothelial cells were treated for 2 hours with fresh medium (a), supernatants from uninfected (b) or CMV infected (c) fibroblasts, or with IL-1 at 10ng/ml as a positive control (d). Endothelial cells were then analysed for the cell surface expression of ICAM-1, PECAM-1, VCAM-1, and E-Selectin by flow cytometry. Flow cytometry profiles from a representative experiment are shown.

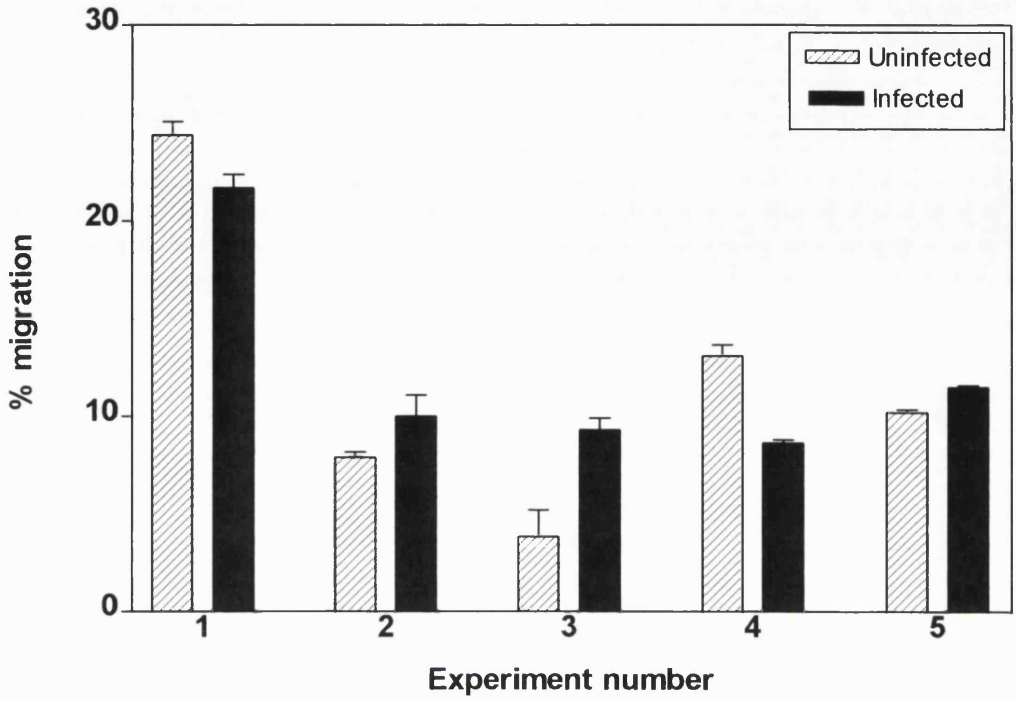


Figure 6.11. Transendothelial migration of CD2+ve lymphocytes towards supernatants from infected or uninfected fibroblasts.

Purified CD2+ve lymphocytes were placed on the upper chamber of a cell culture insert coated with endothelial cells. The lower chamber contained supernatants from infected fibroblasts (24 hours post infection), or uninfected fibroblasts. The migration of CD2+ve lymphocytes across endothelial monolayers towards each supernatant was measured by the counting of migrated lymphocytes using the Cytoron Absolute flow cytometer after migration had proceeded for 18 hours. The results are expressed as percentage migration of the starting population. All supernatant samples were tested in duplicate wells. The data represent the mean ± SD from 5 separate experiments.

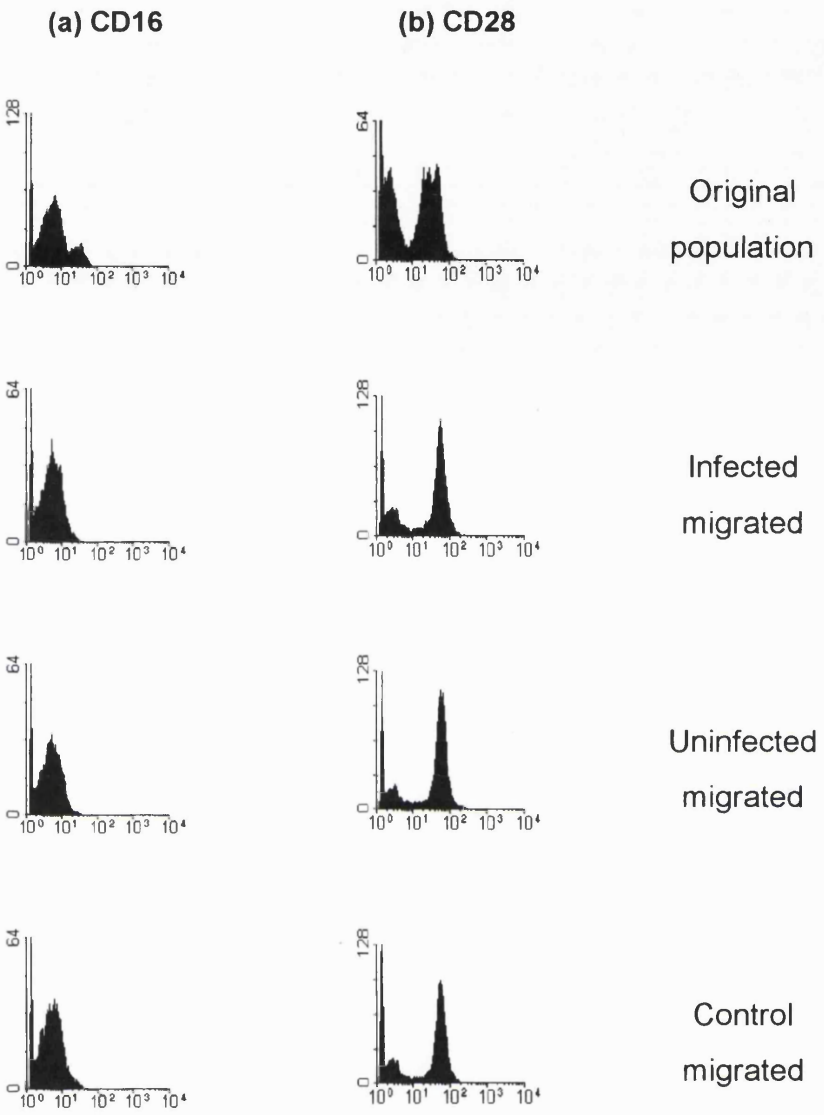


Figure 6.12 (a and b). Analysis of CD16 and CD28 subsets in migrated populations.

Purified CD2+ve lymphocytes were placed on the upper chamber of a cell culture insert coated with endothelial cells. The lower chamber contained supernatants from infected fibroblasts (24 hours post infection), uninfected fibroblasts, or medium only (control migration). After 18 hours the phenotype of each of the migrated lymphocyte populations was compared to the starting population by flow cytometry. Lymphocytes were gated on the basis of their characteristic forward and side scatter profiles, and the expression of CD2. Representative profiles from 3 separate experiments are shown.

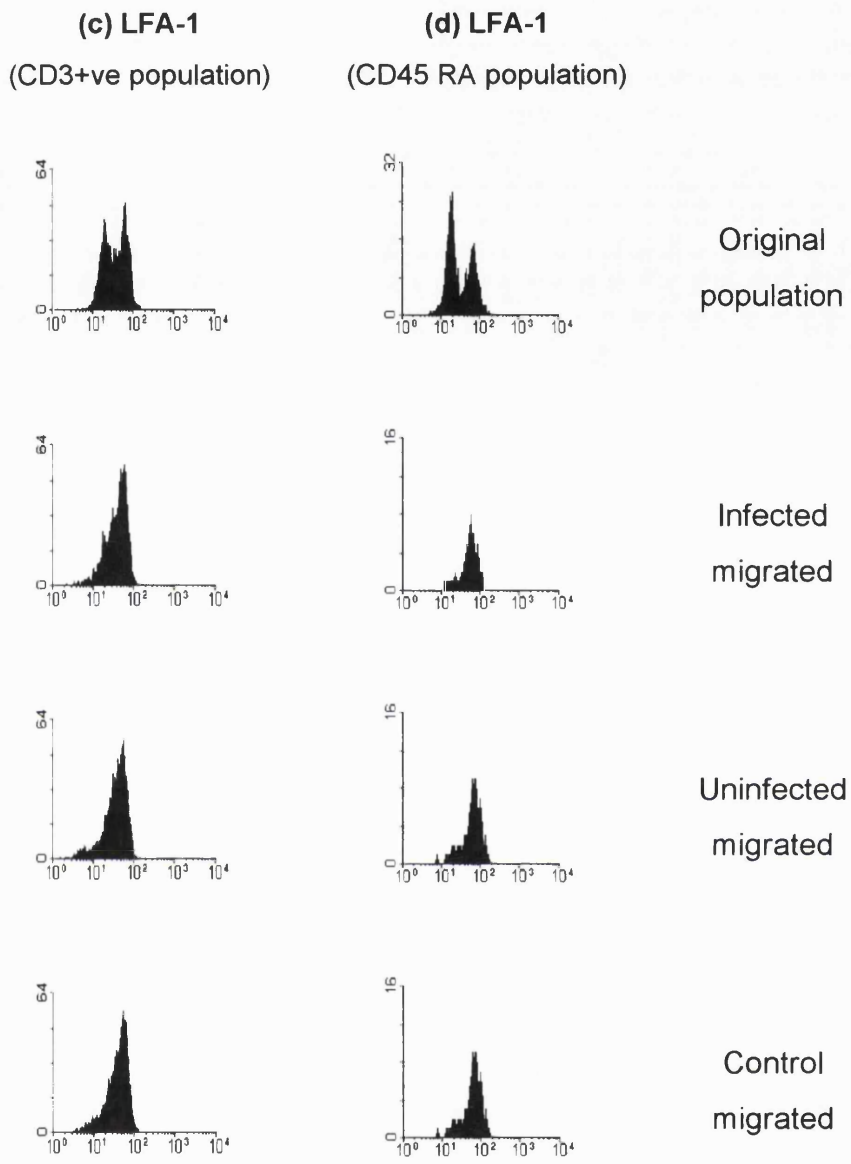


Figure 6.12 (c and d). Analysis of LFA-1 expression in migrated populations.

The same populations described in Figure 6.11 (a and b) were stained for the expression of LFA-1. The experimental conditions were as described in Figure 6.11 (a and b), except that cells were gated on the basis of forward and side scatter and CD3 expression (c) or CD45RA (d), before further analysis of LFA-1 expression.

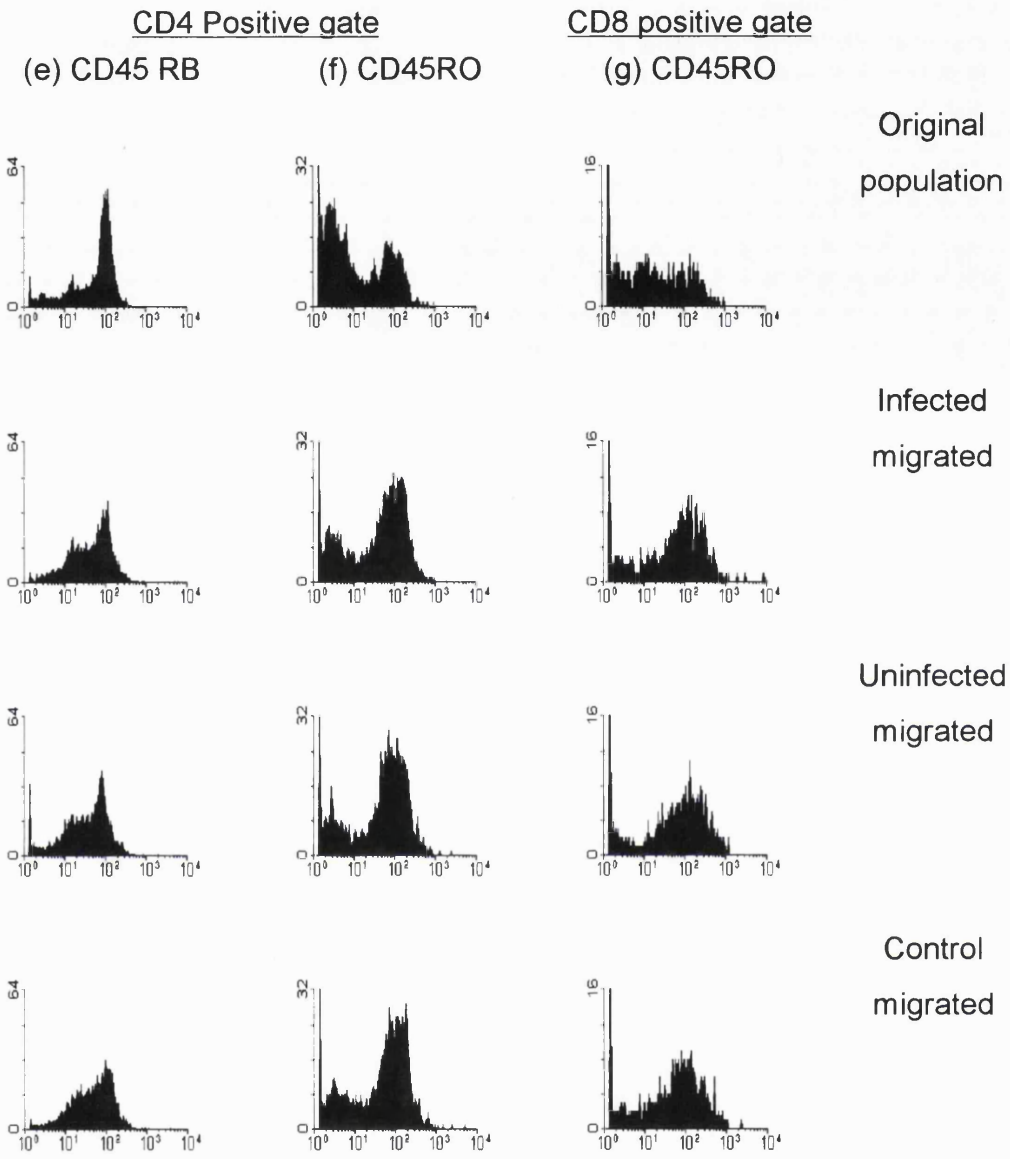


Figure 6.12 (e,f,g). Analysis of CD45RO and RB expression in migrated populations.

The same populations described in Figure 6.11 (a and b) were stained for the expression of CD4, CD8 and CD45RO, or CD4, CD8 and CD45RB. The experimental conditions were as described in Figure 6.11 (a and b). CD4 and CD8 populations were then analysed separately for the expression of CD4 isoforms.

6.3 DISCUSSION

In this study, we have shown that CMV infection of fibroblasts resulted in increased production of IL-8 mRNA and protein. This was a specific effect of the virus and was not attributable to cytokines contained in the viral inoculum. The increased IL-8 produced by CMV infected fibroblasts had functional consequences, as demonstrated by the ability of supernatants from CMV infected cells to significantly enhance neutrophil transendothelial migration above levels achieved using supernatants harvested from uninfected cells. The increase in neutrophil migration occurred in the absence of alterations of adhesion molecule expression on the endothelial cells. The role of IL-8 in inducing neutrophil migration was confirmed by demonstrating that preincubation of fibroblast supernatants with neutralising antibodies to IL-8 reduced neutrophil transmigration to basal levels.

Transcription of the IL-8 gene in fibroblasts is induced by IL-1β, TNFα, and phorbol esters (Strieter *et al.* 1989a). The transcription is regulated by both an NF-κB-like factor, and an NF-IL-6-like factor, which interact co-operatively with two adjacent but distinct cis-elements on the IL-8 promoter (Mukaida *et al.* 1990). The increased IL-8 mRNA levels in CMV infected fibroblasts were first detected at 6 hours post infection, and showed a biphasic pattern, declining at 24 hours but recovering at increased levels by 48 hours, although actin mRNA levels remained stable. We also showed an increase in IL-6 mRNA following a similar time course to that shown for IL-8 in the present study (data not shown), and this has been reported by others. It was suggested that the biphasic pattern of IL-6 induction resulted from early activation of preformed cytoplasmic NF-κB, followed by *de novo* induction of NF-κB later in infection (St.Jeor *et al.* 1993), as CMV infection activates NF-κB DNA-binding and enhancer activities (Kowalik *et al.* 1993). Thus IL-6 and IL-8 may be upregulated by the same mechanism following CMV infection. We have shown in chapter 4 that ICAM-1 expression was upregulated by CMV infection, an effect which was also independent of the production of cytokines, such as IL-1β or TNFα by the infected cells. As NF-κB also acts as a dominant regulator of the ICAM-1 gene (Mukaida *et al.* 1990; Collins *et al.* 1995), a common mechanism may be responsible for the increases in production of IL-8, IL-6 and ICAM-1 following CMV infection.

IL-8 mRNA levels are also increased following herpes simplex virus infection of keratocyte cultures, showing a temporary decline at 6 hours post infection

(Oakes *et al.* 1993). It was suggested that this decline may have been due to fluctuations in the abundance of particular viral proteins at different stages of the replication cycle. The increase in IL-8 protein and mRNA levels in CMV infected cells at 6 hours post infection suggests that a CMV immediate early gene product may be responsible. The CMV immediate early proteins control regulation of their own promoters, as well as those of CMV early and late genes, and can regulate the promoters of other viral and cellular genes (Tevethia *et al.* 1987; Stenberg *et al.* 1990). However, some of the CMV structural proteins which are introduced into the infected cell following virus entry, also have the potential to transactivate cellular genes (Britt and Auger, 1986; Liu and Stinski, 1992), and are thus also possible candidates for the effect on IL-8 production. IL-8 gene expression is induced by the Hepatitis B virus X protein which activates NF-κB (Mahe *et al.* 1991). The induction of IL-8 by respiratory syncitial virus also involves NFκB and NF-IL-6 (Mastronarde *et al.* 1996). A number of other viruses also increase the production of IL-8 in infected cells, including influenza A infection of airway epithelial cells (Choi and Jacoby, 1996), herpes simplex virus infection of corneal keratocytes (Oakes *et al.* 1993), measles virus infection of fibroblasts (Van Damme *et al.* 1989), and rotavirus infection of epithelial cells (Sheth *et al.* 1996). The mechanisms responsible for IL-8 induction have not been clarified for these other viruses.

The major leukocyte subset recruited in the experiments presented in this chapter were the neutrophils. Neutrophil accumulation at sites of CMV infection has been reported (Smyth *et al.* 1993), but is not a common finding. The absence of neutrophil infiltrates at sites of CMV infection *in vivo* may be due to the short time period following infection before the switch to mononuclear infiltration is initiated. Neutrophils are the first leukocytes to be recruited to sites of infection, responding to both increased adhesion molecule expression and presentation of particular chemokines such as IL-8 on the endothelium. IL-8 is also chemotactic for NK cells and NK-like T cells which are also recruited at early stages of infection and accumulate in virally infected tissues (Natuk and Welsh, 1987). The production of IFNβ is inhibitory to IL-8 production, and this may cause the switch in the migration patterns to monocytes and T cells (Oliveira *et al.* 1992). The recruitment of neutrophils by CMV infected fibroblasts and other interstitial cells may therefore be central to the initiation and maintenance of leukocyte migration to sites of infection, as neutrophil-derived cytokines and mediators may act to promote the recruitment and activation of other cell types, including monocytes and T cells, which are

commonly found in infected tissues (Strieter *et al.* 1992; Lloyd and Oppenheim, 1992). As neutrophils, like other leukocytes, are potent sources of chemokines, their recruitment is likely to generate a cascade of chemokine production to orchestrate the subsequent patterns of leukocyte migration.

The earlier reports of chemotactic activity of IL-8 for T cells were somewhat controversial (Larsen *et al.* 1989) until the recent detection of IL-8 receptors on a subset of T cells using radioligand binding and functional assays (Xu *et al.* 1995; Qin *et al.* 1996). The receptors are expressed at lower levels on T cells than neutrophils (Mackay, 1996). IL-8 receptor expression on T cells is reported to be very variable among individuals, and is easily downregulated during T cell purification processes (Xu *et al.* 1995; Qin *et al.* 1996). The concentrations of IL-8 required for T cell migration is also variable among individuals (Qin *et al.* 1996). The detection of T cell migration in response to other α chemokines, in particular IP10, has also yielded discrepant results from different groups, possibly for similar reasons (Taub *et al.* 1993; Roth *et al.* 1995). We have shown in this chapter that IL-8 induced by CMV infection increases the migration of neutrophils towards sites of infection, but the induction of T cell migration by supernatants from infected fibroblasts was not a reproducible finding. Factors such as variations in the composition of the T cell population from different individuals, and variation in IL-8 receptor expression, which determines the concentration of IL-8 required for a chemotactic response, may explain the inconclusive results obtained regarding T cell migration in the present study. There was also no difference in the subsets migrating towards supernatants from infected fibroblasts in comparison to supernatants from uninfected fibroblasts or resting endothelium. A recent study from this laboratory has shown that in an identical experimental system, levels of migration of T lymphocytes are increased in response to IL-15 and RANTES, but the phenotypes of the migrated cells were identical to cells migrating across resting endothelium (Borthwick *et al.* 1996). Other groups have also reported identical percentages of particular phenotypes following migration across resting or IL-1 stimulated endothelium, suggesting that the memory phenotypes have a greater intrinsic capacity to migrate (Cush *et al.* 1992; Pietschmann *et al.* 1992). Earlier studies showing increased migration of memory lymphocytes in response to particular chemokines were carried out in chemotaxis chambers without endothelium (Schall *et al.* 1990; Schall *et al.* 1993). It is now accepted that transendothelial migration is a more accurate representation of lymphocyte migration *in vivo*. Activation of T cells and NK cells alters their migratory activity, probably due to an increase in expression of chemokine receptors in addition to

activation of β_2 integrins (Loetscher *et al.* 1996a). Increased migration has been shown using *in vitro* activated lymphocytes, and *in vivo* activated lymphocytes from patients with acute Epstein Barr virus infection (Borthwick *et al.* 1996). A more comprehensive investigation into the migratory properties of purified T cell subsets, including activated cells, may uncover differences in migration towards infected and uninfected supernatants.

