



2806098713

THESES
WC 500
MAC

**THE EFFECT OF CYTOMEGALOVIRUS INFECTION
OF ENDOTHELIAL CELLS ON CELL SURFACE
MOLECULES INVOLVED IN LEUKOCYTE ADHESION,
MIGRATION AND IMMUNE RECOGNITION.**

A thesis submitted to the University of London

for the degree of

Doctor of Philosophy

in the Faculty of Medicine

by

Luci P. MacCormac, BSc., MSc.

Department of Clinical Immunology

Royal Free and University College Medical School

University of London

September 1998

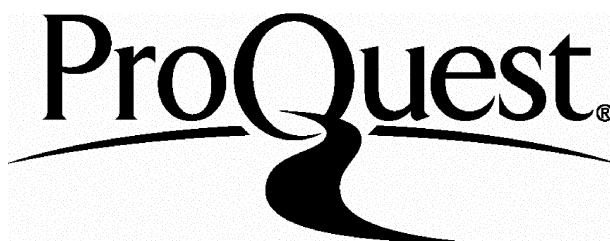
ProQuest Number: U536955

All rights reserved

INFORMATION TO ALL USERS

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

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



ProQuest U536955

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

All rights reserved.

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

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

09992

*Dedicated to my parents,
who were sure I could do it,
and to Robin,
who made sure I did.*

THESES
WC 500
MAC

ABSTRACT

CMV has been detected in a wide variety of cell types *in vivo*. Endothelial cell involvement, in particular, is associated with more severe infections, although the reasons are unclear. An *in vitro* model of virus infection was therefore established, in order to test the effect of CMV infection on endothelial cells. Using this model, a difference in the ability of laboratory strains and clinical isolates of CMV to infect endothelial cells was determined. The laboratory strains only produced a limited infection in a proportion of the cells, despite the induction of virus replication by the short chain fatty acid, sodium butyrate. In contrast, two recent clinical CMV isolates showed continuous infection of endothelial cells in the absence of prior stimulation of the cells. Moreover, the clinical strains were able to spread to surrounding uninfected cells, whereas the laboratory strains were not.

Following infection of endothelial cells with the CMV strains mentioned above, it was found that the expression of a number of cell surface molecules were altered. Thus, all virus strains increased the expression of ICAM-1, but decreased the expression of PECAM-1, ICAM-2, and MHC Class I. Furthermore, the ability of CMV-infected endothelial cells to induce the molecules E-selectin and MHC Class II, in response to cytokine stimulation, was abolished. However, a difference in the ability of the virus strains to increase the expression of LFA-3 was observed. This was not cell-type specific, as a similar phenomenon occurred when the more permissive fibroblast cell type was used. In addition, novel FcγR were also induced on the CMV-infected endothelial cells. All the effects described above were not due to the secretion of soluble factors during the course of the infection.

Finally, CMV infection altered the ability of leukocytes to adhere to, and transmigrate through unactivated or cytokine-activated endothelial cell monolayers. This was partly due to the secretion^{of} soluble factors. Thus, CMV infection of endothelial cells resulted in changes to cell surface molecule expression, and to leukocyte recruitment, which might alter the ability of the host's immune system to respond to the infection.

ACKNOWLEDGMENTS

I would like to express my thanks to my supervisor, Dr. Jane Grundy, for her advice and patience throughout my years in the laboratory, and during the preparation of this thesis. I would also like to thank Dr. Nicola Borthwick, for her collaboration in the T-lymphocyte migration studies, and Miss Jean Fletcher, for her collaboration in the LFA-3 studies. I am also grateful to Dr. Kwee Yong for assistance with the neutrophil migration studies, and to various members of the Immunology department, for donating blood.

Financial support from the British Heart Foundation was gratefully received, and is duly acknowledged.

I would like to thank my friends at the Royal Free Hospital for their continual support, both inside and outside of the work environment, and for keeping in contact throughout the write-up. I would also like to thank Mr. Robin Gupta, for his patience, cheerfulness and help in the last year, and for vacating the premises when asked! Finally, I would like to thank my family, for their love and support throughout my career, and especially to my sister, Lisa, for her patience in answering frantic telephone calls on how to use a computer!

CONTENTS

ABSTRACT.....	I
ACKNOWLEDGEMENTS.....	IV
LIST OF TABLES.....	IX
LIST OF FIGURES.....	X
LIST OF ABBREVIATIONS.....	XII
 CHAPTER 1.....	 1
GENERAL INTRODUCTION	1
1.1. Cytomegalovirus infections in the immunocompetent and immunocompromised population.....	7
1.2. Treatment of CMV infections with antiviral agents.....	7
1.3. CMV association with disease processes.....	7
1.4. CMV classification and virion structure.....	1
1.5. Entry of CMV into host cells.....	11
1.6. CMV replication.....	13
1.7. Cell types infected by CMV in vivo and in vitro, and their contributions to viral dissemination.....	17
1.8. Leukocyte adherence to, and migration through endothelial cells	23
1.9. The innate and adaptive immune response mediated by the host.....	27
1.10. The role of the host immune response in limiting CMV infection.....	31
1.11. CMV interaction with the immune system, and possible contribution to pathogenesis.....	35
1.12. Aims of the project	39
CHAPTER 2.....	41
MATERIALS AND METHODS	41
2.1. Fibroblast and Endothelial Cell Culture.....	42
2.1.1. Fibroblasts.....	42
2.1.2. Endothelial cells.....	42
2.2. Viral Stocks.....	43
2.2.1. AD169, Towne and Davis.....	43
2.2.2. Toledo.....	43
2.2.3. Clinical isolates.....	43
2.2.4. Herpes simplex virus, type 1 (HSV-1).....	44
2.3. Endothelial Cell Adaptation of Viral Stocks.....	44
2.3.1. Clinical isolates.....	44
2.3.2. Toledo.....	45
2.4. Viral Infection of Cell Monolayers.....	46
2.4.1. Fibroblasts.....	46
2.4.2. Endothelial cells.....	46
2.5. Cytoplasmic Immunofluorescent Staining.....	47
2.5.1. Cell monolayers.....	47
2.5.2. Cytocentrifuge preparations.....	47
2.5.3. Neutrophil staining.....	47

2.6.1. Fc gamma receptor (FcγR) analysis.	48
2.6.2. Adhesion molecule and major histocompatibility (MHC) analysis.	48
2.7. Trypsin Sensitivity of the FcγR.	49
2.8. Detection of CMV Immediate Early Antigen by Flow Cytometry.....	49
2.9. Flow Cytometric Acquisition and Analysis.	49
2.10. Monoclonal Antibodies and Related Proteins.	50
2.10.1. FcγR antibodies.	50
2.10.2. Fc fragments and IgG molecules.	50
2.10.3. CMV antigens.....	50
2.10.4. Adhesion molecule antibodies.	51
2.10.5. MHC antibodies.	51
2.10.6. Secondary conjugated antibodies.	51
2.11. Determination of CMV and HSV-1 Titre in Supernatant Fluids.....	52
2.12. Leukocyte Purification.....	52
2.12.1. Peripheral blood lymphocytes (PBLs).....	53
2.12.2. T-Lymphocytes.....	53
2.12.3. Neutrophils.....	53
2.13. Endothelial Cell-Neutrophil Co-Cultures.....	54
2.14. Radioisotope Labelling of Cells.	54
2.15. Transmigration Assays.	55
2.15.1. Preparation of Transwell filters.....	55
2.15.2. Transmigration assay.....	55
2.16. Adhesion Assays.....	55
2.17. Statistical analysis.....	56
CHAPTER 3.....	58
INFECTION OF ENDOTHELIAL CELLS WITH CMV.....	58
3.1. Introduction.	59
3.2. Determination of the optimal conditions under which the laboratory strain of CMV, AD169, leads to the infection of endothelial cells.	60
3.3. Comparison of the ability of the laboratory strain of CMV, AD169, or a low passage clinical isolate, C1F, to be propagated in endothelial cells.	62
3.4. Comparison of the ability of CMV strains AD169 and C1F to produce infectious virus in endothelial cells.	63
3.5. Comparison of the propagation of a clinical isolate of CMV, through either endothelial cells or through fibroblasts, on the ability of the virus to infect these two cell types.	65
3.6. The ability of three other fibroblast-propagated strains of CMV to infect endothelial cells.....	66
3.7. The ability of non-in vitro-passaged CMV to infect endothelial cells.....	68
3.8. Discussion.....	80
CHAPTER 4.....	84
THE EFFECT OF CMV INFECTION ON THE EXPRESSION OF CLASS I AND CLASS II MHC MOLECULES.	84
4.1. Introduction.	85
4.2. The expression of Class I MHC molecules at the surface of endothelial cells following CMV replication.	86
4.3. The effect of CMV infection on the expression of Class I at the surface of endothelial cells at different times post inoculation.....	87