MCP-1 mRNA was present at increased levels in CMV infected fibroblasts. MCP-1 gene expression in fibroblasts can be regulated by similar factors as those regulating IL-8 expression, such as IL-1β and TNF-α (Strieter *et al.* 1989b). MCP-1 protein was constitutively produced by uninfected fibroblasts, and the levels found in infected fibroblast supernatants were similar at 24 hours post infection. However, at 48 and 72 hours post infection, MCP-1 protein levels in infected cultures were reduced significantly in comparison to uninfected cultures. We hypothesised that rather than CMV infection reducing the production of MCP-1 protein, the MCP-1 had bound to the CMV encoded β chemokine receptor and become internalised by the cell, thus reducing the levels in the supernatant. The subsequent demonstration of the sequestering of exogenously added recombinant MCP-1 by infected fibroblasts, but not uninfected controls, suggested that this was the case. The MCP-1 was removed from the supernatant by infected cells only at 48 and 72 hours post infection, but there was little effect at 24 hours post infection. As the CMV gene encoding the β chemokine receptor is expressed at late times post infection (Chee *et al.* 1990b; Welch *et al.* 1991), the depletion of MCP-1 from the supernatant correlates with its predicted time of the expression of this molecule in infected cells. An antibody to the US28 gene product has not yet been produced (T. Schall, personal communication), so the time and levels of expression following infection, at the protein level, cannot be confirmed in any other way at present.

Although the US28 gene has been expressed in transfected cell lines, and is transcribed during CMV infection, the presence of a functional chemokine receptor on CMV infected fibroblasts has not been demonstrated by others (Neote *et al.* 1993; Gao and Murphy, 1994). The pattern of cellular expression of US28 remains to be defined. However, the fact that the protein appears to be expressed at late times post infection means that it is not likely to be expressed in cells which do not support a productive CMV infection, such as monocytes. It is also not known whether the US28 gene product is a component of the virus envelope, but the structure of the protein would predict that this may be a

possibility. Although the assay used in the present study has measured the uptake of MCP-1 by CMV infected fibroblasts, the CMV US28 gene product when expressed in transfected cell lines, is a high affinity receptor for other β chemokines. The binding of MIP-1α, MIP-1β and RANTES to the CMV-encoded β chemokine receptor have been characterised, but other β chemokines may also bind this receptor, as it was shown to be more promiscuous than the other chemokine receptors known at the time (Neote *et al.* 1993; Gao and Murphy, 1994).

The control of chemokine expression and chemokine receptor expression is likely to be important in the control of leukocyte migration to sites of CMV infection *in vivo*. The importance of chemokines in recruiting leukocytes to sites of viral infection has been demonstrated in a MIP-1α deficient mouse model, where delayed clearance of influenza virus was observed in the absence of this chemokine. These mice were also resistant to cocksackievirus B3 induced myocarditis, which is caused by leukocyte infiltration (Cook *et al.* 1995). Histopathological examination of tissues from mice infected with both viruses revealed a profound decrease in the number of mononuclear cells which had accumulated in the tissues. It is interesting that such dramatic effects resulted from the depletion of only one chemokine, as it is often suggested that there is a great deal of redundancy in chemokine function. The MCMV genome has been sequenced, and is reported to contain a US28 homologue, although the sequence of this protein has not yet been published. The existence of a murine CMV homologue for this gene would open the way for investigations into the role of the US28 gene *in vivo* to be initiated.

CHAPTER 7

GENERAL DISCUSSION

Recruitment of leukocytes to sites of infection, and their subsequent recognition of infected cells in tissues is essential for the control of viral infections. The present study has shown that CMV infection of fibroblasts induces expression of the adhesion molecules ICAM-1 and LFA-3 specifically on infected cells, by a cytokine independent mechanism, leading to increased adhesion of both resting and activated lymphocytes. These changes also occurred in the presence of ganciclovir. CMV infected cells also produced increased levels of IL-8, which stimulated the selective transendothelial migration of neutrophils. Expression of MCP-1 mRNA was also increased following CMV infection, but this did not translate to increased expression of MCP-1 protein, possibly due to the sequestering of this chemokine by the expression of a CMV encoded β chemokine receptor on infected cells. Increased expression of adhesion molecules and production of chemokines by infected cells would be expected to enhance the migration and adhesion of leukocytes to sites of infection. Increased expression of class I HLA on uninfected bystander cells was also shown, due predominantly to the release of IFNβ by CMV infected cells. Increased expression of class I HLA, adhesion molecules and chemokines are generally considered to be proinflammatory features. CMV also modulates other factors of the host immune response, such as downregulation of class I HLA expression, which is an immune evasion mechanism, and the expression of a CMV encoded β chemokine receptor, the function of which is not clear at present.

The findings from the present study were derived from a fibroblast model of CMV infection. Recent studies showing that fibroblasts constitute a major target cell population for CMV infection in both lung and gastrointestinal tissues *in vivo* emphasise the importance of determining the effects of CMV infection on normal fibroblast function (Sinzger *et al.* 1995). Fibroblasts are increasingly being recognised as important participants in acute and chronic inflammation, in addition to their traditional role in the production of extracellular matrix components and the maintenance of connective tissue (Strieter *et al.* 1989a). The anatomical location of fibroblasts in the tissues may allow them to actively participate in communication between vascular and interstitial tissues. Mechanisms have been proposed by which chemokines could be rapidly transported from cells such as fibroblasts in the tissues to the endothelium, where they are bound to cell surface proteoglycans and displayed to leukocytes to mark the underlying area of infection (Rot *et al.* 1996). The production of

chemokines and other inflammatory mediators or chemotactic factors by fibroblasts may be an important factor in the initiation of inflammatory events.

ICAM-1 and LFA-3 upregulation following CMV infection was a direct viral effect, and was not mediated by cytokines. Increased expression of these adhesion molecules following CMV infection of endothelial cells has been reported by others and also appears to occur by a cytokine independent mechanism (Sedmak *et al.* 1994b). The fibroblast model for adhesion may therefore be representative of adhesive events between leukocytes and endothelium, and have potential consequences for the extravasation and activation of leukocytes in the initial stages of allograft injury which occur at the endothelium. The accumulating evidence that endothelium is a target for CMV infection *in vivo* underlines the potential importance of adhesion molecule expression in these processes, as leukocyte/endothelium interactions are required for the initial infiltration of leukocytes into tissues.

The increased expression of adhesion molecules and increased chemokine production shown here *in vitro* would be expected promote the recruitment of leukocytes to sites of infection *in vivo*, and prolong their residency in infected tissues. This may be beneficial for immunocompetent hosts, who can mount a rapid immune response and thus control infection in most cases. However, in immunosuppressed patient groups, such as solid organ transplant patients and BMT patients, the spread of the virus is not controlled by the compromised host immune system, so is allowed to progress to a greater extent. The altered balance between the protective immune response, and the capacity of the immune response to mediate tissue damage may therefore contribute to manifestations of certain types of CMV disease in these patient groups. In the following sections, the findings from the present study are discussed in the context of clinical findings from infected individuals, and histopathological findings from both patients with CMV disease and animal models.

CMV infection in normal individuals

In the immunocompetent host, CMV infection rarely results in symptomatic disease, suggesting that in immunocompetent individuals the immune response is able to limit viral replication. The upregulation of adhesion molecules and chemokine expression following virus infection is a process which would be expected to aid the host in the elimination of virally infected cells. It is interesting therefore that the virus itself induces such processes which benefit

the host response, and thus appears to threaten its own survival. The upregulation of adhesion molecules following CMV infection may be a 'by-product' of the stimulation of host transcription factors by CMV which are intended to maximise the transcription of viral genes (Boldogh *et al.* 1993). An example of this is activation of NF-κB by CMV infection (Yurochko *et al.* 1995), which enhances the expression of viral genes, but which may also be responsible for the upregulation of ICAM-1 and IL-8 in CMV infected cells shown here, as the these genes are also regulated by NFκB (Mahe *et al.* 1991; Zamai *et al.* 1994; Ledebur and Parks, 1995). The transcription factors which control the expression of LFA-3 have not been identified, but may also overlap with cellular transcription factors induced by the virus.

Following primary infection, CMV is not cleared from the body by the immune system, but remains in a latent or persistent state which can reactivate when the host is severely immunosuppressed. This is due to the incomplete eradication of virally infected cells by the host immune response, allowing the escape of some infected cells and the establishment of latency. The downregulation of class I HLA appears to be a mechanism used by CMV to evade the immune response. It results in a significant decrease in the killing of infected cells (Hengel *et al.* 1995), and may contribute to the persistence of the virus. The increased expression of adhesion molecules on infected cells may enhance the formation of conjugates between infected cells and CTLs, as has been shown *in vitro* in the present study. This may be particularly important in the generation of effector responses in response to a limited number of viral peptide/TCR complexes.

The role of CMV infection in rejection of solid organ allografts.

In the present study it has been shown that CMV infection leads to increased expression of adhesion molecules on infected cells, and increased expression of class I HLA on uninfected cells surrounding the infected cells. The latter is likely to be an important factor *in vivo*, where uninfected cells outnumber infected cells. Increased expression of MHC antigens and adhesion molecules on endothelial cells is postulated to be a major factor leading to the induction of immune responses to allo-MHC in the transplanted organ (Steinhoff *et al.* 1988; Adams *et al.* 1989; Andersen *et al.* 1992). The induction of adhesion molecule expression on the infected cells, and the upregulation of class I HLA on uninfected bystander cells, both shown in this study, may contribute to the allogenicity of the graft, and be partially responsible for the association between

CMV infection and rejection of the grafted organ. The present study has also shown that increased adhesion of *in vitro* activated T lymphocytes is enhanced following CMV infection of fibroblasts. Many of the cells in the peripheral blood during CMV infection in transplant recipients are activated, so presumably would also show increased capacity to adhere to CMV infected cells. CMV related rejection episodes in renal transplant recipients are associated with an increased incidence of IFNγ-producing activated CD8+ memory cells in the blood (Bertoletti *et al.* 1994a), and the expansion of CD57+ve/LFA-1_{bright}/CD8+ve memory cells (Reinke *et al.* 1994c). Lymphoid activation in CMV infected transplant recipients has been associated with chronic rejection episodes (Lautenschlager *et al.* 1990; Lautenschlager and Hockerstedt, 1993; Bertoletti *et al.* 1994a). Increased expression of adhesion molecules on these *in vivo* activated lymphocytes may allow these cells to enter the graft, and become selectively retained in the infected tissue due to the presence of LFA-3 and ICAM-1 on infected cells. Accumulation of activated lymphocytes is found in biopsies from rejecting organs. Many of these lymphocytes are likely to be activated lymphocytes recruited from the peripheral blood, which are commonly found in CMV and other viral infections (Yang *et al.* 1989; Borthwick *et al.* 1996). CMV associated rejection of cardiac (Koskinen *et al.* 1994) and renal allografts (Richardson *et al.* 1981) is associated with an inflammatory infiltrate consisting predominantly of T lymphocytes and monocytes (Richardson *et al.* 1981; Koskinen *et al.* 1994). In cardiac allograft recipients, this infiltrate consisted primarily of alloreactive T cells (Ouwehand *et al.* 1994). Studies using susceptible mouse strains have shown that CMV-induced myocarditis and hepatitis are also characterised by the presence of CD8+ T cells in the tissues (Lawson *et al.* 1989; Olver *et al.* 1994).

It was shown here that CMV infection of fibroblasts also supported increased adhesion of resting lymphocytes, which appeared to be mediated by the CD2/LFA-3 interaction. Since LFA-3 is a costimulatory molecule for T cell activation, migrated lymphocytes may also be activated *in situ* by the cellular interactions in the allograft. It is well documented that the binding of a combination of ICAM-1, LFA-3 or B7 to their ligands is essential for activation through the TCR to take place, and that the absence of these signals may render the cells anergic (van Seventer *et al.* 1991; Guinan *et al.* 1994). While costimulation through CD28 appears to be essential for primary activation and proliferation of resting T cells (Azuma *et al.* 1992), costimulatory signals from ICAM-1 and LFA-3 are sufficient for the activation of effector cells (Azuma *et al.* 1993a; de Waal Malefyt *et al.* 1993). Increased expression of ICAM-1 and LFA-

3 by CMV infected cells may thus be sufficient to costimulate the generation of allogeneic effector cells in the allograft. Ligation of CD2 on T cells by LFA-3 augments production of IFNγ and IL-2 by T cells, and TNFα and IL-1 by monocytes (Webb *et al.* 1990). The increased production of IL-6 by CMV infected fibroblasts, which has been reported in the present study and by others (St.Jeor *et al.* 1993), may also contribute to CD2 mediated T cell activation, and generation of CTLs as IL-6 has these functions *in vitro* (Lorre *et al.* 1990; Okada *et al.* 1988). IL-6 serum levels are also increased *in vivo* during CMV pneumonitis and allograft rejection (Humbert *et al.* 1993).

Increased levels of ICAM-1 have been correlated with rejection in liver allografts (Adams *et al.* 1989), and the participation of the ICAM-1/LFA-1 interaction in the graft rejection process has been documented by several groups. Monoclonal antibodies which block this interaction have been shown to promote allograft engraftment and prolong allograft survival (Isobe *et al.* 1992). In a xenogeneic model, treatment with ICAM-1 specific antibodies delayed lymphocyte infiltration into the graft, and inhibited rejection (Zeng *et al.* 1994). Both ICAM-1 and LFA-3 are commonly expressed in rejecting organs, either at higher than constitutive levels, or on cells which do not normally express these molecules (Steinhoff *et al.* 1993). The upregulation of these molecules *in vivo* is often attributed to the production of inflammatory cytokines by activated T cells or other lymphoid cells which have entered the graft. However, an *in vivo* study has shown that ICAM-1 upregulation in the liver precedes lymphoid activation during CMV infection in liver allograft recipients, suggesting that ICAM-1 upregulation is not a result of cytokine induction by activated T cells, at least in the early stages of infection (Lautenschlager and Hockerstedt, 1993). The latter finding suggests that the upregulation of ICAM-1 in the absence of activated T cells, reported in the present study, may also be important *in vivo.*

Infection of rats with rat CMV is the animal model most frequently used for investigating transplant-associated complications of CMV infection (Stals *et al.* 1990). Some of the *in vitro* findings from the present study, such as increased adherence of activated T cells, and increased expression of ICAM-1, have also been documented in the rat model. Following allogeneic aortic transplantation, rat CMV infection is associated with chronic rejection (Lemstrom *et al.* 1993), with increased numbers of T cells, particularly activated CD8+ve cells, in perivascular areas and adhering to the blood vessels, and enhanced smooth muscle cell proliferation and intimal thickening (Bruggeman *et al.* 1995; Lemstrom *et al.* 1995). Increased expression of ICAM-1 was also found in cells

of the graft (Bruggeman *et al.* 1995). The inflammatory response and intimal thickening were reduced by immunosuppressive therapy, confirming the role of the immune system in the development of this pathology (Lemstrom *et al.* 1994).

We have also shown increased production of IL-8 in the present study, accompanied by increased transendothelial migration of neutrophils. In addition to its effect on neutrophil migration, IL-8 is also chemotactic for NK cells and a subset of T cells, although possibly in a different concentration range (Sebok *et al.* 1993; Roth *et al.* 1995; Qin *et al.* 1996). The recruitment of neutrophils, which are capable of producing other chemokines to stimulate the recruitment of mononuclear cells (Strieter *et al.* 1992; Liao *et al.* 1995), may be an early step in a cascade of events leading to accumulation of mononuclear cells, and increasing the possibility of allograft rejection. Chemotactic factors are also likely to be important in the migration of smooth muscle cells in chronic rejection. We have shown that the CMV encoded chemokine receptor is functional on infected fibroblasts. The potential expression of this receptor by infected smooth muscle cells may be important in the migration of these cells, and in the proliferative response, due to the activation signals transduced upon engagement of chemokine receptors. The ligation of chemokine receptors leads to the activation of transcription factors and cellular genes (Bokoch, 1995). Binding of RANTES to T cells has also been shown to mediate T cell activation in the absence of an antigen specific stimuli (Bacon *et al.* 1995).

The role of the cellular immune response in CMV pneumonitis.

The clinical data supporting the hypothesis that the T cell response contributes to the pathology of CMV pneumonitis has been discussed in the general introduction. The increased expression of adhesion molecules and the increased adherence of activated T lymphocytes to CMV infected fibroblasts has been reported in the present *in vitro* study. These findings appears to be relevant to histopathological findings from lung tissue during episodes of CMV pneumonitis in allogeneic bone marrow transplant recipients. In particular the increased adherence of differentiated memory cells and cytotoxic T lymphocytes shown here *in vitro* appears to translate to increased accumulation of these cells *in vivo*. The accumulation of activated T lymphocytes (both CD4 and CD8) in the perivascular region in the lungs of CMV pneumonitis patients has been documented (Muller *et al.* 1995). The latter patients also displayed increased expression of ICAM-1 on the alveolar epithelium (Muller *et al.* 1995).

It also has been shown that the lymphocytes present in the BAL of patients with CMV pneumonitis are primed cells, showing a greater proliferative response to various T cell activation stimuli than cells from the peripheral blood from the same patients (Zeevi *et al.* 1992). Increased accumulation of cytotoxic T cells in lung tissue was also shown to occur (Humbert *et al.* 1992; Humbert *et al.* 1994). These cells may be represented by the CD28-ve population which was shown in the present study to be over-represented in the adherent populations.

It was shown here that increased expression of ICAM-1 was important for the binding of activated T lymphocytes to CMV infected fibroblasts. ICAM-1 expression on the endothelium was also increased in a rat CMV model of allogeneic lung transplantation, in comparison to untransplanted control lungs in the same animal, and was accompanied by infiltration of lymphocytes with high expression of LFA-1 and VLA-4, which did not take place in rats receiving syngeneic lung transplants (Steinhoff *et al.* 1996). Increased expression of ICAM-1 following rat CMV infection *in vivo*, in both aortic and pulmonary allografts, thus appears to correlate with the accumulation of activated T cells expressing high levels of adhesion molecules (Steinhoff *et al.* 1996; Bruggeman *et al.* 1995). The severity of CMV disease in the lung in particular may be due to the large number of donor lymphoid cells contained in the bronchus-associated lymphoid tissue, providing a source of migrating donor T lymphocytes and dendritic cells, which may initiate a graft-versus-host response (Prop *et al.* 1989).

The enhanced production of IL-8 by CMV infected cells, and the increased neutrophil migration shown here *in vitro* may also contribute to the pathology of CMV pneumonitis. Neutrophil accumulation in the lung is the hallmark of a number of pulmonary disease states, many of which are infectious in nature (Weiss, 1989), and has also been shown in CMV infected lungs (Smyth *et al.* 1993), although the latter is not a common finding. The recruitment of neutrophils by CMV infected fibroblasts and other interstitial cells may be important both in the initiation and maintenance of an immunopathological response to CMV infection, as neutrophil-derived cytokines and chemokines could then act to recruit and activate other cell types, including monocytes and T cells (Strieter *et al.* 1992).

The use of ganciclovir in treatment of rejection and CMV pneumonitis.

The present study has shown that the increase in adhesion molecule expression on those cells already infected with CMV is not prevented by ganciclovir treatment, and may even become accentuated and prolonged. The concentrations of ganciclovir used in the experiments in the present study (1 to 100 μg/ml) are in line with average peak plasma levels, which are reported to be 6.6μg/ml after a 1 hour infusion of 5mg/kg intravenous ganciclovir (Paul and Dummer, 1992). In the treatment of established CMV pneumonitis, ganciclovir therapy is effective in the reduction of infectious virus release, but this is not necessarily predictive of a favourable outcome (Shepp *et al.* 1985; Churchill *et al.* 1987; Slavin *et al.* 1992), as was also shown in the MCMV model. Ganciclovir was also shown to have no effect on chronic rejection of lung allografts (Duncan *et al.* 1992). Treatment of CMV pneumonitis with ganciclovir may thus suppress further virus replication (Slavin *et al.* 1992; Churchill *et al.* 1987), but the infected cells may remain a focus for the inflammatory response, due to increased expression of adhesion molecules, leading to inflammatory infiltrates and widespread tissue damage. Trials of prophylactic or pre-emptive therapy for CMV pneumonitis have had more success in the reduction of CMV disease than the earlier studies, probably due to the timing of the therapy to suppress viral replication before widespread dissemination of the virus has taken place. The use of CMV IVIG in combination with ganciclovir has dramatically improved the success of treatment of CMV disease (Emanuel, 1993). IVIG has been shown to have a modulatory effect on cytokine production (Andersson *et al.* 1996; Mouthon *et al.* 1996), and may possibly affect chemokine production. Increased mRNA for a range of chemokines has been shown in herpes simplex virus infected cells (Oakes *et al.* 1993). Specific antibody therapy for HSV infection of the cornea has been shown to decrease the expression of transcripts for some chemokines in infected cells (Su *et al.* 1996). It is therefore possible that IVIG exerts its modulatory effect on CMV infection by reducing the expression of chemokines, in addition to other cytokines, and thus decreasing leukocyte infiltration.

The role of chemokines in CMV pathology

The sequestration of β chemokines shown in the present study suggests that the CMV encoded β chemokine receptor was expressed on infected cells, and was functional in the sequestering of β chemokines. Previous studies had used transfected cell lines to examine the function of this receptor. In contrast to the proinflammatory functions of chemokines and adhesion molecules, the

expression of the CMV encoded β chemokine receptor would be expected to have effects in the reduction of the inflammatory response. Whether the expression of this receptor has consequences for the immune response or other aspects of viral replication is not yet clear. There are several possible functions which the chemokine receptor may carry out in infected cells. The obvious function is immune evasion, as the sequestration of chemokines produced by the infected cell or by surrounding immune cells would lead to reduced presentation of chemokines on the endothelium or on the stroma, leading to decreased leukocyte migration.

The CMV encoded β chemokine receptor has a higher affinity for β chemokines than CCR1, the chemokine receptor it was compared to when initially characterised (Neote *et al.* 1993; Gao and Murphy, 1994), so would be expected to compete with the cellular β chemokine receptors for chemokine binding. However, a recent study has shown increased levels of RANTES in the bronchoalveolar lavage fluid during CMV pneumonitis in lung allograft recipients, suggesting that chemokines were not sequestered in sufficient quantities to reduce RANTES to normal levels (Monti *et al.* 1996). The influx of leukocytes found during CMV pneumonitis also suggests that the chemokine receptor does not function in reducing inflammation (Humbert *et al.* 1994; Muller *et al.* 1995). However, it remains possible that the expression of this receptor abrogates the migration of a specific leukocyte subset which is required for resolution of infection. It must also be remembered that T cells migrate in response to chemotactic factors which are not β chemokines, and therefore do not bind to the CMV encoded receptor. A newly defined chemokine, stromal cell derived factor (SDF-1), has been shown to induce high levels of T cell transendothelial migration, and is suggested to be responsible for basal migration of T cells through tissues (Bleul *et al.* 1996). The receptor for SDF-1 is CXCR4 (Feng *et al.* 1996). Interleukin-15 is another cytokine which has strong chemotactic activity for T lymphocytes, although it is not a member of the chemokine family (Borthwick *et al.* 1996; Nieto *et al.* 1996) so uses a different receptor. These chemotactic factors are not influenced by the presence of a β chemokine receptor.

The finding from the present study that increased production of IL-8 following CMV infection leads to increased neutrophil migration may have consequences for the spread of infection throughout the body. Neutrophils are considered to be important in the dissemination of CMV throughout the body, and increased contact between neutrophils and CMV infected cells would be expected to

favour this process. It has been found that although neutrophils cannot be infected with cell-free CMV virions, they can acquire CMV proteins following cell to cell contact with infected fibroblasts (Grefte, 1995) or endothelial cells (data from this laboratory, manuscript in preparation). The increased ICAM-1 expression which takes place following CMV infection may also be important in the interaction between neutrophils and infected cells. Neutrophils have been shown to adhere to CMV infected fibroblasts at increased levels *in vitro* (Span *et al.* 1989). Hence neutrophil adhesion and chemotaxis may play a role in the pathogenesis of CMV disease by the acquisition of virus from the endothelium and its dissemination to distant sites. In addition, it has been reported that giant endothelial cells which have detached from vessel walls are a vehicle for transmission of virus throughout the body (Grefte *et al.* 1993). The potential expression of the CMV encoded chemokine receptor on these cells may increase their capacity to migrate into tissues, and may thus aid virus dissemination. The CMV encoded β chemokine receptor is the product of a CMV gene expressed at late stages of infection (Welch *et al.* 1991), and therefore would probably not be expressed on cells which are abortively infected, such as monocytes and lymphocytes. The CMV encoded β chemokine receptor would therefore not be expected to influence the migration of the latter cells.

The induction of chemokines and chemokine receptors by CMV shown in the present study may have direct consequences for viral replication, since it has recently been shown that the addition of IL-8 to CMV infected cells increased the production of infectious virus (Murayama *et al.* 1994). CMV infection also induces mRNA for the cellular IL-8 receptor type I (CXCR1), suggesting that IL-8 produced by infected cells may potentially act in an autocrine manner to enhance viral replication (Murayama *et al.* 1994). The present study has shown sequestering of β chemokines by CMV infected cells, and studies in transfected cells have shown that signals are transduced on ligation of the CMV encoded β chemokine receptor. As the chemokine receptors signal using similar mechanisms, β chemokines, such as MCP-1 may also have a positive effect on CMV replication, by binding to the CMV encoded chemokine receptor on infected cells. Engagement of a functional chemokine receptor results in G protein mediated signals leading to calcium mobilisation, phospholipid remodelling, increases in cyclic AMP levels, and the generation of second messengers involved in leukocyte activation, leading to the upregulation of transcription factors via the MAP kinase pathway (Bokoch, 1995). Such signals may enhance the expression of viral genes, particularly the CMV IE genes

which have cyclic AMP response elements and binding sites for a myriad of transcription factors in their promoter/regulatory region (Hunninghake *et al.* 1989; Albrecht *et al.* 1990). Human herpesviruses 6 and 7 also express β chemokine receptors (Gompels *et al.* 1995; Isgeawa *et al.* 1996), and herpesvirus saimiri expresses an α chemokine receptor (Ahuja and Murphy, 1993). That chemokine receptors are encoded by herpesviruses infecting different species emphasises the potential importance of these receptors in the herpesvirus life cycle.

Two chemokine receptors (CCCR5 and CXCR4) have recently been shown to be co-receptors for HIV entry into cells (Deng *et al.* 1996; Drajic *et al.* 1996; Feng *et al.* 1996), and β chemokines have been identified as suppressive factors for HIV replication (Paxton *et al.* 1996). It was suggested by the authors that β chemokines function in suppressing HIV replication either by competitively binding to the aforementioned chemokine receptors on target cells, or by downregulating the chemokine receptors by internalising the receptors. The possibility that the expression of the chemokine receptor encoded by CMV may contribute to HIV pathology is intriguing, as CMV has been suggested to be a cofactor for the rapid progression of CMV disease. The CMV encoded chemokine receptor was shown to sequester β chemokines in the present study. A decrease in β chemokine concentrations in the extracellular milieu may allow HIV to replicate more rapidly. It has also been suggested that some isolates of HIV are able to use the CMV encoded chemokine receptor as a co-receptor for viral entry, although this has not yet been established.

In the present study several ways in which infection of cells with CMV may alter the expression of immunologically important molecules and thus modulate the immune response to infection have been identified. The immune evasion mechanisms used by CMV, such as the downregulation of class I HLA are likely to be important in the CMV life cycle, in preventing the eradication of the virus by the host immune system. However, the pro-inflammatory effects of CMV infection, such as chemokine production, adhesion molecule upregulation and upregulation of class I HLA on surrounding uninfected cells may be instrumental in the disruption of the balance between the host and the immune system, and may be responsible for some of the pathology associated with CMV infection.

8. REFERENCES

Abraham, D., Muir, H., and Olsen, I.(1988). Adhesion of T and B lymphocytes to fibroblasts in tissue culture. Immunology 65, 385-392.

Abraham, D., Ince, T., Muir, H., and Olsen, I.(1989). Fibroblast matrix and surface components that mediate cell-to-cell interaction with lymphocytes. J. Invest. Dermatol. 93, 335-340.

Adams, D. H., Hubscher, S. G., Shaw, J., Rothlein, R., and Neuberger, J. M.(1989). Intercellular adhesion molecule 1 on liver allografts during rejection. Lancet 2, 1122-1125.

Adams, D. H. and Shaw, S.(1994). Leucocyte-endothelial interactions and regulation of leucocyte migration. Lancet 343, 831-836.

Adler, S. P.(1983). Transfusion-associated cytomegalovirus infections. Rev. Infect. Dis. 5, 977-993.

Adler, S. P., Starr, S. E., Plotkin, S. A., Hempfling, S. H., Buis, J., and Manning, M. L.(1995). Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. J. Infect. Dis. 171, 26-32.

Ahn, K., Angulo, A., Ghazal, P., Peterson, P. A., Yang, Y., and Fruh, K.(1996). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. 21st Herpesvirus Conference (Abstract)

Ahuja, S. K., Gao, J. L., and Murphy, P. M.(1994). Chemokine receptors and molecular mimicry. Immunol. Today 15, 281-287.

Ahuja, S. K. and Murphy, P. M.(1993). Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. J. Biol. Chem. 268, 20691-20694.

Akbar, A. N., Salmon, M., and Janossy, G.(1991). The synergy between naive and memory T cells during activation. Immunol. Today 12, 184

Akbar, A. N., Borthwick, N., Salmon, M., Gombert, W., Bofill, M., Shamsadeen, N., Pett, S., Grundy, J. E., and Janossy, G.(1993). The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. J. Exp. Med. 178, 427-438.

Albelda, S. M., Muller, W. A., Buck, C. A., and Newman, P. J.(1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J. Cell Biol. 114, 1059-1068.

Albrecht, T., Boldogh, I., Fons, M., AbuBakar, S., and Deng, C. Z.(1990). Cell activation signals and the pathogenesis of human cytomegalovirus. Intervirology 31, 68-75.

Albrecht, T., Boldogh, I., and Fons, M. P.(1992). Receptor-initiated activation of cells and their oncogenes by herpes-family viruses. J. Invest. Dermatol. 98, 29S-35S.

Alcami, J., Barzu, T., and Michelson, S.(1991). Induction of an endothelial cell growth factor by human cytomegalovirus infection of fibroblasts. J. Gen. Virol. 72, 2765-2770.

Alcami, J., Paya, C. V., Virelizier, J. L., and Michelson, S.(1993). Antagonistic modulation of human cytomegalovirus replication by transforming growth factor beta and basic fibroblastic growth factor. J. Gen. Virol. 74, 269-274.

Allavena, P., Paganin, C., Martin-Padura, I., Peri, G., Gaboli, M., Dejana, E., Marchisio, P. C., and Mantovani, A.(1991). Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. J. Exp. Med. 173, 439-448.

Altmann, D. M., Hogg, N., Trowsdale, J., and Wilkinson, D.(1989). Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. Nature 338, 512-514.

Alwan, W. H., Kozlowska, W. J., and Openshaw, P. J.(1994). Distinct types of lung disease caused by functional subsets of antiviral T cells. J. Exp. Med. 179, 81-89.

Andersen, C. B., Blaehr, H., Ladefoged, S., and Larsen, S.(1992). Expression of the intercellular adhesion molecule-1 (ICAM-1) in human renal allografts and cultured human tubular cells. Neph,Dial,Transpl. 7, 147-154.

Andersson, J., Skansen-Saphir, U., Sparrelid, E., and Andersson, U.(1996). Intravenous immune globulin affects cytokine production in T lymphocytes and monocytes/macrophages. Clin. Exp. Immunol. 104 Suppl.1, 10-20.

Appelbaum, F. R., Meyers, J. R., Fefer, A., Flournoy, N., Cheever, M. A., Greenberg, P. D., Hackman, R., and Thomas, E. D.(1982). Nonbacterial nonfungal pneumonia following marrow transplantation in 100 identical twins. Transplantation 33, 265-268.

Arnold, J. C., Portmann, B. C., O'Grady, J. G., Naoumov, N. V., and Alexander, G. J.(1992). Cytomegalovirus infection persists in the liver graft in the vanishing bile duct syndrome. Hepatology 16, 285-292.

Autschbach, F., Meuer, S. C., Moebius, U., Manns, M., Hess, G., Meyer zumBuschenfelde, K. H., Thoenes, W., and Dienes, H. P.(1991). Hepatocellular expression of lymphocyte function-associated antigen 3 in chronic hepatitis. Hepatology 14, 223-230.

Azuma, M., Cayabyab, M., Buck, D., Phillips, J. H., and Lanier, L. L.(1992). CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. 175, 353-360.

Azuma, M., Cayabyab, M., Phillips, J. H., and Lanier, L. L.(1993a). Requirements for CD28-dependent T cell-mediated cytotoxicity. J. Immunol. 150, 2091-2101.

Azuma, M., Phillips, J. H., and Lanier, L. L.(1993b). CD28- lymphoctyes: antigenic and functional properties. J. Immunol. 150, 1147-1159.

Bacon, K. B., Premack, B. A., Gardner, P., and Schall, T. J.(1995). Activation of dual T cell signaling pathways by the chemokine RANTES. Science 269, 1727-1730.

Baggiolini, M., Dewald, B., and Moser, B.(1993). Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. Ann. Rev. Immunol. 97-179

Bailey, T. C.(1993). Prevention of cytomegalovirus disease. Seminars in Respiratory Infections 8, 225-232.

Baracchini, E., Glezer, E., Fish, K., Stenberg, R. M., Nelson, J. A., and Ghazal, P.(1992). An isoform variant of the cytomegalovirus immediate-early auto repressor functions as a transcriptional activator. Virology. 188, 518-529.

Barnes, P. D. and Grundy, J. E.(1992a,b). Down regulation of the Class I HLA heterodimer and b2-microglobulin on the surface of cells infetced with cytomegalovirus. J. Gen. Virol. 73, 2395-2405.

Barry, P. A., Pratt-Lowe, E., Peterlin, B. M., and Luciw, P. A.(1990a). Cytomegalovirus activates transcription directed by the long terminal repeat of human immunodeficiency virus type 1. J. Virol. 64, 2932-2940.

Barry, P. A., Pratt-Lowe, E., Unger, R. E., Marthas, M., Alcendor, D. J., and Luciw, P. A.(1990b). Molecular interactions of cytomegalovirus and the human and simian immunodeficiency viruses. J. Med. Primatol. 19, 327-337.

Beck, S. and Barrell, B.(1991). An HCMV reading frame which has similarity with both the V and C regions of the TCR gamma chain. DNA. Seq. 2, 33-38.

Beck, S. and Barrell, B. G.(1988). Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. Nature 331, 269-272.

Beersma, M. F., Bijlmakers, M. J., and Ploegh, H. L.(1993). Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains. J. Immunol. 151, 4455-4464.

Bell, G. M. and Imboden, J. B.(1995). J. Immunol. 155, 2805-2807.

Berke, G.(1991). T-cell-mediated cytotoxicity. Curr. Opin. Immunol. 3, 320-325.

Berman, M. E., Xie, Y., and Muller, W. A.(1996). Role of platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) in natural killer cell transendothelial migration and b_2 integrin activation. J. Immunol. 156, 1515-1524.

Bertoletti, A., Costanzo, A., Chisari, F. V., Levrero, M., Artini, M., Sette, A., Penna, A., Giuberti, T., Fiaccadori, F., and Ferrari, C.(1994a). Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J. Exp. Med. 180, 933-943.

Bertoletti, A., Sette, A., Chisari, F. V., Penna, A., Levrero, M., De Carli, M., Fiaccadori, F., and Ferrari, C.(1994b). Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. Nature 369, 407-410.

Beschorner, W. E., Hutchins, G. M., Burns, W. H., Saral, R., Tutschka, P. J., and Santos, G. W.(1980). Cytomegalovirus pneumonia in bone marrow transplant recipients: miliary and diffuse patterns. Am. Rev. Respir. Dis. 122, 107-114.

Bird, I. N., Spragg, J. H., Ager, A., and Matthews, N.(1993). Studies of lymphocyte transendothelial migration: analysis of migrated cell phenotypes with regard to CD31 (PECAM), CD45RA and CD45RO. Immunology 80, 553-560.

Biron, K. K., Stanat, S. C., Sorrell, J. B., Fyfe, J. A., Keller, P. M., Lambe, C. U., and Nelson, D. J.(1985). Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. U. S. A. 82, 2473-2477.

Bitsch, A., Kirchner, H., Dupke, R., and Bein, G.(1993). Cytomegalovirus transcripts in peripheral blood leukocytes of actively infected transplant patients detected by reverse transcription-polymerase chain reaction. J. Infect. Dis. 167, 740-743.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C.(1987). Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329, 506-512.

Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A., and Springer, T. A.(1996). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J. Exp. Med. 184, 1101-1109.

Bokoch, G. M.(1995). Chemoattractant signaling and leukocyte activation. Blood 86, 1649-1660.

Boldogh, I., AbuBakar, S., and Albrecht, T.(1990). Activation of proto-oncogenes: an immediate early event in human cytomegalovirus infection. Science 247, 561-564.

Boldogh, I., Fons, M. P., and Albrecht, T.(1993). Increased levels of sequence-specific DNA-binding proteins in human cytomegalovirus-infected cells. Biochem. Biophys. Res. Commun. 197, 1505-1510.

Bolhuis, R. L., Roozemond, R. C., and Van De Griend, R. J.(1986). Induction and blocking of cytolysis in CD2+, CD3- NK and CD2+, CD3+ cytotoxic T lymphocytes via CD2 50kD sheep erythrocyte receptor. J. Immunol. 136, 3939-3945.

Boppana, S. B., Pass, R. F., Britt, W. J., Stagno, S., and Alford, C. A.(1992). Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. Pediatr. Infect. Dis. J. 11, 93-99.

Boppana, S. B., Polis, M. A., Kramer, A. A., Britt, W. J., and Koenig, S.(1995). Virus-specific antibody responses to human cytomegalovirus (HCMV) in human immunodeficiency virus type 1-infected persons with HCMV retinitis. J. Infect. Dis. 171, 182-185.

Borthwick, N. J., Bofill, M., Gombert, W. M., Akbar, A. N., Medina, E., Sagawa, K., Lipman, M. C., Johnson, M. A., and Janossy, G.(1994). Lymphocyte activation in HIV-1 infection. II. Functional defects of CD28- T cells. AIDS 8, 431-441.

Borthwick, N. J., Akbar, A. N., MacCormac, L. P., Lowdell, M., Craigen, J. L., Hassan, I., Grundy, J. E., Salmon, M., and Yong, K. L.(1996). Selective migration of highly differentiated primed T cells, defined by low expression of CD45RB, across human umbilical vein endothelial cells (HUVEC): Effects of viral infection on transmigration. Immunology

Borysiewicz, L. K., Graham, S., Hickling, J. K., Mason, P. D., and Sissons, J. G.(1988a). Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. Eur. J. Immunol. 18, 269-275.

Borysiewicz, L. K., Hickling, J. K., Graham, S., Sinclair, J., Cranage, M. P., Smith, G. L., and Sissons, J. G.(1988b). Human cytomegalovirus-specific cytotoxic T cells. Relative frequency of stage-specific CTL recognizing the 72-kD immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. J. Exp. Med. 168, 919-931.

Boussiotis, V. A., Freeman, G. J., Gray, G. S., Gribben, J. G., and Nadler, L. M.(1993). B7 but not ICAM-1 costimulation prevents the induction of human alloantigen specific tolerance. J. Exp. Med. 178, 1753-1763.

Boussiotis, V. A., Freeman, G. J., Griffin, J. D., Gray, G. S., Gribben, J. G., Nadler, and LM.(1994). CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J. Exp. Med. 180, 1665-1673.

Bowden, R. A., Digel, J., Reed, E. C., and Meyers, J. D.(1987). Immunosuppressive effects of ganciclovir on in vitro lymphocyte responses. J. Infect. Dis. 156, 899-903.

Bowden, R. A., Slichter, S. J., Sayers, M. H., Mori, M., Cays, M. J., and Meyers, J. D.(1991). Use of leukocyte-depleted platelets and cytomegalovirus-seronegative red blood cells for prevention of primary cytomegalovirus infection after marrow transplant. Blood 78, 246-250.

Bower, M., Barton, S. E., Nelson, M. R., Bobby, J., Smith, D., Youle, M., and Gazzard, B. G.(1990). The significance of the detection of cytomegalovirus in the bronchoalveolar lavage fluid in AIDS patients with pneumonia. AIDS 4, 317-320.

Boyce, N. W., Hayes, K., Gee, D., Holdsworth, S. R., Thomson, N. M., Scott, D., and Atkins, R. C.(1988). Cytomegalovirus infection complicating renal transplantation and its relationship to acute transplant glomerulopathy. Transplantation 45, 706-709.

Brandley, B. K., Ross, T. S., and Schnaar, R. L.(1987). Multiple carbohydrate receptors on lymphocytes revealed by adhesion to immobilized polysaccharides. J. Cell. Biol. 105, 991-997.

Britt, W. J. and Auger, D.(1986). Human cytomegalovirus virion-associated protein with kinase activity. J. Virol. 59, 185-188.

Bruggeman, C. A., Li, F., and Stals, F. S.(1995). Pathogenicity: animal models. Scand. J. Infect. Dis. Suppl. 99, 43-50.

Bukowski, J. F., Woda, B. A., Habu, S., Okumura, K., and Welsh, R. M.(1983). Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J. Immunol. 131, 1531-1538.

Bukowski, J. F., Warner, J. F., Dennert, G., and Welsh, R. M.(1994). Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J. Exp. Med. 12, 273-280.