4.4. The effect of ganciclovir on the ability of CMV to alter the expression of Class I at the surface of endothelial cells.	89
4.5. The expression of HLA Class I molecules at the surface of fibroblast cells following infection with CMV.	90
4.6. The effects of supernatants from CMV infected cells on the expression of Class I molecules at the surface of endothelial cells.	92
4.7. The effect of CMV on the expression of Class II at the surface of endothelial cells.	93
4.8. The expression of Class II molecules at the surface of endothelial cells following inoculation of CMV, in the absence or presence of sodium butyrate treatment.	95
4.9. The effect of ganciclovir on the ability of CMV to alter the expression of Class II at the surface of IFN- γ treated endothelial cells.	96
4.10. The ability of supernatants from CMV infected cells on the expression of Class II molecules at the surface of endothelial cells.	97
4.11. Discussion.	108
CHAPTER 5.	115
THE EFFECT OF CMV INFECTION OF ENDOTHELIAL CELLS AND FIBROBLASTS ON THE INDUCTION OF A RECEPTOR FOR THE Fc PORTION OF IgG.	115
5.1. Introduction.	116
5.2. Determination of the optimum conditions necessary to detect the CMV-induced Fc γ R. 1. Observations in the cytoplasm of CMV-infected fibroblasts, with respect to time.	117
5.3. Determination of the optimum conditions necessary to detect the CMV-induced Fc γ R. 2. Conditions required to detect the CMV-induced Fc γ R at the surface of fibroblasts.	117
5.4. Determination of the optimum conditions necessary to detect the CMV-induced Fc γ R. 3. Observations at the surface of CMV-infected fibroblasts, with respect to time.	118
5.5. The expression of an Fc γ R on the surface of endothelial cells.	119
5.6. The effect of virus dose on the surface expression of the CMV induced Fc γ R.	120
5.7. The effect of ganciclovir on the expression of the CMV-induced Fc γ R at the surface of fibroblasts.	121
5.8. The expression of the CMV induced Fc γ R in the cytoplasm of infected fibroblast and endothelial cells.	121
5.9. Reactivity of monoclonal antibodies specific for cellular Fc γ Rs with the cytoplasm of CMV infected fibroblasts.	122
5.10. Binding of IgG and Fc fragments of different species to CMV infected fibroblasts and endothelial cells.	123
5.11. Reactivity of the cell surface form of the CMV induced Fc γ R with antibodies specific for cellular Fc γ Rs.	125
5.12. Reactivity of the cell surface form of the CMV induced Fc γ R with IgG and Fc fragments from different species.	125
5.13. Discussion.	137
CHAPTER 6.	142

THE EFFECT OF CMV INFECTION ON THE EXPRESSION OF ADHESION MOLECULES PRESENT AT THE SURFACE OF ENDOTHELIAL CELLS AND FIBROBLASTS.....	142
6.1. Introduction.....	143
6.2 The effect of CMV infection on the expression of ICAM-1 at the surface of endothelial cells at different times post inoculation.....	144
6.3. The expression of LFA-3 on the surface of endothelial cells following infection with CMV.....	144
6.4. The expression of LFA-3 and ICAM-1 on the surface of fibroblasts following infection with different strains of CMV.....	145
6.5. The expression of LFA-3 and ICAM-1 at the surface of fibroblasts following infection with either an endothelial cell passaged or a fibroblast passaged clinical isolate.....	147
6.6. The expression of ICAM-2 at the surface of endothelial cells following infection with CMV.....	148
6.7. The expression of PECAM-1 at the surface of endothelial cells following infection with CMV.....	148
6.8. The effect of ganciclovir on the ability of CMV to alter the expression of ICAM-1, PECAM-1 and ICAM-2 at the endothelial cell surface.....	149
6.9. The effect of cell-free, CMV-infected cell supernatants on the expression of ICAM-1, ICAM-2 and PECAM-1 at the endothelial cell surface.....	151
6.10. The expression of E-selectin on the surface of endothelial cells following infection with CMV.....	152
6.11. The effect of ganciclovir on the ability of CMV to alter the expression of E-selectin at the surface of cytokine stimulated endothelial cells.....	153
6.11. Discussion.....	164
CHAPTER 7.....	171
THE EFFECT OF CMV INFECTION OF ENDOTHELIAL CELLS ON LEUKOCYTE ADHERENCE AND TRANSENDOTHELIAL CELL MIGRATION.....	171
7.1. Introduction.....	172
7.2. The ability of neutrophils to adhere to endothelial cells following infection with CMV.....	172
7.3 The ability of peripheral blood lymphocytes (PBL) to adhere to endothelial cells following infection with CMV.....	174
7.4. The ability of neutrophils to migrate through CMV infected endothelial cells.....	176
7.5. The ability of T-lymphocytes to transmigrate through CMV infected endothelial cells.....	177
7.6. The ability of culture supernatants to promote leukocyte transmigration through endothelial cells.....	178
7.7. Transmission of CMV to neutrophils.....	179
7.8. Discussion.....	190
CHAPTER 8.....	197
GENERAL DISCUSSION.....	197
CHAPTER 9.....	211
REFERENCES.....	211
LIST OF ABSTRACTS AND PUBLICATIONS.....	246

LIST OF TABLES

CHAPTER 2

Table 2.1. Characteristics of the murine monoclonal antibodies used in this study.....	56
--	----

CHAPTER 3

Table 3.2.1. The effect of increasing concentrations of sodium butyrate on the ability of CMV strain AD169 to infect endothelial cells.....	70
Table 3.2.2. The effect of increasing virus to cell ratio on the ability of CMV strain AD169 to infect endothelial cells.....	71
Table 3.3.1. Comparison of the ability of the low passage clinical isolate C1F, or the high passage laboratory strain of CMV, AD169, to infect endothelial cells.....	72
Table 3.4.1. Comparison of the ability of the low passage clinical isolate C1F, or the high passage laboratory strain of CMV, AD169, to secrete virus into the supernatant fluids of infected endothelial cells.....	73
Table 3.5.1. The ability of a clinical isolate grown through two different cell types to infect either fibroblasts or endothelial cells.....	74
Table 3.6.1. The ability of high and low passage fibroblast-propagated strains of CMV to infect endothelial cells.....	75

CHAPTER 4

Table 4.3.1. The effect of CMV on the expression of Class I at the surface of endothelial cells with respect to time.....	99
Table 4.5.1. The effect of infection with a clinical isolate of CMV on the expression of Class I at the surface of fibroblasts.....	100
Table 4.8.1. The effect of CMV infection on the expression of Class II following stimulation of endothelial cells with interferon gamma.....	101

CHAPTER 5

Table 5.2.1. The expression of the CMV-induced FcγR in the cytoplasm of fibroblasts with respect to time.....	127
Table 5.9.1. The reactivity of a panel of murine monoclonal antibodies specific for human cellular FcγRI, II, and III with the CMV-induced FcγR in the cytoplasm of fibroblasts.....	128
Table 5.10.1. The reactivity pattern of IgG and Fc fragments from various species with the FcγR induced in the cytoplasm of HCMV or HSV-1 infected fibroblasts or endothelial cells.....	129

LIST OF FIGURES

CHAPTER 3

Figure 3.2.1. The effect of sodium butyrate treatment and centrifugal enhancement on CMV IEA expression in endothelial cells.	76
Figure 3.3.1. The ability of different strains of CMV to produce cytopathic effect after sodium butyrate treatment.	77
Figure 3.6.1. The ability of various strains of CMV to infect endothelial cells in the presence or absence of sodium butyrate treatment.	78
Figure 3.7.1. The ability of an ex vivo source of CMV to infect endothelial cells.....	79

CHAPTER 4

Figure 4.2.1. The effect of CMV infection on the expression of Class I MHC molecules at the surface of endothelial cells.	102
Figure 4.4.1. The effect of ganciclovir on the ability of CMV to alter the expression of Class I MHC molecules at the surface of endothelial cells.	103
Figure 4.5.1. The effect of CMV infection on the expression of Class I MHC molecules at the surface of fibroblasts.....	104
Figure 4.6.1. The effect of soluble factors on the expression of Class I MHC molecules at the surface of endothelial cells.	105
Figure 4.7.1. The effect of CMV infection on the expression of Class II MHC at the surface of endothelial cells.....	106
Figure 4.10.1. The effect of soluble factors on the expression of Class II MHC molecules at the surface of endothelial cells.	107

CHAPTER 5

Figure 5.3.1. Trypsin sensitivity of the FcγR induced at the surface of fibroblasts following infection with CMV.....	130
Figure 5.4.1. The induction of an FcγR at the surface of fibroblasts following infection with CMV, with respect to time.....	131
Figure 5.5.1. The effect of CMV infection on the expression of an FcγR at the surface of endothelial cells.....	132
Figure 5.6.1. The effect of virus dose on the ability of CMV to induce an FcγR at the surface of fibroblasts.	133
Figure 5.8.1. The expression of the CMV-induced FcγR in the cytoplasm of endothelial cells or fibroblasts.	134
Figure 5.10.1. The expression of the HSV-1- or CMV-induced FcγR in the cytoplasm of fibroblasts.	135
Figure 5.12.1. The reactivity pattern of Fc fragments and IgG molecules from different species with the surface of CMV infected fibroblasts and endothelial cells.....	136

CHAPTER 6

Figure 6.2.1. The expression of ICAM-1 at the surface of endothelial cells following infection with CMV for various times periods.	154
Figure 6.3.1. The expression of LFA-3 at the surface of endothelial cells following infection with CMV for various times periods.	155
Figure 6.4.1. The effect of infection with different strains of CMV on the expression of LFA-3 or ICAM-1 at the surface of fibroblasts.	156
Figure 6.5.1. The expression of LFA-3 or ICAM-1 on the surface of fibroblasts following infection with either an endothelial-adapted or a fibroblast-adapted strain of CMV.	157
Figure 6.6.1. The expression of ICAM-2 at the surface of endothelial cells following infection with CMV for various time periods.	158
Figure 6.7.1. The expression of PECAM-1 at the surface of endothelial cells following infection with CMV at various times post infection.	159
Figure 6.8.1. The effect of ganciclovir treatment on the expression of ICAM-1, ICAM-2 or PECAM-1 following infection of endothelial cells with CMV.	160
Figure 6.9.1. The effect of soluble factors produced following infection of endothelial cells with CMV on the expression of adhesion molecules.	161
Figure 6.10.1. The effect of CMV infection on the expression of E-selectin at the surface of endothelial cells.	162
Figure 6.11.1. The effect of ganciclovir on the ability of CMV to alter the expression of E-selectin at the surface of IL-1 β stimulated endothelial cells. ...	163

CHAPTER 7

Figure 7.2.1. The adhesion of neutrophils to endothelial cells following infection with CMV.	181
Figure 7.2.2. Photomicrographs showing neutrophil adhesion to CMV infected or uninfected endothelial cells.	182
Figure 7.3.1. The adhesion of PBL to endothelial cells following infection with CMV.	183
Figure 7.3.2. Photomicrograph of PBL adhesion to CMV infected or uninfected endothelial cells.	184
Figure 7.4.1. Neutrophil transmigration through endothelial cells infected with CMV.	185
Figure 7.5.1. T-lymphocyte migration through endothelial cells infected with CMV.	186
Figure 7.6.1. The effect of soluble factors on transendothelial migration following infection of endothelial cells with CMV.	187
Figure 7.6.2. The effect of soluble factors on the expression of adhesion molecules following infection of endothelial cells with CMV.	188
Figure 7.7.1. Photomicrograph of pp65 staining of neutrophils following co-culture with CMV infected endothelial cells.	189

LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
CD	cluster of differentiation
CMV	cytomegalovirus
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
Cr	chromium 51
DNA	deoxyribonucleic acid
Fc γ R	Fc gamma receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
gp,pp,p	glycoprotein, phosphoprotein, protein
HBSS	Hank's balanced saline solution
HIV	human immunodeficiency virus
HSV-1, 2	herpes simplex virus type 1, type 2
HHV	human herpesvirus
ICAM-1,-2,-3	intercellular adhesion molecule-1, -2, -3
IE, IEA	immediate early, immediate early antigen
IFN- α , - β , γ	interferon alpha, beta, gamma
Ig	immunoglobulin
IL-1 β , -8	interleukin-1 beta, interleukin 8
kD	kilodalton
LFA-1, -3	lymphocyte function-associated antigen-1, -3
MHC	major histocompatibility complex
MOI	multiplicity of infection
NF- κ B	nuclear factor κ B
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule-1
PFU	plaque forming unit
RNA/mRNA	ribonucleic acid/messenger ribonucleic acid

TCR	T-cell receptor
TNF- α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1
VZV	varicella zoster virus

CHAPTER 1.

GENERAL INTRODUCTION

1.1. Cytomegalovirus infections in the immunocompetent and immunocompromised population.

Cytomegaloviruses are a highly successful group of ancient viruses which have survived well in nature, infecting many different animal species (Roizman, 1996). The beta-herpesviruses, to which the cytomegaloviruses belong, are highly species specific, and therefore *homo sapiens* are thought to be the only reservoir for human cytomegalovirus (CMV) (Roizman, 1996). CMV infection in the general population is naturally acquired and is endemic world-wide, with all continents being affected. Some variation in the prevalence of infection on the basis of ethnic, geographical, and socio-economic grouping occurs, as judged by the CMV antibody status of surveyed individuals within these societies (Britt, Alford, 1996). In western society, the total incidence of CMV infection has been estimated as 8-60% in the first year of life, 5-10% before the age of 4, and 5% between the age of 4-15. After this, the rate of infection in young adults is estimated at 1% per annum, and gradually declines in adulthood (Naraqi, 1990). Thus the majority of adults in both developed and developing countries have been exposed to CMV.

Primary CMV infection in the general population usually goes unnoticed, with the occasional individual presenting with a mild, mononucleosis-like illness (Britt, Alford, 1996). It has been estimated that 8% of all cases of infectious mononucleosis are a result of CMV infection (Horwitz et al. 1977). CMV mononucleosis, however, is infrequently diagnosed and the disease is self-limiting, rarely being associated with any severe complications (Britt, Alford, 1996). Following primary infection, a latent infection is established which persists in the infected individual for life. After recovery from either an asymptomatic or symptomatic acute infection, the virus may be shed for a prolonged period of time. CMV may also be intermittently shed thereafter due to the reactivation of the latent virus (Ho, 1991b). During an active infection, the sites of viral excretion in the immunocompetent host include the urine, throat, cervix, semen and breast milk (Britt, Alford, 1996; Naraqi, 1990). These exogenous sources of CMV account for the predominate routes of horizontal (extrauterine) viral acquisition through the general population, i.e. lactation, parturition, oral, respiratory and sexual transmission of CMV (Ho, 1991b). Although

CMV may be present in the blood of acutely and latently infected individuals (Stanier et al. 1989), this is not a major source of viral transmission in the general population.

Infection with CMV in individuals with an immature or compromised immune system, however, can cause severe disease. CMV is the most common congenital viral infection in humans, with 0.5-2.5% of neonates being infected at birth (Ho, 1991a; Britt, 1996a). In this setting, acquisition of CMV occurs *in utero*, and therefore the mother is the source of the vertically (intrauterine) transmitted virus. Although the number of pregnant women experiencing either a primary or reactivated infection are similar (1.6% and 1.4%, respectively), spread of the virus through the placenta to the foetus is predominantly associated with a maternal primary infection (transmission rates of 30-50% for primary infection compared with 0.02-1% for recurrent infections) (Britt, 1996). Furthermore, symptomatic disease in the neonate is also associated with a primary infection in the mother, with approximately 5-10% presenting with life threatening disseminated infection at birth (Britt, 1996). Symptoms of this 'cytomegalic inclusion disease' include hepatosplenomegaly, petechiae, microcephaly, chorioretinitis, jaundice and hepatitis (Ho, 1991a). Mortality in these infants is between 11-20%, and is usually associated with pulmonary disease, hepatitis and central nervous system dysfunction (Britt, 1996). The children who survive this viral onslaught usually recover from the organ dysfunctions, although 30-60% will exhibit prolonged hearing loss and sometimes impaired vision (Pass et al. 1980; Britt, 1996). Unfortunately, 5-17% of the congenitally infected neonates who were asymptomatic at birth will also develop hearing loss during the first two years of life (Hanshaw et al. 1976; Britt, 1996). In fact, CMV is the most common cause of non-familial hearing loss in childhood. Both symptomatic and asymptomatic congenitally infected neonates excrete high titres of virus for extended periods of time in the urine and saliva, and therefore are an important reservoir of virus during this period (Britt, 1996).

CMV is also an important pathogen in almost all allograft recipients, being the most frequent infectious cause of morbidity in the post-transplant period (1-3 months) for patients undergoing renal, cardio-thoracic, liver and bone marrow transplantation. The incidence of CMV infection is similar between these groups, with 60-90% of patients showing evidence of the virus (Britt, 1994). The source of CMV transmission

in this group of patients is rarely from person to person contact, as is seen in the general population. In the majority of cases, morbidity from CMV results from a secondary infection (i.e. reactivation of latent virus or re-infection with a different virus strain) (Britt, 1994). For those individuals who have not been exposed to the virus prior to solid organ transplantation, the main source of transmission appears to be from the donor organ itself (Ho et al. 1975; Chou, 1989; Wertheim et al. 1983). In addition, CMV seropositive blood products, which might have been given during the transplantation program, have also been implicated as an important source of virus transmission for CMV seronegative transplant recipients (Przepiorka et al. 1996; Chou et al. 1987).

The majority of solid organ transplant patients will present with few clinical symptoms. However, a proportion (15-30%) of the infected individuals will show overt symptoms of disease, the most common being fever, malaise, leukopenia, lymphocytosis, hepatosplenomegaly and pneumonitis (Britt, 1994). Life threatening complications occur due to severe gastrointestinal disorders, severe hepatitis, and pneumonitis, and less frequently because of encephalitis. Chorioretinitis in the transplanted populations is very rare (Britt, 1994). In addition, the transplanted organ itself appears to have an increased susceptibility to CMV infection. Thus, there is an increased incidence of hepatitis in liver transplant recipients, and pneumonitis in lung transplant recipients, when compared to the other transplant recipient groups (Duncan et al. 1991; Paya et al. 1989; Bronsther et al. 1988). The clinical symptoms which bone marrow transplant patients present with are again similar to those seen in the solid organ transplant population, although in this case, the most common manifestation is pneumonitis (Britt, 1994). This symptom, if untreated, is associated with a high mortality rate (85%), and is responsible for 15-25% of all deaths in this group of patients (Britt, 1994).