Burns, L. J., Waring, J. F., Reuter, J. J., Stinski, M. F., and Ginder, G. D.(1993). Only the HLA class I gene minimal promoter elements are required for transactivation by human cytomegalovirus immediate early genes. Blood 81, 1558-1566.

Cabanas, C. and Hogg, N.(1991). Lymphocyte-fibroblast adhesion. A useful model for analysis of the interaction of the leucocyte integrin LFA-1 with ICAM-1. FEBS Letters 292, 284-288.

Campbell, A. E. and Slater, J. S.(1994). Down-regulation of major histocompatibility complex class I synthesis by murine cytomegalovirus early gene expression. J. Virol. 68, 1805-1811.

Campbell, J. J., Qin, S., Bacon, K. B., Mackay, C. R., and Butcher, E. C.(1996). Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering versus chemotactic responses in the lymphoid cells. J. Cell. Biol. 134, 255-266.

Carney, W. P., Hirsch, M. S., Iacoviello, V. R., Starr, S. E., Fleisher, G., and Plotkin, S. A.(1983). T-lymphocyte subsets and proliferative responses following immunization with cytomegalovirus vaccine. J. Infect. Dis. 147, 958

Carney, W. P. and Hirsch, M. S.(1981). Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. J. Infect. Dis. 144, 47-54.

Carr, M. W., Alon, R., and Springer, T. A.(1996). The C-C chemokine MCP-1 differentially modulates the avidity of b1 and b2 integrins on T lymphocytes. Immunity 4, 179-187.

Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J., and Coughlin, S. R.(1994). Molecular cloning and functional expression of two monocyte chemoattractant protein-1 receptors reveals alternative splicing of the carboxy terminal tails. Proc. Natl. Acad. Sci. U. S. A. 91, 2752-2760.

Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., Kouzarides, T., Martignetti, J. A., and et-al(1990a). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. 154, 125-169.

Chee, M. S., Satchwell, S. C., Preddie, E., Weston, K. M., and Barrell, B. G.(1990b). Human cytomegalovirus encodes three G protein-coupled receptor homologues. Nature 344, 774-777.

Choi, A. M. K. and Jacoby, D. B.(1996). Influenza virus A infection induces interleukin-8 gene expression in human airway epithelial cells. FEBS Letters 309, 327-329.

Chong, A. S., Boussy, I. A., Jiang, X. L., Lamas, M., and Graf, L. H.,Jr.(1994). CD54/ICAM-1 is a costimulator of NK cell-mediated cytotoxicity. Cell. Immunol. 157, 92-105.

Chou, S. W.(1986). Acquisition of donor strains of cytomegalovirus by renal-transplant recipients. N. Engl. J. Med. 314, 1418-1423.

Chou, S. W.(1989). Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors. J. Infect. Dis. 160, 11-15.

Chou, S. W. and Norman, D. J.(1988). The influence of donor factors other than serologic status on transmission of cytomegalovirus to transplant recipients. Transplantation 46, 89-93.

Chrisp, P. and Clissold, S. P.(1991). Foscarnet. A review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs. 41, 104-129.

Churchill, M. A., Zaia, J. A., Forman, S. J., Sheibani, K., Azumi, N., and Blume, K. G.(1987). Quantitation of human cytomegalovirus DNA in lungs from bone marrow transplant recipients with interstitial pneumonia. J. Infect. Dis. 155, 501-509.

Ciccone, E., Grossi, C. E., and Velardi, A.(1996). Opposing functions of activatory T cell receptors and inhibitory NK-cell receptors on cytotoxic T cells. Immunol. Today 17, 450-453.

Cocchi, F., DeVico, A., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P.(1995). Identification of RANTES, MIP-1a, and MIP-1b as the major HIV-1 suppressive factors produced by CD8+ve cells. Science 270, 1811-1815.

Colberg-Poley, A. M., Santomenna, L. D., Harlow, P. P., Benfield, P. A., and Tenney, D. J.(1992). Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. J. Virol. 66, 95-105.

Collins, T., Read, M. A., Neish, A. S., Whitley, M. Z., Thanos, D., and Maniatis, T.(1995). Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB Journal 9, 899-909.

Combadiere, C., Ahuja, S. K., and Murphy, P. M.(1995a). Cloning and functional expression of a human eosinophil CC chemokine receptor. J. Biol. Chem. 270, 16491-16498.

Combadiere, C., Ahuja, S. K., Van Damme, J., Tiffany, H. L., Gao, J. L., and Murphy, P. M.(1995b). Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptors 1 and 2B. J. Biol. Chem. 270, 29671-29675.

Compton, T., Nepomuceno, R. R., and Nowlin, D. M.(1992). Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. Virology. 191, 387-395.

Compton, T.(1993). An immortalized human fibroblast cell line is permissive for human cytomegalovirus infection. J. Virol. 67, 3644-3648.

Compton, T., Nowlin, D. M., and Cooper, N. R.(1993). Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology 193, 834-841.

Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O.(1995). Requirement for MIP-1a for an inflammatory response to viral infection. Science 269, 1583-1585.

Correa, I., Corral, L., and Raulet, D. H.(1994). Multiple natural killer cell-activating signals are inhibited by major histocompatibility complex class I expression in target cells. Eur. J. Immunol. 24, 1323-1331.

Crumpacker, C. S.(1992). Mechanism of action of foscarnet against viral polymerases. Am. J. Med. 92, 3S-7S.

Cush, J. J., Pietschmann, P., Oppenheimer-Marks, N., and Lipsky, P. E.(1992). The intrinsic migratory capacity of memory T cells contributes to their accumulation in rheumatoid synovium. Arthritis Rheum. 35, 1434-1444.

Davignon, D., Martz, E., Reynolds, T., Kurzinger, K., and Springer, T. A.(1981). Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127, 590-595.

Davis, M. G., Kenney, S. C., Kamine, J., Pagano, J. S., and Huang, E. S.(1987). Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc. Natl. Acad. Sci. U. S. A. 84, 8642-8646.

Davis, M. M. and Bjorkman, P. J.(1988). T-cell antigen receptor genes and T cell recognition. Nature 395-402.

Davis, S. J. and van der Merwe, P. A.(1996). The structure and ligand interactions of CD2: implications for T-cell function. Immunol. Today 17, 177-187.

De Fougerolles, A. R., Stacker, S. A., Schwarting, R., and Springer, T. A.(1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J. Exp. Med. 174, 253-267.

De Fougerolles, A. R. and Springer, T. A.(1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. 175, 185-190.

de Waal Malefyt, R., Verma, S., Bejarano, M. T., Ranes-Goldberg, M., and Hill, M.(1993). CD2/LFA-3 or LFA-1/ICAM-1 but not CD28/B7 interactions can augment cytotoxicity by virus-specific CD8+ cytotoxic T lymphocytes. Eur. J. Immunol. 23, 418-424.

Deckert, M., Kubar, J., and Bernard, A.(1992). CD58 and CD59 molecules exhibit potentializing effects in T cell adhesion and activation. J. Immunol. 148, 672-677.

Del-Val, M., Schlicht, H. J., Volkmer, H., Messerle, M., Reddehase, M. J., and Koszinowski, U. H.(1991). Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. J. Virol. 65, 3641-3646.

Del-Val, M., Hengel, H., Hacker, H., Hartlaub, U., Ruppert, T., Lucin, P., and Koszinowski, U. H.(1992). Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J. Exp. Med. 176, 729-738.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R.(1996). Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661-666.

Detmers, P. A., Wright, S. D., Olsen, E., Kimball, B., and Cohn, Z. A.(1987). Aggregation of complement receptors on human neutrophils in the absence of ligand. J. Cell Biol. 105, 1137-1145.

Detmers, P. A., Lo, S. K., Olsen-Egbert, E., Walz, A., Baggiolini, M., and Cohn, Z. A.(1990). Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J. Exp. Med. 171, 1155-1162.

Devergne, O., Peuchmaur, M., Humbert, M., Navratil, E., Leger-Ravet, M. B., Petit, M. A., Galanaud, P., and Emilie, D.(1991). In vivo expression of IL-1 beta and IL-6 genes during viral infections in human. Eur. Cytokine. Netw. 2, 183-194.

Diamond, M. S. and Springer, T. A.(1993). A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J. Cell Biol. 120, 545-556.

Dimmock, N. J.(1987). Multiple mechanisms of neutralization of animal viruses. Trends in Biol. Sci. 12, 70-75.

Dobrescu, D., Ursea, B., Pope, M., Asch, A. S., and Posnett, D. N.(1995). Enhanced HIV-1 replication in V beta 12 T cells due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen. Cell 82, 753-763.

Dokhelar, M. C., Wiels, J., Lipinski, M., Tetaud, C., Devergie, A., Gluckman, E., and Tursz, T.(1981). Natural killer cell activity in human bone marrow recipients: early reappearance of peripheral natural killer activity in graft-versus-host disease. Transplantation 31, 61-65.

Drajic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A.(1996). HIV-1 entry into CD4+ve cells is mediated by the chemokine receptor CC-CKR-5. Nature 381, 667-673.

Dransfield, I., Cabanas, C., Craig, A., and Hogg, N.(1992). Divalent cation regulation of the function of the leukocyte integrin LFA-1. J. Cell Biol. 116, 219-226.

Dransfield, I. and Hogg, N.(1989). Regulated expression of Mg2+ binding epitope on leukocyte integrin a subunits. EMBO J. 8, 3759-3764.

Dubey, C., Croft, M., and Swain, S. L.(1995). Costimulatory requirements of naive CD4+ T cells. ICAM-1 or B7-1 can costimulate naive CD4 T cell activation but both are required for optimum response. J. Immunol. 155, 45-57.

Dummer, J. S., Montero, C. G., Griffith, B. P., Hardesty, R. L., Paradis, I. L., and Ho, M.(1986). Infections in heart-lung transplant recipients. Transplantation 41, 725-729.

Duncan, A. J., Dummer, J. S., Paradis, I. L., Dauber, J. H., Yousem, S. A., Zenati, M. A., Kormos, R. L., and Griffith, B. P.(1991). Cytomegalovirus infection and survival in lung transplant recipients. J. Heart. Lung. Transplant. 10, 638-44; discus.

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

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A.(1986a). Induction by IL-1 and interferon gamma: Tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. 137, 245-254.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A.(1986b). Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. 137, 245-254.

Dustin, M. L., Sanders, M. E., Shaw, S., and Springer, T. A.(1987a). Purified lymphocyte function-associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. J. Exp. Med. 165, 677-692.

Dustin, M. L., Selvaraj, P., Mattaliano, R. J., and Springer, T. A.(1987b). Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature 329, 846-848.

Dustin, M. L., Ferguson, L. M., Chan, P. Y., Springer, T. A., and Golan, D. E.(1996). Visualization of CD2 interaction with LFA-3 and determination of the two-dimensional dissociation constant for adhesion receptors in a contact area. J. Cell Biol. 132, 465-474.

Dustin, M. L. and Springer, T. A.(1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature 341, 619-624.

Dustin, M. L. and Springer, T. A.(1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Ann. Rev. Immunol. 9, 27-66.

Einhorn, L. and Ost, A.(1984). Cytomegalovirus infection of human blood cells. J. Infect. Dis. 149, 207-214.

Emanuel, D., Cunningham, I., Jules-Elysee, K., Brochstein, J. A., Kernan, N. A., Laver, J., Stover, D., White, D. A., Fels, A., Polsky, B., and et-al(1988). Cytomegalovirus pneumonia after bone marrow transplantation successfully treated with the combination of ganciclovir and high-dose intravenous immune globulin. Ann. Intern. Med. 109, 777-782.

Emanuel, D.(1993). Multidisciplinary approach to understanding cytomegalovirus disease. (Michelson, S. and Plotkin, S. A. Eds.) Elsevier Science Publishers, Amsterdam. 295-302.

Epperson, D. E. and Pober, J. S.(1994). Antigen-presenting function of human endothelial cells. Direct activation of resting CD8 T cells. J. Immunol. 153, 5402-5412.

Erlich, K. S., Mills, J., and Shanley, J. D.(1989). Effects of L3T4+ lymphocyte depletion on acute murine cytomegalovirus infection. J. Gen. Virol. 70, 1765-1771.

Ertl, P. F. and Powell, K. L.(1992). Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. J. Virol. 66, 4126-4133.

Farrell, H. E. and Shellam, G. R.(1991). Protection against murine cytomegalovirus infection by passive transfer of neutralizing and non-neutralizing monoclonal antibodies. J. Gen. Virol. 72, 149-156.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A.(1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G-protein coupled receptor. Science 272, 872-877.

Fletcher, C. V. and Balfour, H. H. J.(1989). Evaluation of ganciclovir for cytomegalovirus disease. DICP. 23, 5-12.

Forman, S. J. and Zaia, J. A.(1994). Treatment and prevention of cytomegalovirus pneumonia after bone marrow transplantation: where do we stand? Blood 83, 2392-2398.

Forster, R., Emrich, T., Kremmer, E., and Lipp, M.(1994). Expression of the G protein coupled receptor BLR1 defines mature, recirculating B cells and a subset of T helper memory cells. Blood 84, 830-840.

Fowler, K. B., Stagno, S., Pass, R. F., Britt, W. J., Boll, T. J., and Alford, C. A.(1992). The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. N. Engl. J. Med. 326, 663-667.

Franci, C., Wong, L. M., Van Damme, J., Proost, P., and Charo, I. F.(1995). Monocyte chemoattractant protein-3, but not monocyte chemoattractant protein-2, is a functional ligand of the human monocyte chemoattractant protein-1 receptor. J. Immunol. 154, 6511-6517.

Freitas, V. R., Smee, D. F., Chernow, M., Boehme, R., and Matthews, T. R.(1985). Activity of 9-(1,3-dihydroxy-2-propoxymethyl)guanine compared with that of acyclovir against human, monkey, and rodent cytomegaloviruses. Antimicrob. Agents Chemother. 28, 240-245.

Fujinami, R. S., Nelson, J. A., Walker, L., and Oldstone, M. B.(1988). Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR beta chain: a means for graft rejection and immunosuppression. J. Virol. 62, 100-105.

Gallant, J. E., Moore, R. D., Richman, D. D., Keruly, J., and Chaisson, R. E.(1992). Incidence and natural history of cytomegalovirus disease in patients with advanced human immunodeficiency virus disease treated with zidovudine. The Zidovudine Epidemiology Study Group. J. Infect. Dis. 166, 1223-1227.

Gao, J. L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., and MurphyPM.(1993). Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. J. Exp. Med. 177, 1421-1427.

Gao, J. L. and Murphy, P. M.(1994). Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. J. Biol. Chem. 269, 28539-28542.

Geist, L. J., Monick, M. M., Stinski, M. F., and Hunninghake, G. W.(1992). Cytomegalovirus immediate early genes prevent the inhibitory effect of cyclosporin A on interleukin 2 gene transcription. J. Clin. Invest. 90, 2136-2140.

Geist, L. J., Monick, M. M., Stinski, M. F., and Hunninghake, G. W.(1994). The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-alpha gene expression. J. Clin. Invest. 93, 474-478.

Geppert, T. D. and Lipsky, P. E.(1987). Accessory cell dependent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. J. Immunol. 138, 1660-1664.

Germain, R. N.(1994). MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 76, 287-299.

Gibson, W., Clopper, K. S., Britt, W. J., and Baxter, M. K.(1996). Human cytomegalovirus smallest capsid protein identified as a product of short open reading frame located between HCMV UL48 and UL49. J. Virol. 70, 5680-5683.

Gilat, D., Hershkoviz, R., Mekori, Y. A., Vlodavsky, I., and Lider, O.(1994). Regulation of adhesion of CD4+ve lymphocytes to intact or haparinase-treated subendothelial matrix by diffusible of anchored RANTES and MIP-1b. J. Immunol. 153, 4899-4906.

Gilbert, M. J., Riddell, S. R., Li, C. R., and Greenberg, P. D.(1993). Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. J. Virol. 67, 3461-3469.

Gilbert, M. J., Riddell, S. R., Plachter, B., and Greenberg, P. D.(1996). Cytomegalovirus selectively blocks antigen presentation of its immediate early gene product. Nature 383, 720-722.

Glenn, J.(1981). Cytomegalovirus infections following renal transplantation. Rev. Infect. Dis. 3, 1151-1178.

Gompels, U. A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B. J., Martin, M. E., Efstathiou, S., Craxton, M., and Macaulay, H. A.(1995). The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology 209, 29-51.

Gonczol, E., Andrews, P. W., and Plotkin, S. A.(1985). Cytomegalovirus infection of human teratocarcinoma cells in culture. J. Gen. Virol. 66, 509-515.

Gonwa, T. A., Capehart, J. E., Pilcher, J. W., and Alivizatos, P. A.(1989). Cytomegalovirus myocarditis as a cause of cardiac dysfunction in a heart transplant recipient. Transplantation 47, 197-199.

Gorman, C.(1985). DNA Cloning: A practical approach. (IRL Press, Oxford). 143-165.

Gozlan, J., Salord, J. M., Chouaid, C., Duvivier, C., Picard, O., and Meyohas, M. C.(1993). Human cytomegalovirus (HCMV) late-mRNA detection in peripheral blood of AIDS patients: diagnostic value for HCMV disease compared with those of viral culture and HCMV DNA detection. J. Clin. Microbiol. 31, 1943-1945.

Grattan, M. T., Moreno-Cabral, C. E., Starnes, V. A., Oyer, P. E., Stinson, E. B., and Shumway, N. E.(1989). Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. JAMA 261, 3561-3566.

Greenlaw, R., Robinson, P., Heaton, T., Lombardi, G., and Lechler, R.(1992). Transfection of HLA-DR-expressing DAP.3 cells with a cDNA clone encoding the glycosyl phosphatidylinositol-linked form of lymphocyte function associated antigen-3: biochemical features and functional consequences. Int. Immunol. 4, 673-680.

Grefte, A., van der Giessen, M., van Son, W., and The, T. H.(1993). Circulating cytomegalovirus (CMV)-infected endothelial cells in patients with an active CMV infection. J. Infect. Dis. 167, 270-277.

Grefte, A., Harmsen, M. C., van der Giessen, M., Knollema, S., van Son, W. J., and TheTH.(1994). Presence of human cytomegalovirus (HCMV) immediate early mRNA but not ppUL83 (lower matrix protein pp65) mRNA in polymorphonuclear and mononuclear leukocytes during active HCMV infection. J. Gen. Virol. 75, 1989-1998.

Grefte, A.(1995). Dissemination of human cytomegalovirus: Role of leukocytes and endothelial cells. 117-128.(PhD Thesis, University of Groningen)

Gregory, C. D., Murray, R. J., Edwards, C. F., and Rickinson, A. B.(1988). Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. J. Exp. Med. 167, 1811-1824.

Gretch, D. R., Gehrz, R. C., and Stinski, M. F.(1988a). Characterization of a human cytomegalovirus glycoprotein complex (gcI). J. Gen. Virol. 69, 1205-1215.

Gretch, D. R., Kari, B., Gehrz, R. C., and Stinski, M. F.(1988b). A multigene family encodes the human cytomegalovirus glycoprotein complex gcII (gp47-52 complex). J. Virol. 62, 1956-1962.

Gretch, D. R., Kari, B., Rasmussen, L., Gehrz, R. C., and Stinski, M. F.(1988c). Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. J. Virol. 62, 875-881.

Gribben, J. G., Freeman, G. J., Boussiotis, V. A., Rennert, P., Jellis, C. L., Greenfield, E., Barber, M., Gray, G. S., and Nadler, L. M.(1994). CTLA-4 mediated costimulation induces apoptosis of activated human T lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 92, 811-815.

Grundy, J. E., Shanley, J. D., and Shearer, G. M.(1985). Augmentation of graft-versus-host reaction by cytomegalovirus infection resulting in interstitial pneumonitis. Transplantation 39, 548-553.

Grundy, J. E., Super, M., and Griffiths, P. D.(1986). Reinfection of a seropositive allograft recipient by cytomegalovirus from donor kidney. Lancet 1, 159-160.

Grundy, J. E., Shanley, J. D., and Griffiths, P. D.(1987a). Is cytomegalovirus interstitial pneumonitis in transplant recipients an immunopathological condition? Lancet 2, 996-999.

Grundy, J. E., Super, M., Lui, S., Sweny, P., and Griffiths, P. D.(1987b). The source of cytomegalovirus infection in seropositive renal allograft recipients is frequently the donor kidney. Transplant Proc. 19, 2126-2128.

Grundy, J. E., Ayles, H. M., McKeating, J. A., Butcher, R. G., Griffiths, P. D., and Poulter, L. W.(1988a). Enhancement of class I HLA antigen expression by cytomegalovirus: role in amplification of virus infection. J. Med. Virol. 25, 483-495.

Grundy, J. E., Lui, S. F., Super, M., Berry, N. J., Sweny, P., Fernando, O. N., Moorhead, J., and Griffiths, P. D.(1988b). Symptomatic cytomegalovirus infection in seropositive kidney recipients: reinfection with donor virus rather than reactivation of recipient virus. Lancet 2, 132-135.

Grundy, J. E., Pahal, G. S., and Akbar, A. N.(1993). Increased adherence of CD2 peripheral blood lymphocytes to cytomegalovirus-infected fibroblasts is blocked by anti-LFA-3 antibody. Immunology 78, 413-420.

Grundy, J. E. and Downes, K. L.(1993). Up-regulation of LFA-3 and ICAM-1 on the surface of fibroblasts infected with cytomegalovirus. Immunology 78, 405-412.