Although severe disease in all CMV infected transplant recipients has been associated with primary infection in the affected individual (Duncan et al. 1991; Ho et al. 1975), both the type and level of immunosuppression have been implicated in promoting disease in patients with reactivated CMV, which can be as severe as that seen during a primary infection. Consequently, mortality is lower for those patients who require less immunosuppressive therapy (Goral, Helderman, 1994). Finally, the

severity of symptoms associated with CMV infection also appear to be influenced by transplant rejection episodes. Thus, host versus graft rejection in solid organ transplant recipients or graft versus host disease in bone marrow transplant recipients, together with an active CMV infection, are associated with more aggressive symptoms and a poor prognosis (Goral, Helderman, 1994).

CMV infection is also an important pathogen in HIV positive individuals (Onorato, Pollard, 1996). As prior exposure to CMV is almost universal in individuals at high risk of developing HIV infection (Drew et al. 1981; Gottlieb et al. 1981), the most likely source of CMV in these patients is the reactivation of latent virus, although re-infection with different CMV strains has also been demonstrated (Drew et al. 1984). In fact, the majority (90%) of HIV positive individuals will experience a reactivated CMV infection at some stage after HIV diagnosis, as judged by the development of CMV viraemia (Britt, 1994). However, the majority of these infections will remain subclinical. Syndromes associated with CMV infection in this group of patients are associated with a CD4⁺ cell count falling below 100mm³, and when present, can affect nearly every organ system (Onorato, Pollard, 1996; Britt, 1994). Unlike the clinical manifestations in the transplanted patient group, the most common and disabling symptom is chorioretinitis, which is seen in 20-45% of patients (Onorato, Pollard, 1996; Jacobson, Mills, 1988). Thus, CMV is the most frequent cause of sight threatening disease in patients with AIDS. Gastrointestinal disorders are the second most common feature, with occasional reports of CMV morbidity being due to involvement of the central nervous system and encephalitis (Jacobson, Mills, 1988). CMV-associated pneumonitis appears to be uncommon amongst HIV positive individuals, although specific diagnosis is complicated by the frequent occurrence of other pathogens (Onorato, Pollard, 1996). Disseminated infection, reminiscent of 'cytomegalic inclusion disease' seen in infected neonates, may also occur as end stage disease (Britt, 1994). However, although 90% of HIV positive individuals will show evidence of CMV inclusions in various body tissues at autopsy (Reichert et al. 1983), mortality due to this virus is difficult to determine.

1.2. Treatment of CMV infections with antiviral agents.

Two antiviral agents, ganciclovir and foscarnet, are currently used in the management of CMV infections. Both of these drugs have been used successfully in the therapeutic treatment of AIDS-related CMV retinitis and gastrointestinal disorders (Polis et al. 1993; Dieterich et al. 1988; Bach et al. 1985). However, reactivation of CMV is a common occurrence following withdrawal of the antiviral agents, presumably because AIDS patients do not achieve a sufficient level of immune competence to allow clearance of the virus infection. Thus, long-term maintenance therapy is required to prevent the re-emergence of the original symptoms, which has led to the development of ganciclovir- and foscarnet-resistant CMV strains in this particular patient group (Balfour, Jr. 1992).

Treatment of solid organ transplant recipients with ganciclovir has also been effective in treating tissue invasive CMV disease (Cooper et al. 1991; Jordan et al. 1992; Dunn et al. 1991). In contrast, treatment of CMV pneumonitis in bone marrow transplant recipients, using a single antiviral agent, has generally been ineffective (Aschan et al. 1992; Shepp et al. 1985). More encouraging results have been demonstrated following the combined use of ganciclovir and intravenous globulins (Emanuel et al. 1988; Reed et al. 1988), although subsequent studies have suggested that later complications, due to bacterial or fungal infections, occur following such treatment in the latter patient group (Reusser, 1996).

Due to the poor clinical outcome following treatment of established CMV disease in bone marrow transplant recipients, other strategies have been employed, which involve the prophylactic or pre-emptive use of antiviral drugs to prevent CMV disease. However, although ganciclovir prophylaxis in bone marrow transplant recipients has been shown to reduce the rate of CMV disease, an overall improvement in survival was not demonstrated (Winston et al. 1993). This was due, in part, to a higher number of deaths from non-viral infections, and to incomplete protection from fatal CMV pneumonitis (Winston et al. 1993). Furthermore, there was a delayed restoration of the CMV-specific immune response following ganciclovir prophylaxis, which increased the risk of developing late CMV disease (Li et al. 1994). In contrast to the results obtained with ganciclovir prophylaxis, pre-emptive anti-viral treatment

appears to be effective in decreasing the incidence of CMV disease in both bone marrow and solid organ transplant recipients (Goodrich et al. 1991; Egan et al. 1998). However, effective pre-emptive therapy relies on early detection of the virus, before the onset of disease, and thus the efficiency of this treatment is limited by the surveillance techniques used to identify patients at risk from CMV infection.

1.3. CMV association with disease processes.

CMV has been implicated in the augmentation of a number of disease processes, although many of these associations remain highly controversial. Thus, CMV has been both positively and negatively associated with 'vanishing bile duct syndrome' in liver transplant patients (van den Berg et al. 1996; O'Grady et al. 1988), and with the development of 'glomerulopathy' in renal transplant patients (Herrera et al. 1986; Richardson et al. 1981). Further provocative associations have been made with respect to CMV infection and the development of acute cellular rejection in solid organ transplant patients (Normann et al. 1991; Fernando et al. 1994), and also with graft versus host disease in bone marrow transplant patients (Gratama, 1989; Bostrom et al. 1990). However, given both the ubiquity of CMV in these patient populations, and also the opportunistic nature of these infections in severely immunocompromised individuals, some authors have suggested that the presence of CMV is merely a coincidence and have failed to show a correlation between CMV and the disease processes described above (Bowden et al. 1993; Stovin et al. 1989). Furthermore, the aggressive immunosuppressive therapy used to treat patients undergoing acute rejection episodes may promote an apparent episode of CMV infection by altering the ability of the host's immune system to contain the virus. Thus, CMV infection may occur in transplant recipients who are already rejecting their grafts (Meyers et al. 1986; Miller et al. 1986). Moreover, the generalised haematological abnormalities associated with CMV infections (see section 1.1) has led to the speculation that CMV may cause a generalised immunosuppression *per se* (Carney, Hirsch, 1981; Rinaldo, Jr. et al. 1980), and CMV infection has also been associated with the development of superinfection by bacterial or fungal agents (Chatterjee et al. 1978).

A further disease process which has increasingly been linked to the presence of CMV is the development of atherosclerosis in immunocompetent adults. This suggestion has arisen from the finding that infection of normocholesterolemic chickens with an avian herpesvirus, Marek's disease virus, leads to arterial disease closely resembling chronic human atherosclerosis (Fabricant et al. 1983; Fabricant et al. 1978). Thus, a search for a similar relationship between human herpesvirus infection and atherogenesis was stimulated. In support of this hypothesis, herpesvirus mRNA was detected in the aortic wall of patients undergoing coronary bypass surgery (Gyorkey et al. 1984), whilst herpesvirus virions, CMV antigens or CMV DNA were found in cells recovered from aortic tissues of arteriosclerotic patients (Hendrix et al. 1997; Petrie et al. 1987). In addition, raised levels of CMV specific antibody were found in patients requiring vascular surgery for atherosclerosis (Adam et al. 1987), although a recent study has questioned the validity of the latter finding (Adler et al. 1998b). Furthermore, CMV DNA has been found in both involved and uninvolved areas of blood vessels taken from young trauma victims (Yamashiroya et al. 1988), suggesting that CMV localisation to the blood vessel wall is a common occurrence. Thus, the association between CMV infection and the development of the 'spontaneous' form of atherosclerosis remains provocative.

With regards to the accelerated form of atherosclerosis seen in cardiac transplant patients, the association with CMV infection is more convincing. Cardiac vascular arteriopathy (synonymous with accelerated atherosclerosis or chronic rejection) is a major complication in long term survivors of heart transplantation, being the most frequent cause of death after the first post-transplant year (Billingham, 1994). CMV nucleic acids have been demonstrated in the coronary arteries of heart transplant recipients (Wu et al. 1992), and the presence of CMV in these vessels has been associated with the development of inflammatory aortic diseases, including atherosclerosis (Tanaka et al. 1992). Furthermore, several authors have identified CMV infection as a major factor in developing endothelialitis, an inflammation of the endothelial lining of the blood vessel, characterised by the accumulation of lymphocytes, macrophages, smooth muscle cell proliferation and intimal thickening (Hruban et al. 1990; Paavonen et al. 1992; Koskinen et al. 1994). In addition, CMV infection, as judged by an increase in viral antibody titre or the culture of virus from

blood/urine, in cardiac transplant recipients is associated with more severe graft atherosclerosis and death (McDonald et al. 1989; Loebe et al. 1990; Grattan et al. 1989). Similar associations with CMV infections have been reported for chronic rejection in liver and kidney transplant recipients (synonymous with vanishing bile duct syndrome and glomerulopathy, respectively), as stated above.

In order to understand how CMV may cause disease, it is necessary to study the virus at all levels. The outcome of infection with CMV is a consequence of a complicated series of regulatory events involving the viral genome and the host cells, and this partly determines whether or not CMV infection results in the production of daughter virions.

1.4. CMV classification and virion structure.

There are 3 subfamilies to which all the herpesviruses belong, (the *alpha*, *beta* and *gamma-herpesvirinae*), and each of these subfamilies can be further subdivided into genera. Eight human herpesviruses (HHV) have been isolated to date, and have been termed HHV-1-8 to reflect the order in which they were identified (Roizman, 1996). Members of the subfamily *Alphaherpesvirinae* have a variable host range, a relatively short reproductive cycle, show rapid spread and cell destruction in infected cell cultures, and can establish latency in nervous tissue. HHV-1, 2 and 3 (more commonly known as herpes simplex virus type 1 (HSV-1), HSV-2 and Varicella Zoster virus (VZV), respectively) belong to this subfamily, and are examples of the genera *Simplexvirus* (HSV-1 and HSV-2) and *Varicellovirus* (VZV). The *Betaherpesvirinae* generally display a more restricted host range, have a relatively long reproductive cycle, and consequently the infection progresses more slowly in cell culture. HHV-5, 6 and 7 belong to this subfamily, and are examples of the genera *Cytomegalovirus* (HHV-5, more commonly known as CMV) and *Roseolovirus* (HHV-6 and HHV-7). The *Gammapherpesvirinae* also have a limited host range, and can replicate in lymphocytes. HHV-4 (Epstein Barr Virus, EBV) and HHV-8 (also known as Kaposi's Sarcoma human herpesvirus) belong to this subfamily, and are examples of the genera *Lymphocryptovirus* and *Rhadinovirus*, respectively. Membership of the herpesviruses is based on the architecture of the virion, and CMV exhibits a typical herpesvirus virion structure (Roizman, 1996).

CMV has a large (65-68nm) double stranded DNA genome, which contains approximately 240 kilobase pairs (150×10^6 Daltons), and which is organised according to a 'class E' structure (Roizman, 1996). At either end of the genome are repeated nucleotide sequence, referred to as terminal repeats (TR). The left hand TR (TR_L) contains a variable number of repeated sequences, termed the *a* sequence, whereas the right hand TR (TR_S) usually contains only one *a* sequence (Mocarski, 1996). Variations in the number of the *a* sequences contained at the TR can be used to differentiate one strain of CMV from another (Tamashiro et al. 1984; Mocarski et al. 1987), as can the restriction enzyme profiles of the whole viral DNA molecule (Chou, 1989). These *a* sequences are either followed by a large repeated sequence (the *b* sequence), or a smaller repeated sequence (the *c* sequence) to form the TR_L and TR_S, respectively. The sequences contained in the TR are also found inverted and juxtaposed internally (IR_L and IR_S), and divide the genome into two regions (Mocarski, 1996). These 'unique' regions are of unequal size (approximately 175kbp and 38kbp) and are termed the unique long (UL) and the unique short (US) regions, accordingly. Furthermore, both the TR_L and the IR_L, and the TR_S and the IR_S can also invert, producing four different isomers of the genome, which differ only in the relative orientation of the two components (Stinski, 1990; Mocarski, 1996). The coding sequences contained within these regions are prefixed with either UL or US to establish the position of the gene, and the corresponding proteins are assigned the letters p (protein), pp (phosphoprotein) or gp (glycoprotein), as appropriate (Landini, Spaete, 1993). It has been estimated that the genome of CMV contains over 200 open reading frames (genes) which can each code for a potential protein. However, not all of these proteins are destined to be incorporated into the virion, and many remain to be identified (Mocarski, 1996).

The double stranded DNA genome is contained within an icosahedral capsid approximately 100-110nm in diameter, which is constructed from 162 separate pentameric or hexameric capsomeres. The structural proteins which form this capsid include the 150kD 'major capsid protein' (pUL86), the 34-37kD minor capsid protein (pUL46), and the products of the UL49 and UL85 genes (Mocarski, 1996). The capsid is surrounded by an amorphous and poorly defined structure known as the tegument or matrix. The major constituents of the tegument are the 150kD 'basic

phosphoprotein' pp150 (ppUL32) and the 65kD 'lower matrix protein' pp65 (ppUL83). Other minor constituents of the tegument are the 71kD 'upper matrix protein' pp71 (ppUL82), a 28kD phosphoprotein pp28 (ppUL99), and a phosphoprotein of approximately 130kD (pp130) (Mocarski, 1996). The matrix proteins are thought to anchor the viral capsid to the outer layer of the virion, termed the envelope. This envelope consists of a typical lipid bilayer organisation, and contains numerous virally encoded glycoproteins that protrude from the surface (Mocarski, 1996). The major envelope glycoprotein gB (gpUL55) is a heterodimeric complex of a 55kD and a 93-130kD glycoprotein. This 155kD glycoprotein complex (gc) is also known as gcI (Gretch et al. 1988). A second glycoprotein complex (gcII) is abundant on the envelope, and may contain a 93kD and 450kD protein, although the actual constituents of this complex are unclear (Gretch et al. 1988). A third complex (gcIII) of approximately 240kD is also present at the surface of the viral envelope (Gretch et al. 1988). This consists of the 86kD glycoprotein gH (gpUL75), the 32kD glycoprotein gL (gpUL115) and a third glycoprotein, gp145 (Mocarski, 1996). There are presumably more proteins which are constituents of the virion (currently estimated at exceeding 35) but, as stated above, some of these remain to be unequivocally identified. The mature virion, consisting of the nucleocapsid core, the tegument and the envelope is approximately 150-200nm in diameter, and this pleomorphism is thought to reflect differences in the size of the tegument, rather than interstrain differences (Mocarski, 1996).

1.5. Entry of CMV into host cells.

The initial events which govern successful viral replication are in part determined by the ability of the virus to gain entry into a susceptible cell. This requires that the virus sequentially attaches to the host cell, fuses with cellular membranes and then transfers its genetic material into the appropriate replicative compartment. The mechanism by which CMV gains entry into a cell is incompletely understood, but is thought to involve the interaction of the protruding viral envelope glycoproteins with specific cell surface receptor molecules. The viral envelope constituents which have been implicated in this process are the glycoprotein complexes gcI (gB), gcII and gcIII (gH/gL), whilst the cell surface components

include heparin sulphate containing proteoglycans, and unidentified proteins with molecular weights of 30-34kD, and 92.5kD.

The first step in the entry process appears to be the chance encounter of the viral gCII complex with the heparin sulphate moieties of various cell surface proteoglycans (Kari, Gehrz, 1993; Kari, Gehrz, 1992). Although this initial interaction is weak and reversible (i.e. relatively non-specific), it is thought to rapidly promote a specific, high affinity receptor mediated event (Compton et al. 1993). The second stage in the entry process is characterised as being heparin resistant, and probably involves the firm interaction of an as yet unknown viral glycoprotein with the 30-34kD cell surface protein mentioned above (Adlish et al. 1990; Taylor, Cooper, 1990). This putative viral receptor is present on a wide variety of cell types, and the level of viral attachment correlates with the distribution and relative abundance of this protein at the cell surface (Nowlin et al. 1991).

Although the 30-34kD cellular receptor for CMV remains to be unequivocally identified, recent reports have shown that CMV virions may bind to a 36kD protein present on both endothelial cells and fibroblasts, termed annexin II (Wright et al. 1995; Wright et al. 1994). On the basis of apparent molecular weight, proteolytic fragmentation pattern and partial sequence data, annexin II appears to correspond with the previously mentioned 30-34kD viral receptor. Annexin II is associated with the surface of the virions through calcium-dependant interactions with the viral envelope phospholipids (Wright et al. 1995), or calcium-independent associations with the viral envelope glycoprotein gB (Pietropaolo, Compton, 1997). These findings suggest that CMV may acquire annexin II concomitantly with the cellular lipid bilayer during virion envelopment, and thus it is not clear whether this cellular protein plays a role in viral entry or viral egress. Interestingly, annexin II has been implicated in the bridging and fusion of biological membranes (Emans et al. 1993; Creutz, 1992), as has the viral glycoprotein gB (Navarro et al. 1993), and thus it is speculated that the interaction of the two proteins may be involved in one of the many membrane fusion events required during CMV infection (Pietropaolo, Compton, 1997).