Guckel, B., Berek, C., Lutz, M., Altevogt, P., Schirrmacher, V., and Kyewski, B. A.(1991). Anti-CD2 antibodies induce T cell unresponsiveness in vivo. J. Exp. Med. 174, 957-967.

Guglielmo, B. J., Wong-Beringer, A., and Linker, C. A.(1994). Immune globulin therapy in allogeneic bone marrow transplant: a critical review. Bone Marrow Transplant. 13, 499-510.

Guinan, E. C., Gribben, J. G., Boussiotis, V. A., Freeman, G. J., and Nadler, L. M.(1994). Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. Blood 84, 3261-3282.

Hahn, W. C., Menu, E., Bothwell, A. L., Sims, P. J., and Bierer, B. E.(1992a). Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. Science 256, 1805-1807.

Hahn, W. C., Rosenstein, Y., Calvo, V., Burakoff, S. J., and Bierer, B. E.(1992b). A distinct cytoplasmic domain of CD2 regulates ligand avidity and T-cell responsiveness to antigen. Proc. Natl. Acad. Sci. U. S. A. 89, 7179-7183.

Hahn, W. C. and Bierer, B. E.(1993). Separable portions of the CD2 cytoplasmic domain involved in signaling and ligand avidity regulation. J. Exp. Med. 178, 1831-1836.

Harrison, C. J., Britt, W. J., Chapman, N. M., Mullican, J., and Tracy, S.(1995). Reduced congenital cytomegalovirus (CMV) infection after maternal immunization with a guinea pig CMV glycoprotein before gestational primary CMV infection in the guinea pig model. J. Infect. Dis. 172, 1212-1220.

Haverstick, D. M. and Gray, L. S.(1992). Lymphocyte adhesion mediated by lymphocyte function associated antigen-1: II. Interaction between phorbol ester and cAMP sensitive pathways. J. Immunol. 149, 397-402.

Hengel, H., Eblinger, C., Pool, J., Goulmy, E., and Koszinowski, U. H.(1995). Cytokines restore MHC class I complex formation and control antigen presentation in human cytomegalovirus-infected cells. J. Gen. Virol. 76, 2987-2997.

Hermiston, T. W., Malone, C. L., and Stinski, M. F.(1990). Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. J. Virol. 64, 3532-3536.

Herrera, G. A., Alexander, R. W., Cooley, C. F., Luke, R. G., Kelly, D. R., Curtis, J. J., and Gockerman, J. P.(1986). Cytomegalovirus glomerulopathy: a controversial lesion. Kidney Int. 29, 725-733.

Hibbs, M. L., Jakes, S., Stacker, S. A., Wallace, R. W., and Springer, T. A.(1991). The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 beta subunit: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. J. Exp. Med. 174, 1227-1238.

Hirsch, M. S. and Felsenstein, D.(1984). Cytomegalovirus-induced immunosuppression. Ann. N. Y. Acad. Sci. 437, 8-15.

Ho, M., Suwansirikul, S., Dowling, J. N., Youngblood, L. A., and Armstrong, J. A.(1975). The transplanted kidney as a source of cytomegalovirus infection. N. Engl. J. Med. 293, 1109-1112.

Ho, M.(1994). Advances in understanding cytomegalovirus infection after transplantation. . Transplant Proc. 26, 7-11.

Hollander, N.(1992). Membrane dynamics of the phosphatidylinositol-anchored form and the transmembrane form of the cell adhesion protein LFA-3. J. Biol. Chem. 267, 5663-5667.

Holmes, W. E., Lee, J., Kuang, W., Rice, G. C., and Wood, W. I.(1991). Structure and functional expression of a human interleukin-8 receptor. Science 253, 1278-1280.

Hu, K. Q., Yu, C. H., and Vierling, J. M.(1992). Up-regulation of intercellular adhesion molecule 1 transcription by hepatitis B virus X protein. Proc. Natl. Acad. Sci. U. S. A. 89, 11441-11445.

Huber, A. R., Kunkel, S. L., Todd, R. F., and Weiss, S. J.(1991). Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 254, 99-102.

Hughes, C. C., Savage, C. O., and Pober, J. S.(1990). Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. J. Exp. Med. 171, 1453-1467.

Humbert, M., Devergne, O., Cerrina, J., Rain, B., Simonneau, G., Dartevelle, P., Duroux, P., Galanaud, P., and Emilie, D.(1992). Activation of macrophages and cytotoxic cells during cytomegalovirus pneumonia complicating lung transplantations. Am. Rev. Respir. Dis. 145, 1178-1184.

Humbert, M., Delattre, R. M., Fattal, S., Rain, B., Cerrina, J., Dartevelle, P., Duroux, P., Galanaud, P., and Emilie, D.(1993). In situ production of interleukin-6 within human lung allografts displaying rejection or cytomegalovirus pneumonia. Transplantation 56, 623-627.

Humbert, M., Magnan, A., Ladurie, F. L., Dartevelle, P., Simonneau, G., Duroux, P., and Emilie, D.(1994). Perforin and granzyme B gene-expressing cells in bronchoalveolar lavage fluids from lung allograft recipients displaying cytomegalovirus pneumonitis. Transplantation 57, 1289-1292.

Hunig, T., Tiefenthaler, G., Meyer zum Buschenfelde, K. H., and Meuer, S. C.(1987). Alternative pathway activation of T cells by binding of CD2 to its cell-surface ligand. Nature 326, 298-301.

Hunninghake, G. W., Monick, M. M., Liu, B., and Stinski, M. F.(1989). The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J. Virol. 63, 3026-3033.

Hynes, R. O.(1987). Integrins: a family of cell surface receptors. . Cell 48, 549-554.

Hynes, R. O.(1992). Integrins: versatility, modulation, and signaling in cell adhesion. . Cell 69, 11-25.

Ibanez, C. E., Schrier, R., Ghazal, P., Wiley, C., and Nelson, J. A.(1991). Human cytomegalovirus productively infects primary differentiated macrophages. J. Virol. 65, 6581-6588.

Imai, T., Tanaka, Y., Fukudome, K., Takagi, S., Araki, K., and Yoshie, O.(1993). Enhanced expression of LFA-3 on human T-cell lines and leukemic cells carrying human T-cell-leukemia virus type 1. Int. J. Cancer 55, 811-816.

Irani, D. N. and Griffin, D. E.(1996). Regulation of lymphocyte homing into the brain during viral encephalitis at various stages of infection. J. Immunol. 156, 3850-3856.

Isgeawa, Y., Ping, Z., and Yamanishi, K.(1996). Human herpesvirus 6 open reading frame U12 encodes a functional chemokine receptor. Abstracts of the 21st International Herpesvirus Workshop (Abstract)

Isobe, M., Yagita, H., Okumura, K., and Ihara, A.(1992). Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science 255, 1125-1127.

Ito, M., Watanabe, M., Ihara, T., Kamiya, H., and Sakurai, M.(1995a). Increased expression of adhesion molecules (CD54, CD29 and CD44) on fibroblasts infected with cytomegalovirus. Microbiology & Immunology 39, 129-133.

Ito, M., Watanabe, M., Kamiya, H., and Sakurai, M.(1995b). Changes of adhesion molecule (LFA-1, ICAM-1) expression on memory T cells activated with cytomegalovirus antigen. Cell. Immunol. 160, 8-13.

Iwamoto, G. K., Monick, M. M., Clark, B. D., Auron, P. E., Stinski, M. F., and Hunninghake, G. W.(1990). Modulation of interleukin 1 beta gene expression by the immediate early genes of human cytomegalovirus. J. Clin. Invest. 85, 1853-1857.

Jabs, D. A., Enger, C., and Bartlett, J. G.(1989). Cytomegalovirus retinitis and acquired immunodeficiency syndrome. Arch. Ophthalmol. 107, 75-80.

Jacobson, M. A., Mills, J., Rush, J., Peiperl, L., Seru, V., Mohanty, P. K., Hopewell, P. C., Hadley, W. K., Broadus, V. C., Leoung, G., and et-al(1991). Morbidity and mortality of patients with AIDS and first-episode Pneumocystis carinii pneumonia unaffected by concomitant pulmonary cytomegalovirus infection. Am. Rev. Respir. Dis. 144, 6-9.

Jacobson, M. A. and Mills, J.(1988). Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. Ann. Intern. Med. 108, 585-594.

Jacobson, S., Sekaly, R. P., Jacobson, C. L., McFarland, H. F., and Long, E. O.(1989). HLA class II restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. J. Virol. 63, 1756-1761.

Janossy, G., Montano, L., Selby, W. S., Duke, O., Panayi, G., Lampert, I., Thomas, J. A., Granger, S., Bofill, M., Tidman, N., Thomas, H. C., and Goldstein, G.(1982). T cell subset abnormalities in tissue lesions developing during autoimmune disorders, viral infection, and graft-vs.-host disease. J. Clin. Immunol. 2, 42S-56S.

Janossy, G. and Amlot, P. A.(1987). Lymphocytes. A practical approach IRL Press, Oxford. 70

Jenkins, D. E., Martens, C. L., and Mocarski, E. S.(1994). Human cytomegalovirus late protein encoded by ie2: a trans-activator as well as a repressor of gene expression. J. Gen. Virol. 75, 2337-2348.

Jones, E. Y., Davis, S. J., Williams, A. F., Harlos, K., and Stuart, D. I.(1992). Crystal structure of a soluble form of the cell adhesion molecule CD2. Nature 360, 232-239.

Jones, T. R., Hanson, L. K., Sun, L., Slater, J. S., Stenberg, R. M., and Campbell, A. E.(1995). Multiple independent loci within the human cytomegalovirus short region down-regulate expression of major histocompatibility complex class I heavy chains. J. Virol. 69, 4830-4841.

Jones, T. R. and Muzithras, V. P.(1992). A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. J. Virol. 66, 2541-2546.

Jones, T. R. and Sun, L.(1996). The HCMV US2 gene product causes downregulation of MHC class I heavy chains. Abstracts of the 21st International Herpesvirus Workshop (Abstract)

Jonjic, S., Del-Val, M., Keil, G. M., Reddehase, M. J., and Koszinowski, U. H.(1988). A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. J. Virol. 62, 1653-1658.

Jonjic, S., Mutter, W., Weiland, F., Reddehase, M. J., and Koszinowski, U. H.(1989). Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J. Exp. Med. 169, 1199-1212.

Jonjic, S., Pavic, I., Lucin, P., Rukavina, D., and Koszinowski, U. H.(1990). Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes. J. Virol. 64, 5457-5464.

Jonjic, S., Pavic, I., Polic, B., Crnkovic, I., Lucin, P., and Koszinowski, U. H.(1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J. Exp. Med. 179, 1713-1717.

June, C. H., Ledbetter, J. A., Linsley, P. S., and Thomson, C. B.(1990). Role of CD28 receptor in T cell activation. Immunol. Today 11, 211-216.

Kapasi, K. and Rice, G. P.(1988). Cytomegalovirus infection of peripheral blood mononuclear cells: effects on interleukin-1 and -2 production and responsiveness. J. Virol. 62, 3603-3607.

Kari, B., Goertz, R., and Gehrz, R.(1990). Characterization of cytomegalovirus glycoproteins in a family of complexes designated gC-II with murine monoclonal antibodies. Arch. Virol. 112, 55-65.

Kari, B., Li, W., Cooper, J., Goertz, R., and Radeke, B.(1994). The human cytomegalovirus UL100 gene encodes the gC-II glycoproteins recognized by group 2 monoclonal antibodies. J. Gen. Virol. 75, 3081-3086.

Kari, B. and Gehrz, R.(1992). A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. J. Virol. 66, 1761-1764.

Kari, B. and Gehrz, R.(1993). Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II. J. Gen. Virol. 74, 255-264.

Keay, S., Merigan, T. C., and Rasmussen, L.(1989). Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc. Natl. Acad. Sci. U. S. A. 86, 10100-10103.

Keay, S. and Baldwin, B.(1991). Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. J. Virol. 65, 5124-5128.

Keenan, R. J., Lega, M. E., Dummer, J. S., Paradis, I. L., Dauber, J. H., Rabinowich, H., Yousem, S. A., Hardesty, R. L., Griffith, B. P., Duquesnoy, R. J., and et-al(1991). Cytomegalovirus serologic status and postoperative infection correlated with risk of developing chronic rejection after pulmonary transplantation. Transplantation 51, 433-438.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and et al.(1994). Lymphotactin: a cytokine that represents a new class of chemokine. Science 266, 1395-1399.

Kern, F., Ode-Hakim, S., Vogt, K., Holfich, C., Reinke, P., and Volk, H. D.(1996). The enigma of CD57+ve CD28-ve T cell expansion-anergy or activation? Clin. Exp. Immunol. 104, 180-184.

Kersh, G. J. and Allen, P. M.(1996). Essential flexibility in the T-cell recognition of antigen. Nature 380, 495-498.

Killeen, N., Stuart, S. G., and Littman, D. R.(1992). Development and function of T cells in mice with a disrupted CD2 gene. EMBO J. 11, 4329-4336.

Kirby, J. A., Rajasekar, M. R., Lin, Y., Proud, G., and Taylor, R. M.(1993). Interaction between T lymphocytes and kidney epithelial cells during renal allograft rejection. Kidney International - Supplement 39, S124-S128.

Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D, Koppe, B., Rosenberg, W., Boyd, D., Edwards, A., and et al.(1994). Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature 369, 403-407.

Kobayashi, T., Yokoyama, I., Nishiyama, Y., Hayashi, S., Uchida, K., and Takigi, H.(1995). Strong enhancement of HLA Class I surface expression by incomplete cytomegalovirus particles: Analysis by dual colour flow cytometry using saponin. Transplant Proc. 27, 966-969.

Kohl, S., Springer, T. A., Schmalsteig, F. C., Loo, L. S., and Anderson, D. C.(1984). Defective natural killer cell cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. J. Immunol. 133, 2972-2978.

Koskinen, P., Lemstrom, K., Bruggeman, C., Lautenschlager, I., and Hayry, P.(1994). Acute cytomegalovirus infection induces a subendothelial inflammation (endothelialitis) in the allograft vascular wall. A possible linkage with enhanced allograft arteriosclerosis. Am. J. Pathol. 144, 41-50.

Koszinowski, U. H., Keil, G. M., Schwarz, H., Schickedanz, J., and Reddehase, M. J.(1987). A nonstructural polypeptide encoded by immediate-early transcription unit 1 of murine cytomegalovirus is recognized by cytolytic T lymphocytes. J. Exp. Med. 166, 289-294.

Koszinowski, U. H., Thale, R., Lucin, P., Ziegler, H., Hengel, H., and Farell, H.(1996). A mouse cytomegalovirus glycoprotein arrests MHC class I molecule export from the ER. Abstracts of the 21st International Herpesvirus Workshop (Abstract)

Kowalik, T. F., Wing, B., Haskill, J. S., Azizkhan, J. C., Baldwin, A. S.,Jr., and Huang, E. S.(1993). Multiple mechanisms are implicated in the regulation of NF-kappa B activity during human cytomegalovirus infection. Proc. Natl. Acad. Sci. U. S. A. 90, 1107-1111.

Kraus, E., Schneider-Schaulies, S., Miyasaka, M., Tamatani, T., and Sedgwick, J.(1992). Augmentation of major histocompatibility complex class I and ICAM-1 expression on glial cells following measles virus infection: evidence for the role of type-1 interferon. Eur. J. Immunol. 22, 175-182.

Krensky, A. M., Robbins, E., Springer, T. A., and Burakoff, S. J.(1984). LFA-1, LFA-2 and LFA-3 antigens are involved in CTL target conjugation. J. Immunol. 132, 2180-2185.

Krensky, A. M., Mentzer, S. J., Clayberger, C., Anderson, D. C., Schmalstieg, F. C., and Springer, T. A.(1985). Heritable lymphocyte function-associated antigen-1 deficiency: abnormalities of cytotoxicity and proliferation associated with abnormal expression of LFA-1. J. Immunol. 135, 3102-3108.

Labalette, M., Salez, F., Pruvot, F. R., Noel, C., and Dessaint, J. P.(1994). CD8 lymphocytosis in primary cytomegalovirus (CMV) infection of allograft recipients: expansion of an uncommon CD8+ CD57- subset and its progressive replacement by CD8+ CD57+ T cells. Clin. Exp. Immunol. 95, 465-471.

Landini, M. and Spaete, R. R.(1993). Multidisciplinary approach to understanding cytomegalovirus disease. (Michelson, S. and Plotkin, S. A. Eds.) Elsevier Science Publishers, Amsterdam. 65-74.

Lapierre, L. A., Fiers, W., and Pober, J. S.(1988). Three distinct classes of regulatory cytokines control endothelial cell MHC antigen expression. Interactions with immune gamma interferon differentiate the effects of tumor necrosis factor and lymphotoxin from those of leukocyte alpha and fibroblast beta interferons. J. Exp. Med. 167, 794-804.

Larsen, C. G., Anderson, A. O., Appella, E., Oppenheim, J. J., and Matsushima, K.(1989). The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science 243, 1464-1466.

Lathey, J. L., Spector, D. H., and Spector, S. A.(1994). Human cytomegalovirus-mediated enhancement of human immunodeficiency virus type-1 production in monocyte-derived macrophages. Virology 199, 98-104.

Lathey, J. L. and Spector, S. A.(1991). Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. J. Virol. 65, 6371-6375.

Lautenschlager, I., Hockerstedt, K., Salmela, K., Isoniemi, H., Holmberg, C., Jalanko, H., and Hayry, P.(1990). Fine-needle aspiration biopsy in the monitoring of liver allografts. Different cellular findings during rejection and cytomegalovirus infection. Transplantation 50, 798-803.

Lautenschlager, I. and Hockerstedt, K.(1993). Induction of ICAM-1 on hepatocytes precedes the lymphoid activation of acute liver allograft rejection and cytomegalovirus infection. Transplant Proc. 25, 1429-1430.

Lawson, C. M., O'Donoghue, H., and Reed, W. D.(1989). The role of T cells in mouse cytomegalovirus myocarditis. Immunology 67, 132-134.

Ledebur, H. C. and Parks, T. P.(1995). Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. Essential roles of a variant NF-kappa B site and p65 homodimers. J. Biol. Chem. 270, 933-943.

Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., and Wood, W. I.(1992). Characterization of two high affinity human interleukin-8 receptors. J. Biol. Chem. 267, 16283-16287.

Lemstrom, K., Persoons, M., Bruggeman, C., Ustinov, J., and Lautenschlager, I.(1993). Cytomegalovirus infection enhances allograft arteriosclerosis in the rat. Transplant Proc. 25, 1406-1407.

Lemstrom, K., Bruning, J., Koskinen, P., Bruggeman, C., and Lautenschlager, I.(1994). Triple-drug immunosuppression significantly reduces chronic rejection in noninfected and RCMV-infected rats. Transplant Proc. 26, 1727-1728.

Lemstrom, K., Koskinen, P., Krogerus, L., Daemen, M., Bruggeman, C., and Hayry, P.(1995). Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection. Circulation 92, 2594-2604.

Liao, F., Rabin, R. L., Yannelli, J. R., Koniaris, L. G., Vanguri, P., and Farber, J. M.(1995). Human Mig chemokine: biochemical and functional characterization. J. Exp. Med. 182, 1301-1314.

Lindsley, M. D., Torpey, D. J., and Rinaldo, C. R.,Jr.(1986). HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by Leu-3-positive T cells. J. Immunol. 136, 3045-3051.

Linsley, P. S., Bradley, W., Grosmaire, L., Aruffo, A., Damle, N. K., and Ledbetter, J. A.(1991). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin-2 mRNA accumulation. J. Exp. Med. 173, 721-729.

Littler, E., Stuart, A. D., and Chee, M. S.(1992). Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature. 358, 160-162.

Liu, B. and Stinski, M. F.(1992). Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. J. Virol. 66, 4434-4444.

Ljungman, P., Engelhard, D., Link, H., Biron, P., Brandt, L., Brunet, S., Cordonnier, C., Debusscher, L., de-Laurenzi, A., Kolb, H. J., and et-al(1992). Treatment of interstitial pneumonitis due to cytomegalovirus with ganciclovir and intravenous immune globulin: experience of European Bone Marrow Transplant Group. Clin. Infect. Dis. 14, 831-835.

Ljungman, P., Biron, P., Bosi, A., Cahn, J. Y., Goldstone, A. H., Gorin, N. C., LinkH., Messina, C., Michallet, M., Richard, C., and et al.(1994). Cytomegalovirus interstitial pneumonia in autologous bone marrow transplant recipients. Infectious Disease Working Party of the European Group for Bone Marrow Transplantation. Bone Marrow Transplant. 13, 209-212.

Lloyd, A. R., Oppenheim, J. J., Kelvin, D. J., and Taub, D. D.(1996). Chemokines regulate T cell adherence to recombinant adhesion extracellular matrix proteins. J. Immunol. 156, 932-938.

Lloyd, A. R. and Oppenheim, J. J.(1992). Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immunol. Today 13, 169-172.

Loebe, M., Schuler, S., Zais, O., Warnecke, H., Fleck, E., and Hetzer, R.(1990). Role of cytomegalovirus infection in the development of coronary artery disease in the transplanted heart. J. Heart. Transplant. 9, 707-711.