Regardless of the actual proteins involved in receptor-mediated entry, this event is quickly followed by the fusion of the viral envelope with the cellular membrane. This occurs by direct pH independent fusion of the two envelopes at the

cell surface, rather than within cytoplasmic vesicles during endocytosis (Compton et al. 1992). Penetration is mediated in part by the interaction of the viral gH (gpUL75, a constituent of the gCIII complex) with the unidentified 92.5kD cellular component (Keay et al. 1989), which is thought to be a phosphorylated glycoprotein and may be a kinase (Keay, Baldwin, 1992). The use of anti-idiotypic antibodies demonstrated that gH-mediated binding was separate from viral attachment, but crucial for cell fusion (Keay, Baldwin, 1991). The glycoprotein gB (gpUL55, part of the gCI complex) is thought to play a role in these events, as it has been proposed that gB both binds heparin (Compton et al. 1993), and annexin II (Pietropaolo, Compton, 1997). Ultimately, however, fusion of the viral and cellular lipid membranes results in the penetration of the nucleocapsid into the cytoplasm of the cell, and allows the initiation of the replicative cycle.

1.6. CMV replication.

Once CMV has successfully penetrated the outer cell membrane, the naked viral nucleocapsids are quickly transported through the cytoplasm, along with a limited number of tegument constituents such as pp71 and pp65 (Yamauchi et al. 1985). Shortly after this, the capsid proteins are removed and the genome of CMV circularises to form an episomal DNA structure in the nucleus of the infected cell. The organisation of the CMV genome and the steps by which the genome is expressed in the individual cell type now plays a crucial role in determining the pattern of the virus-host interaction, i.e. in determining whether a permissive, abortive or latent infection is established. In susceptible cells, a series of events known as transcription, translation, DNA replication, assembly and egress are now initiated, which ultimately result in virus propagation. In a productive infection (such as occurs in cultured human fibroblasts), there is a sequential expression of the viral genome which is temporally regulated. The viral genes are expressed in a cascade fashion, and can be approximated into three phases, termed immediate early (IE) or α , early or β , and late or γ , according to the timing of transcription and gene expression (Stinski, 1990; Mocarski, 1996). In general, the IE genes are necessary for regulatory events involved in permissive or latent infection, the early genes are necessary for the replication of DNA, and the late genes produce the structural components of the

virion shell. The IE genes are expressed quickly after infection (0-4 hours), whilst the expression of the early and late genes are prolonged (4-24 hours, and 24-48 hours, respectively). Due to the delay required for the expression of these last two phases, several days are required for maximum virion production in cell culture (Stinski, 1990).

Viral gene expression and part of the maturation of virus particles occur in the nucleus of the infected cells. The first genes to be expressed are the IE genes, and these are transcribed and translated prior to the onset of *de novo* viral DNA and protein synthesis. The expression of the IE genes is restricted to discrete regions of the viral genome, although the major site of IE transcription is in the UL component of the genome (Mocarski, 1996). As stated in Section 1.3, CMV DNA has highly conserved sets of repetitive elements, interspersed with irregular stretches of non-repetitive sequences. One highly repetitive region makes up the CMV major IE enhancer/promoter. This region contains sequences that bind to pre-existing cellular proteins which promote transcription from the IE genes, and also protect the viral DNA from digestion by cellular enzymes (Mocarski, 1996). In addition, the viral upper matrix protein pp71 (ppUL82) is also involved in *trans*-activating IE gene expression (Liu, Stinski, 1992). Four regulatory IE genes are driven by the major IE promoter/enhancer. IE1 (UL123) and IE2 (UL122) mRNA transcripts arise from alternative splicing and therefore share common sequences. The proteins which are transcribed from these genes (pp72 and pp86, respectively) are involved in stimulating subsequent viral, and possibly host cell macromolecule synthesis (Stinski, 1990; Mocarski, 1996). The IE1 pp72 is the most abundant viral protein expressed immediately after infection, and is expressed throughout the viral replicative cycle, although its transcription is down-regulated at late times (Mocarski, 1996). The IE2 pp86 is a promiscuous *trans*-activator, and associates with a number of viral (e.g. pUL84) and cellular products involved in transcriptional and cell cycle regulation (Mocarski, 1996). Furthermore, IE2 pp86, either acting alone or augmented by the IE1 protein, greatly stimulates transcription from early gene promoters, whereas later products of this gene may be involved in down-regulating transcription from the IE region. Thus, the IE gene products are multifunctional proteins which influence

cellular and viral pathways in different ways to provide optimal conditions for CMV infection (Stinski, 1990; Mocarski, 1996).

After the synthesis of the IE gene products, there is a switch from restricted to extensive transcription of the viral genome. Many of the early genes are involved in viral DNA synthesis, and early gene expression is blocked by agents which interfere with protein synthesis (Mocarski, 1996). Early genes involved in the replication of CMV include the 140kD viral DNA polymerase (pUL54), the 140kD single stranded DNA binding protein (pUL57), and proteins of 65kD and 34kD (pUL84 and pUL112-113, respectively). These both promote viral DNA replication and may edit newly synthesised DNA (Stinski, 1990; Mocarski, 1996). Viral DNA synthesis is initiated on the episomal structure from the origin of replication (*ori_{ht}*) and proceeds in both directions to form a rolling circle, yielding 'head to tail' concatameric DNA. This is then cut into genome sized lengths for packaging, although the actual process is unclear (Mocarski, 1996). After the first round of DNA synthesis (approximately 12 hours), abundant transcription of early genes continues throughout the infection. Some of these products are required for the transcriptional stimulation of the late gene products, e.g. a glycoprotein of 48kD (gpUL4) and the products of the UL122-113 genes. In addition, cellular transcription factors are thought to be stimulated during this period, and selectively bind to the transcription units of the late genes (Mocarski, 1996). Some of the viral gene products, such as pp71 (ppUL82), pUL69 and a 52kD protein (pUL44), are expressed at the early stage but are most abundant at the late times of infection. The accumulation of these proteins at this latter stage may therefore be responsible for the second peak of viral DNA synthesis which occurs at the late times (72-96 hours).

The anti-viral agents, ganciclovir and foscarnet, act at this stage in the virus replication cycle. The pro-drug ganciclovir is a nucleoside analogue of guanosine, and requires phosphorylation to exert its effect (Crumpacker, 1996). The CMV UL97 gene product phosphorylates ganciclovir to the monophosphate form, and cellular enzymes then convert the monophosphate to ganciclovir triphosphate, which is the actual inhibitor of viral DNA synthesis (Crumpacker, 1996; Littler et al. 1992). The active form of ganciclovir competitively inhibits the incorporation of deoxyguanosine triphosphate into elongating viral DNA (Crumpacker, 1996). Once incorporated, the

ganciclovir-terminated DNA is a poor substrate for chain elongation, and thus viral DNA chains^{elongate} slowly and terminate prematurely (Crumpacker, 1996). Foscarnet (phosphonoformic acid) 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 (Crumpacker, 1992). This inhibits the formation of the 3'-5'-phosphodiester bond between the primer and the substrate, thus preventing chain elongation (Crumpacker, 1992).

The final genes to be produced are the late genes, and these are sensitive to inhibitors of viral DNA synthesis, such as ganciclovir and foscarnet. These genes are mainly the structural genes of the capsid, tegument and envelope, which are required to form the virion coat (described in Section 1.2). In addition, some of these proteins may play an important role in regulating transcription, viral DNA synthesis or viral assembly, as well as modifying host cell proteins (Stinski, 1990; Mocarski, 1996). The overall level of transcription of these late genes is directionally proportional to the amount of viral DNA synthesis, although most, but not all, of the late genes do not require DNA synthesis to be transcribed (Mocarski, 1996). Thus it is thought that some of these genes, although transcribed in the presence of viral DNA inhibitors, require a viral or cellular product, which is only induced after viral DNA synthesis, in order to be expressed (Mocarski, 1996).

Concomitant with the synthesis of the virion components and late DNA replication, the assembly of virus particles is initiated within the cell nucleus. The assembly proteins formed during this period are scaffolding proteins, which are required to form the virion structure, but are absent from the final product (Harper, 1994). The major assembly protein (pUL80) contains two components, one the actual scaffolding protein and the other a protease that is necessary to cleave the protein into a 35kD structure. The assembly protein is removed as DNA is packaged into the mature capsid particle (Harper, 1994). Assembly of the tegument is thought to occur as the nucleocapsid particles bud through the nuclear membrane, where initial envelopment may take place. However, it is unclear whether the final envelope is derived from the nuclear membrane or from cytoplasmic membrane structures (Mocarski, 1996). Insertion of the viral envelope glycoproteins is thought to occur in the endosomal compartment of the cell, and egress of the fully mature virions is

thought to be mediated by early endosomal compartments (Tooze et al. 1993). However, two other enveloped particles, in addition to the mature virion, may be produced during this period. Non-infectious virus particles produced during *in vitro* culture are composed mainly of the assembly protein, and thus arise from immature capsid structures (Irmiere, Gibson, 1985). A second defective particle, which is thought to occur from incorrect packaging arrangements, is termed the dense body. This is composed of abundant lower matrix protein (pp65), with very little other constituents except the envelope and the associated viral glycoproteins (Stinski, 1976). Both these particles do not contain DNA, and the function *in vivo* is unclear.

Once produced, the virus may now be shed into the surrounding body fluid, or may infect adjacent cells via cell to cell spread. In addition to the role in viral attachment, gB appears to play an important role in the cell to cell spread of internal virion progeny, and in the fusion of infected cells (Tugizov et al. 1994; Navarro et al. 1993). This spread is not affected by the addition of heparin sulphate to the medium, and is thus separate from the entry of cell free virus into host cells (Kari, Gehrz, 1992; Compton et al. 1993). Other proteins may also be responsible for the spread of the virus in specialised situations, such as the polar transmission of CMV in retinal epithelial cells via the US9 gene product (Maidji et al. 1996). Regardless of the mode of viral spread, however, many different cell types are either abortively, latently or permissively infected by the virus *in vivo*.

1.7. Cell types infected by CMV *in vivo* and *in vitro*, and their contributions to viral dissemination.

As stated above (Section 1.3), CMV receptors are found on a variety of cell types *in vitro*, but not all of these cells support permissive infection in culture. Therefore, it is assumed that a number of factors associated with the cells themselves determine the outcome of CMV infection following viral entry. This is perhaps not surprising considering CMV is, in part, dependant upon cellular factors in order to initiate the transcription of viral proteins, particularly those of the IE genes (Section 1.4). In fact, it is thought that induction of gene expression from the IE loci is important in determining the ability of CMV to replicate or to become latent in a variety of cell types, but is not thought to be the only determinant. The actual site(s)

of CMV latency in the host is still obscure, as are the cell types most commonly involved in disseminating the virus within immunocompromised patients. Although explanted autopsy tissues from individuals suffering from acute CMV disease have been shown to produce infectious virus when grown in culture (Hedley-White, Craighead, 1965), few distinct cell types *ex vivo* have proved capable of transmitting the virus to permissive cell types growing *in vitro*. Furthermore, isolation of the cells responsible for the reactivation of latent virus within the normal population has proved to be even more complicated. The only cells which were consistently capable of transmitting infectious virus to cultured cells were obtained from the mononuclear or polymorphonuclear fractions of peripheral blood from acutely infected immunocompromised individuals (Turtinen et al. 1987; Rinaldo, Jr. et al. 1977). These fractions have also been implicated in transmitting CMV via transfused blood obtained from the general population (Hersman et al. 1982; Winston et al. 1980).

In vivo, however, the presence of CMV has been detected in every major organ of the body by a variety of methods. The classical method involved histological identification of infected cells by specific nuclear and cytoplasmic 'inclusion bodies' (hence the name 'cytomegalic inclusion disease'). These are areas of microscopically visible sites of virus assembly or cellular damage, and are part of the distinctive changes which some viruses incur in susceptible cells as part of their generalised 'cytopathic effect' (CPE). Another classical CPE incurred by CMV is the enlargement of the cell in question (hence the name *cyto*=cell *megalo*=large virus), and this characteristic is associated with late viral antigen expression and virion production. The major sites of viral replication which have been identified by this method include the brain, placenta, lung, GI tract and associated organs, liver, heart, kidney, cornea, and peripheral blood of individuals suffering from CMV disease (Bevilacqua et al. 1985; Roberts et al. 1989; Lin et al. 1981; Furukawa et al. 1994; Egbert et al. 1980; Bronsther et al. 1988; Wu et al. 1992). However, in the latter site, the cells which manifested characteristic CPE were found to be of stromal cell origin, in particular, circulating endothelial cells (Grefte et al. 1993). This finding was surprising because endothelial cells are not usually associated with the peripheral blood circulation. Moreover, circulating endothelial cells have so far been isolated only from immunocompromised patients with either acute CMV viraemia or clinical disease

manifestation (Percivalle et al. 1993; Grefte et al. 1993; Salzberger et al. 1997), and are not present in the blood from normal individuals. With regards to the cells involved in the other sites associated with viral CPE, the most common cell types were also of stromal cell origin, in particular, endothelial cells and epithelial cells (Goodman, Porter, 1973; Foucar et al. 1981; Hedley-White, Craighead, 1965). Only a small number of 'non-stromal' inflammatory cells were identified by the presence of CPE, and these were found to be predominantly of macrophage origin (Craighead, 1971). The identification of cell types by these methods, whilst informative, did lead to a conceptual problem. This problem centred on the fact that, as stated above, epidemiological studies implicated transfused blood products as an important source of CMV infection, but the peripheral blood cells of normal individuals did not show any characteristic morphological changes.

The use of more recent techniques, which include immunocytochemical double staining methods, *in situ* hybridisation, and the polymerase chain reaction, have largely solved this problem. This is partly because these latter methods identify cells that are either 'transiently' infected during the acute stage of CMV infection, or that do not express the full range of viral/cellular factors which necessitate CPE. Thus, cells that harbour the virus, but only express a limited array of viral antigens (such as during a latent infection), may be detected. Using these methods, the organ distribution of CMV in young, otherwise healthy trauma victims, has been found to be similar to that seen in patients suffering from acute CMV infection, except that gene expression was limited to the IE stage of viral replication (Hendrix et al. 1997; Toorkey, Carrigan, 1989). Moreover, mononuclear cells from the peripheral blood of healthy individuals have also shown evidence that the viral genome is present (Hendrix et al. 1997; Stanier et al. 1989). This lack of complete CMV replication (and thus infectious virus) in the peripheral blood cells did not explain how the virus could be transmitted during transfusion procedures. An early report using the murine model of CMV infection showed that transfusion of blood from mice latently infected with murine CMV resulted in activation of the virus in allogeneic hosts, but rarely in isogenic recipients (Cheung, Lang, 1977). A recent isolated finding that circulating peripheral mononuclear blood cells from healthy CMV seropositive donors may also reactivate and transmit the virus under conditions of allogeneic stimulation further

suggests that an immunological reaction may be responsible (Soderberg Naucner et al. 1997). This, of course, does not explain how the polymorphonuclear fraction of blood cells from normal individuals can transmit the virus.

Limited CMV gene expression has also been found in the mononuclear fraction of peripheral blood from immunocompromised individuals (Gerna et al. 1992; Saltzman et al. 1988), and thus these mononuclear cells are a prime candidate for being the reservoir of latent virus. Likewise, the differentiated macrophage cell (which is a derivative of the monocyte) express IE and E genes, and a limited percentage of these cells also display late viral antigens (Gnann, Jr. et al. 1988; Sinzger et al. 1996; Taylor Wiedeman et al. 1994). Therefore, as the macrophage can apparently support viral replication, this lineage is implicated in viral dissemination. In addition, the polymorphonuclear fraction of peripheral blood from immunosuppressed individuals has also been shown to harbour the virus by DNA, RNA and IE antigen detection (Saltzman et al. 1988; Gerna et al. 1992; Turtinen et al. 1987), as have infiltrating neutrophils in tissues from seropositive patients (Sinzger et al. 1996; Sinzger et al. 1995). Indeed, in immunocompromised patients displaying CMV viraemia, the identification of CMV in the circulating neutrophils has often been associated with severe disease manifestation and poor clinical prognosis (Niubo et al. 1996; Podzamczar et al. 1997; Koskinen et al. 1993). However, most of these studies only monitored the structural protein, pp65, as a marker of infection (Revello et al. 1992). This fact has led to the suggestion that phagocytic neutrophils may acquire both the virion phosphoprotein from the ingestion of debris from virus-infected cells, and also assimilate viral DNA by similar methods. Other studies, however, have found that the virus was in fact harboured in an infectious form (Gerna et al. 1992; Turtinen et al. 1987), thus the ability of the virus to cause an infection in neutrophils remains controversial.

With regards to other cell types capable of supporting the full CMV replicative cycle *in vivo*, smooth muscle cells and fibroblasts have been identified, in addition to endothelial cells and epithelial cells described above (Sinzger et al. 1995). The finding that CMV does indeed infect fibroblasts *in vivo* provides relevance for the *in vitro* studies involving this cell type, as the fibroblast is the preferential, and in the majority of cases to date, the only cell type used to propagate the virus *in vitro*.

Bone marrow stromal cells derived from a patient who died of CMV related complications also expressed viral antigen and shed virus after culture (Boeckh et al. 1998), and viral DNA has been detected in early haematopoietic progenitor cells from the bone marrow of healthy individuals (Minton et al. 1994).