Loetscher, M. L., Gerber, B., Loetscher, P., Jones, S. A., Piali, L., Clark Lewis, M., Bagglioni, M., and Moser, B.(1996). Chemokine receptor specific for IP-10 and Mig: structure, function and expression in activated T lymphocytes. J. Exp. Med. 184, 963-969.

Loetscher, P., Seitz, M., Baggiolini, M., and Moser, B.(1996a). Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. J. Exp. Med. 184, 569-577.

Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M., and Moser, B.(1996b). Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. J. Immunol. 156, 322-327.

Lollo, B. A., Chan, K. W. H., Hanson, E. M., Moy, V. T., and Brian, A. A.(1993). Direct evidence for two affinity states for lymphocyte function-associated antigen-1 on activated T cells. J. Biol. Chem. 268, 21693-21700.

Lopez, C., Simmons, R. L., Mauer, S. M., Najarian, J. S., Good, R. A., and Gentry, S.(1974). Association of renal allograft rejection with virus infections. Am. J. Med. 56, 280-289.

Lorre, K., Van Damme, J., Verwilghen, J., Baroja, M. L., and Ceuppens, J. L.(1990). IL-6 is an accessory signal in the alternative CD2-mediated pathway of T cell activation. J. Immunol. 144, 4681-4687.

Lubon, H., Ghazal, P., Hennighausen, L., Reynolds-Kohler, C., Lockshin, C., and Nelson, J.(1989). Cell-specific activity of the modulator region in the human cytomegalovirus major immediate-early gene. Mol. Cell Biol. 9, 1342-1345.

Lucin, P., Pavic, I., Polic, B., Jonjic, S., and Koszinowski, U. H.(1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J. Virol. 66, 1977-1984.

MacCormac, L. P. and Grundy, J. E.(1996). Human cytomegalovirus induces an Fc gamma receptor in endothelial cells and fibroblasts that is distinct from the human cellular Fc gamma receptors. J. Infect. Dis. In press,

Mackay, C. R.(1996). Chemokine receptors and T cell chemotaxis. J. Exp. Med. 184, 799-802.

Mahe, Y., Mukaida, N., Kuno, K., Akiyama, M., Ikeda, N., and Matsushima, K.(1991). Hepatitis B virus X protein transactivates human interleukin-8 gene through acting on nuclear factor kB and CCAAT/enhancer-binding protein-like cis-elements. J. Biol. Chem. 266, 13759-13763.

Maher, P., O'Toole, C. M., Wreghitt, T. G., Spiegelhalter, D. J., and English, T. A.(1985). Cytomegalovirus infection in cardiac transplant recipients associated with chronic T cell subset ratio inversion with expansion of a Leu-7+ TS-C+ subset. Clin. Exp. Immunol. 62, 515-524.

Makgoba, M. W., Sanders, M. E., Ginther Luce, G. E., Gugel, E. A., Dustin, M. L., and Shaw, S.(1988). Functional evidence that intercellular adhesion molecule-1 (ICAM-1) is a ligand for LFA-1-dependent adhesion in T cell-mediated cytotoxicity. Eur. J. Immunol. 18, 637-640.

Malone, C. L., Vesole, D. H., and Stinski, M. F.(1990). Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. 64, 1498-1506.

Mar, E. C., Chiou, J. F., Cheng, Y. C., and Huang, E. S.(1985). Inhibition of cellular DNA polymerase alpha and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine. J. Virol. 53, 776-780.

Marker, O., Scheynius, A., Christensen, J. P., and Thomsen, A. R.(1995). Virus activated T cells regulate expression of adhesion molecules on endothelial cells at sites of infection. Journal of Neuroimmunology 62, 35-42.

Marlin, S. D. and Springer, T. A.(1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell 51, 813-819.

Marshalko, S. J., Schweitzer, B. I., and Beardsley, G. P.(1995). Chiral chemical synthesis of DNA containing (S)-9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and effects on thermal stability, duplex structure, and thermodynamics of duplex formation. Biochemistry 34, 9235-9248.

Marshall, C. R., Raff, E. C., and Raff, R. A.(1994). Dollo's law and the death and resurrection of genes. Proc. Natl. Acad. Sci. U. S. A. 91, 12283-12287.

Mastronarde, J. G., He, B., Monick, M. M., Mukaida, N., Matsushima, K., and Hunninghake, G. W.(1996). Induction of IL-8 gene expression by respiratory syncitial virus involves activation of NF-kappa B and NF-IL-6. J. Infect. Dis. 174, 262-267.

Matthews, T. and Boehme, R.(1988). Antiviral activity and mechanism of action of ganciclovir. Rev. Infect. Dis. 10 Suppl 3, S490-S494.

McDonald, K., Rector, T. S., Braulin, E. A., Kubo, S. H., and Olivari, M. T.(1989). Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus infection. Am. J. Cardiol. 64, 359-362.

McGavran, M. H. and Smith, M. G.(1965). Ultrastructural, cytochemical and microchemical observations on cytomegalovirus (salivary gland virus) infection of human cells in tissue culture. Exp. Mol. Pathol. 4, 1-10.

McKeithen, T. W.(1995). Kinetic proofreading in T-cell receptor signal transduction. Proc. Natl. Acad. Sci. U. S. A. 92, 5042-5046.

McLaughlin-Taylor, E., Pande, H., Forman, S. J., Tanamachi, B., Li, C. R., Zaia, J. A., and Riddell, S. R.(1994). Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J. Med. Virol. 43, 103-110.

McMichael, A. J. and Gotch, F. M.(1987). Leukocyte Typing III. Oxford University Press, Oxford. 31-62.

Meyers, J. D., Flournoy, N., and Thomas, E. D.(1986). Risk factors for cytomegalovirus infection after human marrow transplantation. J. Infect. Dis. 153, 478-488.

Michelson, S., Alcami, J., Kim, S. J., Danielpour, D., Bachelerie, F., Picard, L., Paya, C., and Virelizier, J. L.(1994). Human cytomegalovirus infection induces transcription and secretion of transforming growth factor beta 1. J. Virol. 68, 5730-5737.

Miles, P. R., Baughman, R. P., and Linnemann, C. C. J.(1990). Cytomegalovirus in the bronchoalveolar lavage fluid of patients with AIDS. Chest 97, 1072-1076.

Millar, A. B., Patou, G., Miller, R. F., Grundy, J. E., Katz, D. R., Weller, I. V., and Semple, S. J.(1990). Cytomegalovirus in the lungs of patients with AIDS. Respiratory pathogen or passenger? Am. Rev. Respir. Dis. 141, 1474-1477.

Miller, G. T., Hochman, P. S., Meier, W., Tizard, R., Bixler, S. A., and Rosa, M. D.(1993). Specific interaction of lymphocyte function-associated antigen 3 with CD2 can inhibit T cell responses. J. Exp. Med. 178, 211-222.

Miller, W., Flynn, P., McCullough, J., Balfour, H. H. J., Goldman, A., Haake, R., McGlave, P., Ramsay, N., and Kersey, J.(1986). Cytomegalovirus infection after bone marrow transplantation: an association with acute graft-v-host disease. Blood 67, 1162-1167.

Moingeon, P., Chang, H. C., Wallner, B. P., Stebbins, C., Frey, A. Z., and Reinherz, E. L.(1989). CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. Nature 339, 312-314.

Monti, G., Magnan, A., Fattal, M., Rain, B., Humbert, M., Mege, J., Noirclerc, M., Dartevelle, P., Cerrina, J., Simmoneau, G., Galanaud, P., and Emile, D.(1996). Intrapulmonary production of RANTES during rejection and CMV pneumonitis after lung transplantation. Transplantation 61, 1757-1762.

Moretta, L., Ciccone, E., Mingari, M. C., Biassoni, R., and Moretta, A.(1994). Human natural killer cells: origin, clonality, specificity, and receptors. Adv. Immunol. 55, 341-380.

Moses, A. V. and Garnett, H. M.(1990). The effect of human cytomegalovirus on the production and biologic action of interleukin-1. J. Infect. Dis. 162, 381-388.

Mouthon, I., Kaveri, S. V., Spalter, S. H., Lacroix-Desmazes, S., Lefranc, C., Desai, R., and Kazatchkine, M. D.(1996). Mechanisms of action of intravenous immune globulin in immune-mediated diseases. Clin. Exp. Immunol. 104 Suppl. 1, 3-9.

Mukaida, N., Mahe, Y., and Matsushima, K.(1990). Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. J. Biol. Chem. 265, 21128-21133.

Muller, C. A., Hebart, H., Roos, A., Roos, H., Steidle, M., and Einsele, H.(1995). Correlation of interstitial pneumonia with human cytomegalovirus-induced lung infection and graft-versus-host disease after bone marrow transplantation. Med. Microbiol. Immunol. 184, 115-121.

Muller, W. A., Weigl, S. A., Deng, X., and Phillips, D. M.(1993). PECAM-1 is required for transendothelial migration of leukocytes. J. Exp. Med. 178, 449-456.

Murayama, T., Kuno, K., Jisaki, F., Obuchi, M., Sakamuro, D., Furukawa, T., Mukaida, N., and Matsushima, K.(1994). Enhancement of human cytomegalovirus replication in a human lung fibroblast cell line by interleukin-8. J. Virol. 68, 7582-7585.

Murphy, P. M. and Tiffany, H. L.(1991). Cloning of a complementary DNA encoding a functional interleukin-8 receptor. Science 253, 1280-1283.

Nakano, T., Ito, M., Mizuno, T., Mizutani, K., Ihara, T., Kamiya, H., and Sakurai, M.(1993). Increase of interleukin 2 receptor and CD45RO antigen on lymphocytes cultured with human cytomegalovirus. Cell. Immunol. 147, 73-80.

Natuk, R. J. and Welsh, R. M.(1987). Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. J. Immunol. 138, 877-883.

Navarro, D., Paz, P., Tugizov, S., Topp, K., La Vail, J., and Pereira, L.(1993). Glycoprotein B of human cytomegalovirus promotes virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells. Virology 197, 143-158.

Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J., and Ploegh, H. L.(1990). The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. Cell 61, 171-183.

Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J.(1993). Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell 72, 415-425.

Neote, K., Mak, J. Y., Kolakowski, L. F.,Jr., and Schall, T. J.(1994). Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. Blood 84, 44-52.

Ng-Bautista, C. L. and Sedmak, D. D.(1995). Cytomegalovirus infection is associated with absence of alveolar epithelial cell HLA class II antigen expression. J. Infect. Dis. 171, 39-44.

Nieto, M., Angel del Pozo, M., and Sanchez-Madrid, F.(1996). Interleukin-15 induces adhesion receptor redistribution in T lymphocytes. Eur. J. Immunol. 26, 1302-1307.

Nowlin, D. M., Cooper, N. R., and Compton, T.(1991). Expression of a human cytomegalovirus receptor correlates with infectibility of cells. J. Virol. 65, 3114-3121.

O'Grady, J. G., Alexander, G. J., Sutherland, S., Donaldson, P. T., Harvey, F., Portmann, B., Calne, R. Y., and Williams, R.(1988). Cytomegalovirus infection and donor/recipient HLA antigens: interdependent co-factors in pathogenesis of vanishing bile-duct syndrome after liver transplantation. Lancet 2, 302-305.

Oakes, J. E., Monteiro, C. A., Cubitt, C. L., and Lausch, R. N.(1993). Induction of interleukin-8 gene expression is associated with herpes simplex virus infection of human corneal keratocytes but not human corneal epithelial cells. J. Virol. 67, 4777-4784.

Oberg, B.(1989). Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmacol. Ther. 40, 213-285.

Oh, C. S., Stratta, R. J., Fox, B. C., Sollinger, H. W., Belzer, F. O., and Maki, D. G.(1988). Increased infections associated with the use of OKT3 for treatment of steroid-resistant rejection in renal transplantation. Transplantation 45, 68-73.

Okada, M., Kitahara, M., Kishimoto, S., Matsuda, T., Hirano, T., and Kishimoto, T.(1988). IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J. Immunol. 141, 1543-1549.

Okamoto, S., Mukaida, N., Yasumoto, K., Horiguchi, H., and Matsushima, K.(1993). The Chemokines Plenum Press, New York. 87-97.

Oliveira, I. C., Sciavolino, P. J., Lee, T. H., and Vilcek, J.(1992). Downregulation of interleukin 8 gene expression in human fibroblasts: unique mechanism of transcriptional inhibition by interferon. Proc. Natl. Acad. Sci. U. S. A. 89, 9049-9053.

Olver, S. D., Price, P., and Shellam, G. R.(1994). Cytomegalovirus hepatitis: characterisation of the inflammatory infiltrate in resistant and susceptable mice. Clin. Exp. Immunol. 98, 375-381.

Openshaw, P. J.(1995). Immunity and immunopathology to respiratory syncytial virus. The mouse model. . Am. J. Resp. Crit. Care Med. 152, S59-62.

Oppenheimer-Marks, N., Davis, L. S., Bogue, D. T., Ramberg, J., and Lipsky, P. E.(1991). Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J. Immunol. 147, 2913-2921.

Osborn, L., Day, E. S., Miller, G. T., Karpusas, M., Tizard, R., Meuer, S. C., Hochman, and PS.(1995). Amino acid residues required for binding of lymphocyte function-associated antigen 3 (CD58) to its counter-receptor CD2. J. Exp. Med. 181, 429-434.

Ouwehand, A. J., Balk, A. H., Baan, C. C., Vaessen, L. M., Jutte, N. H., Bos, E., ClaasFH., and Weimar, W.(1994). Cytomegalovirus infection and allospecific cytotoxic activity of graft-infiltrating cells after heart transplantation. J. Med. Virol. 42, 175-181.

Palacios, R. and Martinez-Maza, O.(1982). Is the E receptor on human T lymphocytes a 'negative signal receptor'? J. Immunol. 12, 2479-2485.

Pande, H., Terramani, T., Tressel, T., Churchill, M. A., Hawkins, G. G., and Zaia, J. A.(1990). Altered expression of fibronectin gene in cells infected with human cytomegalovirus. J. Virol. 64, 1366-1369.

Pardi, R., Inverardi, L., and Bender, J. R.(1992). Regulatory mechanisms in leukocyte adhesion: flexible receptors for sophisticated travelers . . Immunol. Today 13, 224-230.

Pari, G. S., Field, A. K., and Smith, J. A.(1995). Potent antiviral activity of an antisense oligonucleotide complementary to the intron-exon boundary of human cytomegalovirus genes UL36 and UL37. Antimicrob. Agents Chemother. 39, 1157-1161.

Pari, G. S. and Anders, D. G.(1993). Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. J. Virol. 67, 6979-6988.

Parry, G. J.(1988). Peripheral neuropathies associated with human immunodeficiency virus infection. Ann. Neurol. 23 Suppl, S49-S53.

Patel, J. A., Kunimoto, M., Sim, T. C., Garofalo, R., Eliott, T., Baron, S., Ruuskanen, O., Chonmaitree, T., Ogra, P. L., and Schmalstieg, F.(1995). Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. Am. J. Resp. Cell Molec. Biol. 13, 602-609.

Paul, S. and Dummer, S.(1992). Topics in clinical pharmacology: ganciclovir. Am. J Med. Sci. 304, 272-277.

Paxton, W. A., Martin, S. R., Tse, D., O'Brien, T. R., Skurnick, J., VanDevanter, N. L., Padian, N., Braun, J. F., Kotler, D. P., Wolinsky, S. M., and Koup, R. A.(1996). Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nature Medicine 2, 412-417.

Paya, C. V., Hermans, P. E., Wiesner, R. H., Ludwig, J., Smith, T. F., Rakela, J., and Krom, R. A.(1989). Cytomegalovirus hepatitis in liver transplantation: prospective analysis of 93 consecutive orthotopic liver transplantations. J. Infect. Dis. 160, 752-758.

Paya, C. V., Wiesner, R. H., Hermans, P. E., Larson-Keller, J. J., Ilstrup, D. M., Krom, R. A., Moore, S. B., Ludwig, J., and Smith, T. F.(1992). Lack of association between cytomegalovirus infection, HLA matching and the vanishing bile duct syndrome after liver transplantation. Hepatology. 16, 66-70.

Pertel, P., Hirschtick, R., Phair, J., Chmiel, J., Poggensee, L., and Murphy, R.(1992). Risk of developing cytomegalovirus retinitis in persons infected with the human immunodeficiency virus. J. Acquir. Immune. Defic. Syndr. 5, 1069-1074.

Peter, K. and O'Toole, T. E.(1995). Modulation of cell adhesion by changes in a_L b_2 (LFA-1, CD11a/CD18) cytoplasmic domain/cytoskeleton interaction. J. Exp. Med. 181, 315-326.

Peterson, A. and Seed, B.(1987). Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). Nature 329, 842-846.

Peterson, P. K., Balfour, H. H. J., Marker, S. C., Fryd, D. S., Howard, R. J., and Simmons, R. L.(1980). Cytomegalovirus disease in renal allograft recipients: a prospective study of the clinical features, risk factors and impact on renal transplantation. Medicine Baltimore. 59, 283-300.

Peterson, P. K., Gekker, G., Chao, C. C., Hu, S. X., Edelman, C., Balfour, H. H.,Jr., and Verhoef, J.(1992). Human cytomegalovirus-stimulated peripheral blood mononuclear cells induce HIV-1 replication via a tumor necrosis factor-alpha-mediated mechanism. J. Clin. Invest. 89, 574-580.

Piela-Smith, T. H., Broketa, G., Hand, A., and Korn, J. H.(1992). Regulation of ICAM-1 expression and function in human dermal fibroblasts by IL-4. J. Immunol. 146, 1375-1381.

Pietschmann, P., Cush, J. J., Lipsky, P. E., and Oppenheimer-Marks, N.(1992). Identification of subsets of human T cells capable of enhanced transendothelial migration. J. Immunol. 149, 1170-1178.

Pitzalis, C., Kingsley, G., Haskard, D., and Panayi, G.(1988). The preferential accumulation of helper-inducer T lymphocytes in inflammatory lesions: evidence for regulation by selective endothelial and homotypic adhesion. Eur. J. Immunol. 18, 1397-1404.

Plotkin, S. A., Starr, S. E., Friedman, H. M., Brayman, K., Harris, S., Jackson, S., Tustin, N. B., Grossman, R., Dafoe, D., and Barker, C.(1991). Effect of Towne live virus vaccine on cytomegalovirus disease after renal transplant. A controlled trial . Ann. Intern. Med. 114, 525-531.

Portela, D., Patel, R., Larson-Keller, J. J., Ilstrup, D. M., Wiesner, R. H., Steers, JL, Krom, R. A., and Paya, C. V.(1995). OKT3 treatment for allograft rejection is a risk factor for cytomegalovirus disease in liver transplantation. J. Infect. Dis. 171, 1014-1018.

Power, C. A., Meyer, A., Nemeth, K., Proudfoot, A. E., and Wells, T. N.(1995). Molecular cloning and functional expression of a novel CC chemokine receptor. J. Biol. Chem. 270, 19495-19501.

Prop, J., Wildevuur, C. R., and Nieuwenhuis, P.(1989). Acute graft-versus-host disease after lung transplantation. Transplant Proc. 21, 2603-2604.

Qin, S., LaRosa, G., Campbell, J. J., Smith-Heath, H., Kassam, N., Shi, X., Zeng, L, Buthcher, E. C., and Mackay, C. R.(1996). Expression of monocyte chemoattractant protein-1 and interleukin-8 receptors on subsets of T cells: correlation with transendothelial chemotactic potential. Eur. J. Immunol. 26, 640-647.

Quinnan, G. V., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Santos, G. W., Saral, R., and Burns, W. H.(1982). Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T- lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone marrow transplant recipients. N. Engl. J. Med. 307, 7-13.

Rapp, M., Messerle, M., Lucin, P., and Koszinowski, U. H.(1993). Multidisciplinary approach to understanding cytomegalovirus disease. (Michelson, S. and Plotkin, S. A. Eds.) Elsevier Science Publishers, Amsterdam. 327-332.

Rasmussen, L., Kelsall, D., Nelson, R., Carney, W., Hirsch, M., Winston, D., Preiksaitis, J., and Merigan, T. C.(1982). Virus-specific IgG and IgM antibodies in normal and immunocompromised subjects infected with cytomegalovirus. J. Infect. Dis. 145, 191-199.

Rasmussen, L.(1994). Deficiency in antibody response to human cytomegalovirus glycoprotein gH in human immunodeficiency virus-infected patients at risk for cytomegalovirus retinitis. J. Infect. Dis. 170, 673-677.

Razvi, E. S. and Welsh, R. M.(1993). Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency. J. Virol. 67, 5754-5765.

Reddehase, M. J., Keil, G. M., and Koszinowski, U. H.(1984). The cytolytic T lymphocyte response to the murine cytomegalovirus. II. Detection of virus replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur. J. Immunol. 14, 56-61.

Reddehase, M. J., Jonjic, S., Weiland, F., Mutter, W., and Koszinowski, U. H.(1988). Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors. J. Virol. 62, 1061-1065.

Reddehase, M. J. and Koszinowski, U. H.(1984). Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature 312, 369-371.

Reddehase, M. J. and Lucin, P.(1993). Multidisciplinary approach to understanding cytomagalovirus disease. (Michelson, S. and Plotkin, S. A. Eds.) Elsevier Science Publishers, Amsterdam. 129-138.

Reed, E. C., Bowden, R. A., Dandliker, P. S., Lilleby, K. E., and Meyers, J. D.(1988). Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. Ann. Intern. Med. 109, 783-788.

Reid, R., Mar, E. C., Huang, E. S., and Topal, M. D.(1988). Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human alpha and human beta polymerases. A unique inhibition mechanism for 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. J. Biol. Chem. 263, 3898-3904.

Reilly, P. L., Woska, J. R., Jeanfavre, D. D., McNally, E., Rothlein, R., and Bormann, B. J.(1995). The native structure of ICAM-1 is a dimer: correlation with binding to LFA-1. J. Immunol. 155, 529-532.

Reinke, P., Fietze, E., Docke, W. D., Kern, F., Ewert, R., and Volk, H. D.(1994a). Late acute rejection in long-term renal allograft recipients. Diagnostic and predictive value of circulating activated T cells. Transplantation 58, 35-41.

Reinke, P., Fietze, E., Ode-Hakim, S., Prosch, S., Lippert, J., Ewert, R., and VolkHD.(1994b). Late-acute renal allograft rejection and symptomless cytomegalovirus infection. Lancet 344, 1737-1738.

Reinke, P., Fietze, E., Ode-Hakim, S., Prosch, S., Lippert, J., Ewert, R., and VolkHD.(1994c). Late-acute renal allograft rejection and symptomless cytomegalovirus infection. Lancet 344, 1737-1738.

Reipert, B., Scheuch, C., Lukowsky, A., Reinke, P., Fietze, E., Docke, W. D., Czerlinksi, S., Hetzer, R., and Volk, H. D.(1992). CD3+ CD57+ lymphocytes are not likely to be involved in antigen-specific rejection processes in long-term allograft recipients. Clin. Exp. Immunol. 89, 143-147.

Rene, E., Marche, C., Regnier, B., Saimot, A. G., Vilde, J. L., Perrone, C., Michon, C., Wolf, M., Chevalier, T., Vallot, T., and et-al(1989). Intestinal infections in patients with acquired immunodeficiency syndrome. A prospective study in 132 patients. Dig. Dis. Sci. 34, 773-780.

Reusser, P., Riddell, S. R., Meyers, J. D., and Greenberg, P. D.(1991). Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 78, 1373-1380.

Rice, G. P., Schrier, R. D., and Oldstone, M. B.(1984). Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. Proc. Natl. Acad. Sci. U. S. A. 81, 6134-6138.

Richardson, W. P., Colvin, R. B., Cheeseman, S. H., Tolkoff-Rubin, N. E., Herrin, J. T., Cosimi, A. B., Collins, A. B., Hirsch, M. S., McCluskey, R. T., Russell, P. S., and Rubin, R. H.(1981). Glomerulopathy associated with cytomegalovirus viremia in renal allografts. N. Engl. J. Med. 305, 57-63.

Riddell, S. R., Rabin, M., Geballe, A. P., Britt, W. J., and Greenberg, P. D.(1991). Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J. Immunol. 146, 2795-2804.

Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E., and Greenberg, P. D.(1992). Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones . Science. 257, 238-241.

Riddell, S. R. and Greenberg, P. D.(1990). The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J. Immunol. Methods 128, 189-201.

Rinaldo, C. R.(1994). Modulation of major histocompatibility complex antigen expression by viral infection. Am. J. Pathol. 144, 637-650.

Ringden, O., Sundberg, B., Markling, L., and Tollemar, J.(1987). Polyclonal antibody secretion during acute graft-versus-host disease. Scand. J. Immunol. 26, 469-476.

Robey, E. and Allison, J. P.(1995). T cell activation: integration of signals from the antigen receptor and costimulatory molecules. Immunol. Today 16, 306-310.

Rodgers, B. C., Scott, D. M., Mundin, J., and Sissons, J. G.(1985). Monocyte-derived inhibitor of interleukin 1 induced by human cytomegalovirus. J. Virol. 55, 527-532.

Roenhorst, H. W., Beelen, J. M., Middeldorp, J. M., Schirm, J., Tegzess, A. M., and The, T. H.(1985). Maintenance of cytomegalovirus (CMV) latency and host immune responses of long term renal allograft survivors. II. Secondary CMV infections associated with impaired in vitro proliferative responses to mitogens, allogeneic lymphocytes and CMV infected cells. Clin. Exp. Immunol. 61, 72-79.

Roizman, B.(1993). The human herpesviruses. (Roizman, B. N., Whitley, R. J., and Lopez, C. Eds.) Raven Press, New York. 1-9.

Rook, A. H.(1988). Interactions of cytomegalovirus with the human immune system. Rev. Infect. Dis. 10 Suppl 3, S460-S467.

Rot, A.(1992). Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. Immunol. Today 13, 291-294.

Rot, A., Hub, E., Middleton, J., Pons, F., Rabeck, C., Thierer, K., Wintle, J., Wolff, B., Zsak, M., and Dukor, P.(1996). Some aspects of IL-8 pathophysiology. III: Chemokine interaction with endothelial cells. . J. Leukoc. Biol. 59, 39-44.

Roth, S. J., Carr, M. W., and Springer, T. A.(1995). C-C chemokines, but not the C-X-C chemokines IL-8 and IP-10, stimulate transendothelial chemotaxis of T lymphocytes. Eur. J. Immunol. 25, 3482-3488.

Rothlein, R., Czajkowski, M., O'Neill, M. M., Marlin, S. D., Mainolfi, E., and MerluzziVJ.(1988). Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. J. Immunol. 141, 1665-1669.

Rothlein, R. and Springer, T. A.(1986). The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. 163, 1132-1149.

Rousset, F., Billaud, M., Blanchard, D., Figdor, C., Lenoir, G. M., Spits, H., and De Vries, J. E.(1989). IL-4 induces LFA-1 and LFA-3 expression on Burkitt's lymphoma cell lines. J. Immunol. 143, 1490-1498.

Rubin, R. H., Tolkoff-Rubin, N. E., Oliver, D., Rota, T. R., Hamilton, J., Betts, R. F., Pass, R. F., Hillis, W., Szmuness, W., Farrell, M. L., and et-al(1985). Multicenter seroepidemiologic study of the impact of cytomegalovirus infection on renal transplantation. Transplantation 40, 243-249.

Rubin, R. H.(1990). Impact of cytomegalovirus infection on organ transplant recipients. Rev. Infect. Dis. 12 Suppl 7, S754-S766.

Sakuma, S., Furukawa, T., and Plotkin, S. A.(1977). The characterization of IgG receptor induced by human cytomegalovirus. Proc. Soc. Exp. Biol. Med. 155, 168-172.

Salmon, M., Pilling, D., Borthwick, N. J., Viner, N., Janossy, G., Bacon, P. A., and Akbar, A. N.(1994). The progressive differentiation of primed T cells is associated with an increasing susceptability to apoptosis. Eur. J. Immunol. 24, 892-899.

Saltzman, R. L., Quirk, M. R., and Jordan, M. C.(1988). Disseminated cytomegalovirus infection. Molecular analysis of virus and leukocyte interactions in viremia. J. Clin. Invest. 81, 75-81.

Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M.(1996). Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry 35, 3362-3367.

Sanchez-Madrid, F., Krensky, A. M., Ware, C. F., Robbins, E., Strominger, J. L., Burakoff, S. J., and Springer, T. A.(1982). Three distinct antigens associated with human T lymphocyte mediated cytolysis; LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. U. S. A. 79, 7489-7493.

Sanders, M. E., Makgoba, M. W., Sharrow, S. O., Stephany, D., Springer, T. A., YoungHA., and Shaw, S.(1988). Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J. Immunol. 140, 1401-1407.

Sansom, D. M., Wilson, A., Boshell, M., Lewis, J., and Hall, N. D.(1993). B7/CD28 but not LFA-3/CD2 interactions can provide 'third-party' co-stimulation for human T-cell activation. Immunology 80, 242-247.

Sayers, M. H., Anderson, K. C., Goodnough, L. T., Kurtz, S. R., Lane, T. A., Pisciotto, P., and Silberstein, L. E.(1992). Reducing the risk for transfusion-transmitted cytomegalovirus infection. Ann. Intern. Med. 116, 55-62.

Schall, T. J., Bacon, K., Toy, K. J., and Goeddel, D. V.(1990). Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347, 669-671.

Schall, T. J., Bacon, K., Camp, R. D., Kaspari, J. W., and Goeddel, D. V.(1993). Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta chemokines attract distinct populations of lymphocytes. J. Exp. Med. 177, 1821-1826.

Schmidt, G. M., Kovacs, A., Zaia, J. A., Horak, D. A., Blume, K. G., Nademanee, A. P., O'Donnell, M. R., Snyder, D. S., and Forman, S. J.(1988). Ganciclovir/immunoglobulin combination therapy for the treatment of human cytomegalovirus-associated interstitial pneumonia in bone marrow allograft recipients. Transplantation 46, 905-907.

Schmidt, G. M., Horak, D. A., Niland, J. C., Duncan, S. R., Forman, S. J., and Zaia, J. A.(1991). A randomized, controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants; The City of Hope-Stanford-Syntex CMV Study Group . N. Engl. J. Med. 324, 1005-1011.

Schrier, R. D., Rice, G. P., and Oldstone, M. B.(1986). Suppression of natural killer cell activity and T cell proliferation by fresh isolates of human cytomegalovirus. J. Infect. Dis. 153, 1084-1091.

Schrier, R. D. and Oldstone, M. B.(1986a). Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. J. Virol. 59, 127-131.

Schrier, R. D. and Oldstone, M. B.(1986b). Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. J. Virol. 59, 127-131.

Schulz, M., Schuurman, H. J., Joergensen, J., Steiner, C., Meerloo, T., Kagi, D., Hengartner, H., Zinkernagel, R. M., Schreier, M. H., Burki, K., and et al.(1995). Acute rejection of vascular heart allografts by perforin-deficient mice. Eur. J. Immunol. 25, 474-480.

Sebok, K., Woodside, D., al-Aoukaty, A., Ho, A. D., Gluck, S., and Maghazachi, A. A.(1993). IL-8 induces the locomotion of human IL-2 activated natural killer cells. J. Immunol. 150, 1524-1534.

Sedmak, D. D., Roberts, W. H., Stephens, R. E., Buesching, W. J., Morgan, L. A., Davis, D. H., and Waldman, W. J.(1990). Inability of cytomegalovirus infection of cultured endothelial cells to induce HLA class II antigen expression. Transplantation 49, 458-462.

Sedmak, D. D., Guglielmo, A. M., Knight, D. A., Birmingham, D. J., Huang, E. H., and WaldmanWJ.(1994a). Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells . Am. J. Pathol. 144, 683-692.

Sedmak, D. D., Knight, D. A., Vook, N. C., and Waldman, W. J.(1994b). Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. Transplantation 58, 1379-1385.

Seed, B.(1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature 329, 840-842.

Selvaraj, P., Plunkett, M. L., Dustin, M., Sanders, M. E., Shaw, S., and Springer, T. A.(1987). The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. Nature 326, 400-403.

Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W.(1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science 261, 609-612.

Shanley, J. D., Jordan, M. C., and Stevens, J. G.(1981). Modification by adoptive humoral immunity of murine cytomegalovirus infection. J. Infect. Dis. 143, 231-237.

Shanley, J. D., Via, C. S., Sharrow, S. O., and Shearer, G. M.(1987). Interstitial pneumonitis during murine cytomegalovirus infection and graft-versus-host reaction. Characterization of bronchoalveolar lavage cells. Transplantation 44, 658-662.

Sharples, L. D., Tamm, M., McNeil, K., Higenbottam, T. W., Stewart, S., and Wallwork, J.(1996). Development of bronchiolitis obliterans syndrome in recipients of heart-lung transplantation--early risk factors. Transplantation 61, 560-566.

Shaw, S., Luce, G. E., Quinones, R., Gress, R. E., Springer, T. A., and Sanders, M. E.(1986). Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature 323, 262-264.

Shelbourn, S. L., Kothari, S. K., Sissons, J. G., and Sinclair, J. H.(1989). Repression of human cytomegalovirus gene expression associated with a novel immediate early regulatory region binding factor. Nucleic. Acids. Res. 17, 9165-9171.

Shen, J., Devery, J. M., and King, N. J.(1995). Early induction of interferon-independent virus-specific ICAM-1 (CD54) expression by flavivirus in quiescent but not proliferating fibroblasts--implications for virus-host interactions. Virology 208, 437-449.

Shepp, D. H., Dandliker, P. S., de-Miranda, P., Burnette, T. C., Cederberg, D. M., Kirk, L. E., and Meyers, J. D.(1985). Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine in the treatment of cytomegalovirus pneumonia. Ann. Intern. Med. 103, 368-373.

Sheth, R., Anderson, J., Sato, T., Oh, B., Hempson, S. J., Rollo, E., Mackow, E. R., and Shaw, R. D.(1996). Rotavirus stimulates IL-8 secretion from cultured epithelial cells. Virology 221, 251-259.

Shi-Wen, X., Panesar, M., Vancheeswaran, R., Mason, J., Haskard, D., Black, C., Olsen, I., and Abraham, D.(1994). Expression and shedding of intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3 by normal and scleroderma fibroblasts. Effects of interferon-gamma, tumor necrosis factor alpha, and estrogen. Arthritis Rheum. 37, 1689-1697.

Siliciano, R. F., Pratt, J. C., Schmidt, R. E., Ritz, J., and Reinherz, E. L.(1985). Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature 317, 428-430.

Simmons, D., Makgoba, M. W., and Seed, B.(1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature 331, 624-627.

Sinclair, J. H., Baillie, J., Bryant, L. A., Taylor-Wiedeman, J. A., and Sissons, J. G.(1992). Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line. J. Gen. Virol. 73, 433-435.

Singh, N., Dummer, J. S., Kusne, S., Breinig, M. K., Armstrong, J. A., Makowka, L., Starzl, T. E., and Ho, M.(1988). Infections with cytomegalovirus and other herpesviruses in 121 liver transplant recipients: transmission by donated organ and the effect of OKT3 antibodies. J. Infect. Dis. 158, 124-131.

Sinzger, C., Grefte, A., Plachter, B., Gouw, A. S. H., The, T. H., and Jahn, G.(1995). Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J. Gen. Virol. 76, 741-750.

Sinzger, C., Plachter, B., Grefte, A., The, T. H., and Jahn, G.(1996). Tissue macrophages are infected by human cytomegalovirus in vivo. J. Infect. Dis. 173, 240-245.

Slavin, M. A., Gleaves, C. A., Schoch, H. G., and Bowden, R. A.(1992). Quantification of cytomegalovirus in bronchoalveolar lavage fluid after allogeneic marrow transplantation by centrifugation culture. J. Clin. Microbiol. 30, 2776-2779.

Smiley, M. L., Wlodaver, C. G., Grossman, R. A., Barker, C. F., Perloff, L. J., Tustin, N. B., Starr, S. E., Plotkin, S. A., and Friedman, H. M.(1985). The role of pretransplant immunity in protection from cytomegalovirus disease following renal transplantation. Transplantation 40, 157-161.

Smith, G. L.(1996). Virus proteins that bind cytokines, chemokines or interferons. Curr. Opin. Immunol. 8, 467-471.

Smith, P. D., Saini, S. S., Raffeld, M., Manischewitz, J. F., and Wahl, S. M.(1992). Cytomegalovirus induction of tumor necrosis factor-alpha by human monocytes and mucosal macrophages. J. Clin. Invest. 90, 1642-1648.

Smith, S. H., Brown, M. H., Rowe, D., Callard, R. E., and Beverley, P. C. L.(1986). Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. Immunology 58, 63-70.

Smyth, R. L., Scott, J. P., Higenbottam, T. W., Wreghitt, T. G., Stewart, S., Fradet, G., Clelland, C. A., and Wallwork, J.(1990). Experience of cytomegalovirus infection in heart-lung transplant recipients. Transplant Proc. 22, 1820-1821.

Smyth, R. L., Scott, J. P., Borysiewicz, L. K., Sharples, L. D., Stewart, S., WreghittTG., Gray, J. J., Higenbottam, T. W., and Wallwork, J.(1991). Cytomegalovirus infection in heart-lung

transplant recipients: risk factors, clinical associations, and response to treatment. J. Infect. Dis. 164, 1045-1050.

Smyth, R. L., Higenbottam, T. W., Scott, J. P., and Wallwork, J.(1993). Molecular aspects of human cytomegalovirus diseases. (Becker, Y., Darai, G., and Huang, E.-S. Eds.) Springer-Verlag, Berlin. 205-221.

Snydman, D. R., Werner, B. G., Heinze-Lacey, B., Berardi, V. P., Tilney, N. L., Kirkman, R. L., Milford, E. L., Cho, S. I., Bush, H. L. J., Levey, A. S., and et-al(1987). Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. N. Engl. J. Med. 317, 1049-1054.

Snydman, D. R.(1990). Cytomegalovirus immunoglobulins in the prevention and treatment of cytomegalovirus disease. Rev. Infect. Dis. 12 Suppl 7, S839-S848.

Snydman, D. R., Werner, B. G., Dougherty, N. N., Griffith, J., Rubin, R. H., DienstagJL., Rohrer, R. H., Freeman, R., Jenkins, R., Lewis, W. D., and et al.(1993). Cytomegalovirus immune globulin prophylaxis in liver transplantation. A randomized, double-blind, placebo-controlled trial. The Boston Center for Liver Transplantation CMVIG Study Group. Ann. Intern. Med. 119, 984-991.

Soderberg, C., Larsson, S., Bergstedt-Lindqvist, S., and Moller, E.(1993). Definition of a subset of human peripheral blood mononuclear cells that are permissive to human cytomegalovirus infection. J. Virol. 67, 3166-3175.

Somersalo, K., Tarkkanen, J., Patarroyo, M., and Saksela, E.(1992). Involvement of b_2-integrins in the migration of human natural killer cells. J. Immunol. 149, 590-598.

Somoza, C., Driscoll, P. C., Cyster, J. G., and Williams, A. F.(1993). Mutational analysis of the CD2/CD58 interaction: the binding site for CD58 lies on one face of the first domain of human CD2. J. Exp. Med. 178, 549-558.

Spaete, R. R., Gehrz, R. C., and Landini, M. P.(1994). Human cytomegalovirus structural proteins. . J. Gen. Virol. 75, 3287-3308.

Span, A. H., van-Boven, C. P., and Bruggeman, C. A.(1989). The effect of cytomegalovirus infection on the adherence of polymorphonuclear leucocytes to endothelial cells. Eur. J. Clin. Invest. 19, 542-548.

Span, A. H., Mullers, W., Miltenburg, A. M., and Bruggeman, C. A.(1991). Cytomegalovirus induced PMN adherence in relation to an ELAM-1 antigen present on infected endothelial cell monolayers. Immunology 72, 355-360.

Spector, S. A., Hirata, K. K., and Newman, T. R.(1984). Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome. J. Infect. Dis. 150, 953-956.

Springer, T. A.(1990). Adhesion receptors of the immune system. . Nature 346, 425-434.

Springer, T. A.(1995). Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. . Ann. Rev. Physiol. 57, 827-872.

St.Jeor, S., Admirand, J., Bruening, E., and Riolo, J.(1993). Multidisciplinary approach to understanding cytomegalovirus disease (Michelson, S. and Plotkin, S. A. Eds.) Elsevier Science Publishers B.V. Amsterdam. 123-126.

Stagno, S., Pass, R. F., Cloud, G., Britt, W. J., Henderson, R. E., Walton, P. D., Veren, D. A., Page, F., and Alford, C. A.(1986). Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. JAMA 256, 1904-1908.

Stals, F. S., Bosman, F., van-Boven, C. P., and Bruggeman, C. A.(1990). An animal model for therapeutic intervention studies of CMV infection in the immunocompromised host. Arch. Virol. 114, 91-107.

Staunton, D. E., Dustin, M. L., and Springer, T. A.(1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature 339, 61-64.

Staunton, D. E., Dustin, M. L., Erickson, H. P., and Springer, T. A.(1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus Cell 61, 243-254.

Steinhoff, G., Wonigeit, K., and Pichlmayr, R.(1988). Analysis of sequential changes in major histocompatibility complex expression in human liver grafts after transplantation. Transplantation 45, 394-401.

Steinhoff, G., Behrend, M., Schrader, B., and Pichlmayr, R.(1993). Intercellular immune adhesion molecules in human liver transplants: overview on expression patterns of leukocyte receptor and ligand molecules. Hepatology 18, 440-453.

Steinhoff, G., You, X. M., Steinmuller, C., Bauer, D., Lohmann-Matthes, M. L., Bruggeman, C. A., and Haverich, A.(1996). Enhancement of cytomegalovirus infection and acute rejection after allogeneic lung transplantation in the rat. Transplantation 61, 1250-1260.

Steinmassl, M. and Hamprecht, K.(1994). Double fluorescence analysis of human cytomegalovirus (HCMV) infected human fibroblast cultures by flow cytometry: increase of class I MHC expression on uninfected cells and decrease on infected cells. Arch. Virol. 135, 75-87.

Stenberg, R. M., Fortney, J., Barlow, S. W., Magrane, B. P., Nelson, J. A., and Ghazal, P.(1990). Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. 64, 1556-1565.

Stewart, M. P., Cabanas, C., and Hogg, N.(1996). T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. J. Immunol. 156, 1810-1817.

Stratta, R. J., Shaefer, M. S., Markin, R. S., Wood, R. P., Kennedy, E. M., Langnas, A. N., Reed, E. C., Woods, G. L., Donovan, J. P., Pillen, T. J., and et-al(1989). Clinical patterns of cytomegalovirus disease after liver transplantation. Arch. Surg. 124, 1443-9; discuss.

Strieter, R. M., Phan, S. H., Showell, H. J., Remick, D. G., Lynch, J. P., Genord, M., Raiford, C., Eskandari, M., Marks, R. M., and Kunkel, S. L.(1989a). Monokine-induced neutrophil chemotactic factor gene expression in human fibroblasts. J. Biol. Chem. 264, 10621-10626.

Strieter, R. M., Wiggins, R., Phan, S. H., Wharram, B. L., Showell, H. J., Remick, D. G., Chensue, S. W., and Kunkel, S. L.(1989b). Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. Biochem. Biophys. Res. Commun. 162, 694-700.

Strieter, R. M., Kasahara, K., Allen, R. M., Standiford, T. J., Rolfe, M. W., BeckerFS., Chensue, S. W., and Kunkel, S. L.(1992). Cytokine-induced neutrophil-derived interleukin-8. Am. J. Pathol. 141, 397-407.

Strieter, R. M., Koch, A. E., Antony, V. B., Fick, R. B., Standiford, T. J., and Kunkel, S. L.(1994). The immunopathology of chemotactic cytokines: The role of interleukin-8 and monocyte chemoattractant protein-1. Journal of Laboratory & Clinical Medicine 123, 183-197.

Su, H. C., Ishikawa, R., and Biron, C. A.(1993). Transforming growth factor beta expression and natural killer cell responses during virus infection of normal, nude, and SCID mice. J. Immunol. 151, 4874-4890.

Su, Y. H., Yan, X. T., Oakes, J. E., and Lausch, R. N.(1996). Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection. J. Virol. 70, 1277-1281.

Sugamura, K. and Hinuma, Y.(1993). The Retroviridae (Levy, J. A. Ed.) Plenum Press, New York. 399-435.

Sullivan, K. M.(1996). Immunomodulation in allogeneic marrow transplantation: use of intravenous immune globulin to suppress acute graft-versus-host disease. . Clin. Exp. Immunol. 104 Suppl 1, 43-48.

Sullivan, K. M. and Parkman, R.(1983). The pathophysiology and treatment of graft-versus-host disease. Clin. Haematol. 12, 775-789.

Sullivan, V., Talarico, C. L., Stanat, S. C., Davis, M., Coen, D. M., and Biron, K. K.(1992). A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature. 359, 85

Tanaka, K., Koga, Y., Lu, Y. Y., Zhang, X. Y., Wang, Y., Kimura, G., and Nomoto, K.(1994). Murine cytomegalovirus-associated pneumonitis in the lungs free of the virus. J. Clin. Invest. 94, 1019-1025.

Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S.(1993a). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta . Nature 361, 79-82.

Tanaka, Y., Adams, D. H., and Shaw, S.(1993b). Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. . Immunol. Today 14, 111-115.

Tanaka, Y., Fukudome, K., Hayashi, M., Takagi, S., and Yoshie, O.(1995). Induction of ICAM-1 and LFA-3 by Tax1 of human T-cell leukemia virus type 1 and mechanism of down-regulation of ICAM-1 or LFA-1 in adult-T-cell-leukemia cell lines. Int. J. Cancer 60, 554-561.

Tarakhovsky, A., Muller, W., and Rajewsky, K.(1994). Lymphocyte populations and immune responses in CD5-deficient mice. Eur. J. Immunol. 24, 1678-1684.

Tatum, E. T., Sun, P. C., and Cohn, D. L.(1989). Cytomegalovirus vasculitis and colon perforation in a patient with the acquired immunodeficiency syndrome. Pathology. 21, 235-238.

Taub, D. D., Lloyd, A. R., Conlon, K., Wang, J. M., Ortaldo, J. R., Harada, A., Matsushima, K., Kelvin, D. J., and Oppenheim, J. J.(1993). Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J. Exp. Med. 177, 1809-1814.

Taub, D. D., Sayers, T. J., Carter, C. R., and Ortaldo, J. R.(1995). Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. J. Immunol. 155, 3877-3888.

Taylor, H. P. and Cooper, N. R.(1990). The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. J. Virol. 64, 2484-2490.

Taylor-Wiedeman, J., Sissons, J. G., Borysiewicz, L. K., and Sinclair, J. H.(1991). Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. J. Gen. Virol. 72, 2059-2064.

Taylor-Wiedeman, J., Hayhurst, G. P., Sissons, J. G., and Sinclair, J. H.(1993). Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. J. Gen. Virol. 74, 265-268.

Testi, R., D'Ambrosio, D., De Maria, R., and Santoni, A.(1994). The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol. Today 15, 479-483.

Tevethia, M. J., Spector, D. J., Leisure, K. M., and Stinski, M. F.(1987). Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology 161, 276-285.

Thale, R., Lucin, P., Schneider, K., Eggers, M., and Koszinowski, U. H.(1994). Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. J. Virol. 68, 7757-7765.

Thale, R., Szepan, U., Hengel, H., Geginat, G., Lucin, P., and Koszinowski, U. H.(1995). Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility complex class I molecule transport. J. Virol. 69, 6098-6105.

The, T. H., van den Berg, A. P., Harmsen, M. C., Van Der Bij, W., and van Son, W. J.(1995). The cytomegalovirus antigenemia assay: A plea for standardization. Scand. J. Infect. Dis. Suppl 99, 25-29.

Tiefenthaler, G., Hunig, T., Dustin, M. L., Springer, T. A., and Meuer, S. C.(1987). Purified lymphocyte function-associated antigen-3 and T11 target structure are active in CD2-mediated T cell stimulation. Eur. J. Immunol. 17, 1847-1850.

Timon, M., Arnaiz-Villena, A., Ruiz-Contreras, J., Ramos-Amador, J. T., PachecoA., and Regueiro, J. R.(1993). Selective impairment of T lymphocyte activation through the T cell receptor/CD3 complex after cytomegalovirus infection. Clin. Exp. Immunol. 94, 38-42.

Tosi, M. F., Stark, J. M., Hamedani, A., Smith, C. W., Gruenert, D. C., and Huang, Y. T.(1992). Intercellular adhesion molecule-1 (ICAM-1)-dependent and ICAM-1-independent adhesive interactions between polymorphonuclear leukocytes and human airway epithelial cells infected with parainfluenza virus type 2. J. Immunol. 149, 3345-3349.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H., Foster, L., and Karre, K.(1989). Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature 340, 443-448.

Tschopp, J. and Hofmann, K.(1996). Cytotoxic T cells: more weapons for new targets? Trends in Microbiology 4, 91-94.

Tuder, R. M., Weinberg, A., Panajotopoulos, N., and Kalil, J.(1991). Cytomegalovirus enhances PBMC binding and HLA class I expression on cultured endothelial cells. Transplant Proc. 23, 91-92.

Tuder, R. M., Weinberg, A., Panajotopoulos, N., and Kalil, J.(1994). Cytomegalovirus infection amplifies class I major histocompatibility complex expression on cultured human endothelial cells. Journal of Heart and Lung Transplantation 13, 129-138.

Tumilowicz, J. J., Gawlik, M. E., Powell, B. B., and Trentin, J. J.(1985). Replication of cytomegalovirus in human arterial smooth muscle cells. J. Virol. 56, 839-845.

Turtinen, L. W., Saltzman, R., Jordan, M. C., and Haase, A. T.(1987). Interactions of human cytomegalovirus with leukocytes in vivo: analysis by in situ hybridization. Microb. Pathog. 3, 287-297.

Ustinov, J., Bruggeman, C., Hayry, P., and Lautenschlager, I.(1994). Cytomegalovirus-induced class II expression in rat kidney. Transplant Proc. 26, 1729

Ustinov, J. A., Loginov, R. J., Mattila, P. M., Nieminen, V. K., Suni, J. I., Hayry, P. J., and Lautenschlager, I. T.(1991). Cytomegalovirus infection of human kidney cells in vitro. Kidney Int. 40, 954-960.

Valitutti, S., Muller, S., Cella, M., Padovan, E., and Lanzavecchia, A.(1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature 375, 148-151.

Van Damme, J., Decock, B., Conings, R., Lenaerts, J. P., Opdenakker, G., and Billiau, A.(1989). The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte-derived interleukin. Eur. J. Immunol. 19, 1189-1194.

van den Berg, A. P., van Son, W. J., Janssen, R. A., Brons, N. H., Heyn, A. A., Postma, S., van der Giessen, M., Tegzess, A. M., de LeijLH., and et al.(1992). Recovery from cytomegalovirus infection is associated with activation of peripheral blood lymphocytes. J. Infect. Dis. 166, 1228-1235.

van den Berg, A. P., Meyaard, L., Otto, S. A., van Son, W. J., Klompmaker, I. J., Mesander, G., de Leij, L. H., Miedema, F., and The, T. H.(1995). Cytomegalovirus infection associated with a decreased proliferative capacity and increased rate of apoptosis of peripheral blood lymphocytes. Transplant Proc. 27, 936-938.

van der Merwe, P. A., McNamee, P. N., Davies, E. A., Barclay, A. N., and Davis, S. J.(1995). Topology of the CD2-CD48 adhesion complex: implications for antigen recognition by T cells. Current Biology 5, 74-84.

van Dorp, W. T., van Wieringen, P. A., Marselis-Jonges, E., Bruggeman, C. A., DahaMR., van Es, L. A., and van der Woude, F.(1993a). Cytomegalovirus directly enhances MHC class I and intercellular adhesion molecule-1 expression on cultured proximal tubular epithelial cells. Transplantation 55, 1367-1371.

van Dorp, W. T., van Wieringen, P. A., Marselis-Jonges, E., Bruggeman, C. A., DahaMR., van Es, L. A., and van der Woude, F.(1993b). Cytomegalovirus directly enhances MHC class I and intercellular adhesion molecule-1 expression on cultured proximal tubular epithelial cells. Transplantation 55, 1367-1371.

van Kooyk, Y., van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T. W., and FigdorCG.(1989). Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature 342, 811-813.

van Kooyk, Y., Weder, P., Hogervorst, F., Verhoeven, A. J., van Seventer, G. A., te Velde, A. A., Borst, J., Keizer, G. D., and Figdor, C. G.(1991). Activation of LFA-1 through a Ca-dependent epitope stimulates lymphocyte adhesion. J. Cell. Biol. 112, 345-354.

van Kooyk, Y., Weder, P., Heije, K., and Figdor, C. K.(1994). Extracellular Ca^{2+} modulates leukocyte function associated antigen-1 cell surface distribution on T lymphocytes and consequently affects cell adhesion. J. Cell Biol. 124, 1061-1070.

van Seventer, G. A., Shimizu, Y., and Shaw, S.(1991). Roles of multiple accessory molecules in T-cell activation. . Curr. Opin. Immunol. 3, 294-303.

van-Dorp, W. T., Jonges, E., Bruggeman, C. A., Daha, M. R., van-Es, L. A., and van-Der-Woude, F. J.(1989). Direct induction of MHC class I, but not class II, expression on endothelial cells by cytomegalovirus infection. Transplantation 48, 469-472.

Vere-hodge, R. A. and Perkins, R. M.(1989). Mode of action of 9-(4-hydroxy-3-hydroxy-methylbut-1-yl)guanine (BRL 39123) against herpes simplex virus in MRC-5 cells. Antimicrob. Agents Chemother. 33, 223-229.

Villareal, D. T., Murphy, W. A., Teitelbaum, S. L., Arens, M. Q., and Whyte, M. P.(1992). Painful diffuse osteosclerosis after intravenous drug abuse. Am. J. Med. 93, 371-381.

Volpes, R., van den Oord, J. J., and Desmet, V. J.(1990). Hepatic expression of intercellular adhesion molecule-1 (ICAM-1) in viral hepatitis B. Hepatology 12, 148-154.

Volpes, R., van den Oord, J. J., Desmet, V. J., and Yap, S. H.(1992). Induction of intercellular adhesion molecule-1 (CD54) on human hepatoma cell line HepG2: influence of cytokines and hepatitis B virus-DNA transfection. Clin. Exp. Immunol. 87, 71-75.

von-Willebrand, E., Pettersson, E., Ahonen, J., and Hayry, P.(1986). CMV infection, class II antigen expression, and human kidney allograft rejection. Transplantation 42, 364-367.

Voraberger, G., Schafer, R., and Stratowa, C.(1991). Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5'-regulatory region. Induction by cytokines and phorbol ester. J. Immunol. 147, 2777-2786.

Waldman, W. J., Adams, P. W., Orosz, C. G., and Sedmak, D. D.(1992). T lymphocyte activation by cytomegalovirus-infected, allogeneic cultured human endothelial cells. Transplantation 54, 887-896.

Waldman, W. J., Knight, D. A., VanBuskirk, A., Adams, P. W., Orosz, C. G., and Sedmak, D. D.(1993). Endothelial HLA class II induction mediated by allogeneic T cells activated by cytomegalovirus-infected cultured endothelial cells. Transplant Proc. 25, 1439-1440.

Waldman, W. J., Knight, D. A., Adams, P. W., Orosz, C. G., and Sedmak, D. D.(1995a). In vitro induction of endothelial adhesion molecule and MHC antigen expression by cytomegalovirus-activated CD4+ T cells. Transplant Proc. 27, 1269-1271.

Waldman, W. J., Knight, D. A., Huang, E. H., and Sedmak, D. D.(1995b). Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: an in vitro model. J. Infect. Dis. 171, 263-272.

Wallner, B. P., Frey, A. Z., Tizard, R., Mattaliano, R. J., Hession, C., Sanders, M. E., and Springer, T. A.(1987). Primary structure of lymphocyte function-associated antigen 3 (LFA-3). The ligand of the T lymphocyte CD2 glycoprotein. J. Exp. Med. 166, 923-932.

Walter, E. A., Greenberg, P. D., Gilbert, M. J., Finch, R. J., Watanabe, K. S., Thomas, ED, and Riddell, S. R.(1995). Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N. Engl. J. Med. 333, 1038-1044.

Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, M., Thompson, C. B., and Bluestone, J. A.(1994). CTLA-4 can function as a negative regulator of T cell activation. Immunity 1, 405-413.

Wang, E. C., Moss, P. A., Frodsham, P., Lehner, P. J., Bell, J. I., and Borysiewicz, L. K.(1995). CD8highCD57+ T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus. J. Immunol. 155, 5046-5056.

Wang, E. C. and Borysiewicz, L. K.(1995). The role of CD8+ve, CD57+ve cells in human cytomegalovirus and other viral infections. Scand. J. Infect. Dis. Suppl. 99, 69-77.

Warren, A. P., Ducroq, D. H., Lehner, P. J., and Borysiewicz, L. K.(1994). Human cytomegalovirus-infected cells have unstable assembly of major histocompatibility complex class I complexes and are resistant to lysis by cytotoxic T lymphocytes. J. Virol. 68, 2822-2829.

Watson, J. V.(1992). Flow cytometry data analysis, Basic concepts and statistics. Cambridge University Press, Cambridge. 101

Webb, D. S., Shimizu, Y., van Seventer, G. A., Shaw, S., and Gerrard, T. L.(1990). LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. Science 249, 1295-1297.

Webster, A., Lee, C. A., Cook, D. G., Grundy, J. E., Emery, V. C., Kernoff, P. B., and Griffiths, P. D.(1989). Cytomegalovirus infection and progression towards AIDS in haemophiliacs with human immunodeficiency virus infection. Lancet 2, 63-66.

Webster, A., Phillips, A. N., Lee, C. A., Janossy, G., Kernoff, P. B., and Griffiths, P. D.(1992). Cytomegalovirus (CMV) infection, CD4+ lymphocyte counts and the development of AIDS in HIV-1-infected haemophiliac patients. Clin. Exp. Immunol. 88, 6-9.

Weinshenker, B. G., Wilton, S., and Rice, G. P.(1988). Phorbol ester-induced differentiation permits productive human cytomegalovirus infection in a monocytic cell line. J. Immunol. 140, 1625-1631.

Weiss, S. J.(1989). Tissue destruction by neutrophils. N. Engl. J. Med. 320, 365-376.

Welch, A. R., McGregor, L. M., and Gibson, W.(1991). Cytomegalovirus homologs of cellular G protein-coupled receptor genes are transcribed. J. Virol. 65, 3915-3918.

Welsh, R. M.(1986). Regulation of virus infections by natural killer cells. A review. . Nat. Immun. Cell Growth Regul. 5, 169-199.

Welsh, R. M., Brubaker, J. O., Vargas-Cortes, M., and O'Donnell, C. L.(1991). Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. J. Exp. Med. 173, 1053-1063.

Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L.(1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell 84, 769-779.

Wiertz, E. J. H. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L.(1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell 84, 769-779.

Wingard, J. R., Chen, D. Y., Burns, W. H., Fuller, D. J., Braine, H. G., Yeager, A. M., Kaiser, H., Burke, P. J., Graham, M. L., Santos, G. W., and et-al(1988a). Cytomegalovirus infection after autologous bone marrow transplantation with comparison to infection after allogeneic bone marrow transplantation. Blood 71, 1432-1437.

Wingard, J. R., Mellits, E. D., Sostrin, M. B., Chen, D. Y., Burns, W. H., Santos, G. W., Vriesendorp, H. M., Beschorner, W. E., and Saral, R.(1988b). Interstitial pneumonitis after allogeneic bone marrow transplantation. Nine-year experience at a single institution. Medicine Baltimore. 67, 175-186.

Xu, L., Kelvin, D. J., Ye, G. Q., Taub, D. D., Ben-Baruch, A., Oppenheim, J. J., Wang, and JM.(1995). Modulation of IL-8 receptor expression on purified human T lymphocytes is associated with changed chemotactic responses to IL-8. J. Leukoc. Biol. 57, 335-342.

Xu-Bin, Murayama, T., Ishida, K., and Furukawa, T.(1989). Characterization of IgG Fc receptors induced by human cytomegalovirus. J. Gen. Virol. 70, 893-900.

Yakushijin, Y., Yasukawa, M., and Kobayashi, Y.(1992). Establishment and functional characterization of human herpesvirus 6-specific CD4+ human T-cell clones. J. Virol. 66, 2773-2779.

Yamashita, Y., Shimokata, K., Mizuno, S., Yamaguchi, H., and Nishiyama, Y.(1993). Down-regulation of the surface expression of class I MHC antigens by human cytomegalovirus. Virology 193, 727-736.

Yamashita, Y., Shimokata, K., Saga, S., Mizuno, S., Tsurumi, T., and Nishiyama, Y.(1994). Rapid degradation of the heavy chain of class I major histocompatibility complex antigens in the endoplasmic reticulum of human cytomegalovirus-infected cells. J. Virol. 68, 7933-7943.

Yang, H. Y., Dundon, P. L., Nahill, S. R., and Welsh, R. M.(1989). Virus-induced polyclonal cytotoxic T lymphocyte stimulation. J. Immunol. 142, 1710-1718.

Yang, S. Y., Chouaib, S., and Dupont, B.(1986). A common pathway for T lymphocyte activation involving both the CD3-Ti complex and CD2 sheep erythrocyte receptor determinants. J. Immunol. 137, 1097-1100.

Yang, W. C., Carreno, M., Esquenazi, V., Fuller, L., Ranjan, D., Burke, G., Roth, D., and Miller, J.(1991). Evidence that antibodies to cytomegalovirus and the T cell receptor (TCR)/CD3 complex may have common ligands. Transplantation 51, 490-498.

Yeager, A. S., Grumet, F. C., Hafleigh, E. B., Arvin, A. M., Bradley, J. S., and Prober, C. G.(1981). Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J. Pediatr. 98, 281-287.

Yssel, H., Aubrey, J. P., De Waal Malefijt, R., De Vries, J. E., and Spits, H.(1987). Regulation by anti-CD2 monoclonal antibody of the activation of a human T cell clone induced by anti-CD3 or anti-TCR antibodies. J. Immunol. 139, 2850-2902.

Yurochko, A. D., Kowalik, T. F., Huong, S. M., and Huang, E. S.(1995). Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. J. Virol. 69, 5391-5400.

Zamai, L., Rana, R., Mazzotti, G., Centurione, L., Di Pietro, R., and Vitale, M.(1994). Lymphocyte binding to K562 cells: effect of target cell irradiation and correlation with ICAM-1 and LFA-3 expression. European Journal of Histochemistry 38 Suppl 1, 53-60.

Zarcone, D., Viale, O., Cerruti, G., Tenca, C., Malorni, W., Arancia, G., Iosi, F., Galandrini, R., Velardi, A., Moretta, A., and Grossi, C. E.(1992). Antibodies to adhesion molecules inhibit the lytic function of MHC-unrestricted cytotoxic cells by preventing their activation. Cell. Immunol. 143, 389-404.

Zeevi, A., Uknis, M. E., Spichty, K. J., Tector, M., Keenan, R. J., Rinaldo, C., Duncan, S., Paradis, I., Dauber, J., and et al.(1992). Proliferation of cytomegalovirus-primed lymphocytes in bronchoalveolar lavages from lung transplant patients. Transplantation 54, 635-639.

Zeng, Y., Gage, A., Montag, A., Rothlein, R., Thistlethwaite, J. R., and BluestoneJA.(1994). Inhibition of transplant rejection by pretreatment of xenogeneic pancreatic islet cells with anti-ICAM-1 antibodies. Transplantation 58, 681-689.