Further paradoxes exist between these *in vivo* studies, and the data obtained *in vitro*. Whereas the endothelial and epithelial cell were identified as major sites of infection *in vivo*, these were only poorly infected *in vitro*. Likewise, although circulating monocytes express a limited array of viral antigens *in vivo*, reproducing this infection in culture also proved difficult. One explanation for these discrepancies centres on the fact that the differentiation state of the cell in question influences viral susceptibility. Teratocarcinoma cells, embryonic cancer cells which can be induced to differentiate into distinct cell types, have been important in elucidating the latter finding. Whereas non-differentiated teratocarcinoma cells have been shown to be non-permissive for CMV infection, the induction of cell differentiation by phorbol esters and thymidine analogues appears to promote productive infection (St.Jeor, Rapp, 1973; Gonczol et al. 1984). This phenomenon has putatively been identified as being controlled by the 'modulator' region of the CMV IE genes, which repress transcription in non-differentiated cells, but possibly promote transcription in differentiated cells (Meier, Stinski, 1997). A similar finding has been observed in monocytes, where differentiation into macrophages supports full viral gene expression and replication (Weinshenker et al. 1988; Ibanez et al. 1991). In addition, treatment with short chain fatty acids, such as sodium butyrate, has been shown to induce CMV replication in a proportion of endothelial cells and epithelial cells (Radsak et al. 1989; Wu et al. 1994; Tanaka et al. 1991). The early finding that CMV has a propensity to infect replicating murine endothelial cells *in vitro* supports this view (Vogel, 1958). Thus under conditions of cellular differentiation, CMV may in fact be induced to express viral antigen *in vitro*.

Most of the above *in vitro* studies have been performed using laboratory strains of virus which have been passaged many times in fibroblasts, and this may have adversely affected the tropism for other cell types. Other studies have indicated that low passaged strains of CMV can productively infect cultured endothelial cells without further treatment (Waldman et al. 1989; Ho et al. 1984), whilst a recent

clinical isolate of CMV was found to replicate more efficiently in epithelial cells than the higher passaged laboratory virus strains (Mocarski et al. 1993). Similar results have also been obtained for cells of monocyte origin (Einhorn, Ost, 1984). Moreover, different clinical isolates of CMV targeted distinct components of the haemopoietic system, namely stromal cells or myeloid progenitor cells, whereas laboratory strains infected these cell types poorly (Sing, Ruscetti, 1990; Simmons et al. 1990). That different tissue tropism may occur *in vivo* has been indicated by a number of findings. One study, which investigated the *in vivo* importance of haemopoietic cells in acute CMV infections, found differences in both the distribution and level of CMV infection in leukocytes from tissues of individual patients (Sinzger et al. 1996). Another study found that separate clinical isolates obtained from bone marrow transplant patients had specific tropisms for different target cells (fibroblasts, macrophages, adipocytes and endothelial cells). Moreover, the latter study suggested that these different tropisms may have been responsible for differences in pathogenicity, and hence clinical outcome (Torok Storb et al. 1993). In support of this proposal, different gB genotypes of CMV have also been implicated in different tissue distributions of virus and clinical outcome (Rasmussen et al. 1997; Torok Storb et al. 1997).

Finally, the origin of the virus carried in the peripheral blood cell remains obscure, although inferences can be made from the above data. A number of reports indicate that CMV can infect haematopoietic progenitor cells early in their development (Minton et al. 1994; Kondo et al. 1994; Reiser et al. 1986). Furthermore, these multipotent bone marrow derived cells do not succumb to lytic infection, but rather retain their ability to proliferate and differentiate (Kondo et al. 1994; Zhuravskaya et al. 1997). Thus it has been proposed that the CMV genome is able to replicate in these immature blood cells during normal cellular proliferation (Maciejewski et al. 1992), but upon differentiation into macrophages, the virus reactivates and disseminates the infection (Minton et al. 1994; Zhuravskaya et al. 1997). Similarly, infection of stromal cells within the bone marrow microenvironment might allow the virus to spread to the haematopoietic progenitors during the maturation process (Simmons et al. 1990). An alternative suggestion is that the virus may be picked up during monocyte interactions with infected endothelial cells. One

in vitro study showed a bi-directional acquisition of CMV during endothelial cell and monocyte co-culture, thus supporting this presumption (Waldman et al. 1995b). The origin of the virus in the neutrophil might be a result of a similar interaction (Grundy et al. 1998).

Regardless of the origin of the virus, permissive infection usually triggers an immune response to CMV. Thus, not only the cell type, but also the quality of the immune response determines the outcome of the infection. This immune response depends on the recruitment of leukocytes from the peripheral blood to the site of infection, and the mechanisms responsible for this process are discussed below.

1.8. Leukocyte adherence to, and migration through endothelial cells .

Under normal conditions, most leukocytes circulate in the blood without interacting with other blood cells, or the endothelial cells which line the blood vessels. However, monocytic cells sporadically interact with the vessel wall to seed the underlying tissues with the resident macrophages that are essential for the development of both the innate and adaptive immune responses. This occurs at a low frequency, and once in the tissues, the macrophage does not recirculate (Johnston, Jr. 1988). In addition, there is continuous recirculation of both naïve and memory lymphocytes across the blood vessel wall, through the underlying tissues and into the lymphatic system, where transport to the lymphatic organs ensures that the lymphocyte will encounter any specific antigen which has become trapped there (Janeway, Travers, 1996b). This occurs at specialised sites, termed high endothelial venules, which are comprised of a distinct type of endothelium (Woodruff et al. 1987). If no specific antigen is encountered, the lymphocytes re-enter the blood circulatory system and continue to mediate immune surveillance of the body tissues. In contrast, when an inflammatory signal has been generated, it is necessary for various leukocyte types to enter the body tissues through venular endothelium at multiple sites (Shimizu et al. 1992; McEver, 1992). The endothelial cell thus provides an important barrier between the blood and the underlying tissues, and interaction with the endothelium is critical in regulating the leukocyte response to physical injury or to invading micro-organisms.

Leukocyte movement during either a non-inflammatory or an inflammatory response involves the expression of three classes of adhesion molecules at both the endothelial cell and the leukocyte cell surface; these are the selectins, the integrins and the members of the immunoglobulin superfamily (Hogg, 1992; Butcher, 1991). In addition, small secreted chemoattractant proteins, collectively termed chemokines, are also important in this process (Kunkel et al. 1997). All these molecules interact to form a regulated series of events which begin with initial weak adhesion of the leukocytes to the endothelium, followed by a firmer adhesive interaction, and finally diapedesis between endothelial cell junctions and movement of leukocytes through the underlying tissues (Butcher, 1991).

The initial weak interaction is mediated by members of the selectin class of adhesion molecules expressed on either the leukocyte or the endothelial cell. There are three members of this glycoprotein family, all belonging to the same cluster of differentiation (CD). These three members are termed L-selectin (CD62^L), E-selectin (CD62^E) and P-selectin (CD62^P), and their ligands are carbohydrate epitopes on the surface of certain cell surface glycoproteins (McEver, 1992). L-selectin is present at the surface of naïve T-lymphocytes, B-lymphocytes, monocytes and natural killer cells, and binds to the sulphated carbohydrates of GlyCAM-1 and CD34, which are present on endothelial cells. P-selectin and E-selectin are expressed at the surface of endothelial cells in response to the inflammatory mediators leukotriene B₄, the complement protein C5a, or histamine in the case of P-selectin, and tumour necrosis factor alpha (TNF- α), bacterial lipopolysaccharide, or interleukin (IL-) 1 beta in the case of E-selectin (Butcher, 1991; McEver, 1992). Expression either occurs immediately (P-selectin) or a few hours after (E-selectin) exposure to the appropriate stimuli, but both molecules recognise the sialyl-Lewis X moiety (CD15^S) of certain leukocyte glycoproteins which are present on neutrophils, eosinophils and monocytes (Kuijpers, 1993). Regardless of the initial stimuli, interaction of the selectins with the appropriate ligand leads to reversible binding which is characterised by the leukocyte 'rolling' across the endothelium in the direction of the blood flow (Janeway, Travers, 1996b). This tethering interaction, whilst insufficient to promote diapedesis through the endothelium, is thought to prolong the exposure of the leukocyte to the vessel

wall, thus increasing the chance of interacting with the molecules necessary for firmer binding.

The firmer adhesive interactions are mediated, in part, by the chemokines which both attract and activate the leukocytes, and by the adhesion molecules of both the integrin and immunoglobulin superfamily. The chemokines are small monomeric proteins (<80 amino acids) that are secreted by various cell types (e.g. macrophages, endothelial cells and fibroblasts) (Kunkel et al. 1997). Members of the chemokine family are separated into four broad categories, the C-X-C chemokines (e.g. interleukin (IL)-8), the C-C chemokines (eg monocyte chemoattractant protein 1), the C chemokine, lymphotactin, and a chemokine-like structure bearing a CX₃X motif (Baggiolini, 1998). In general, the C-X-C chemokines are responsible for promoting the migration of neutrophils, and C-C chemokines are responsible for promoting the migration of monocytes. Both, however, may also be responsible for promoting the migration of a T-lymphocyte subset, whereas the C chemokine lymphotactin appears to act preferentially on lymphocytes (Baggiolini, 1998). Rolling of the leukocyte across the endothelial cell surface allows contact with chemokine molecules which are locally bound to proteoglycans at the endothelial cell surface (Tanaka et al. 1993b). The leukocytes possess distinct receptors for these different chemokine molecules (Baggiolini, 1998), which upon encounter, induce a conformational change in certain integrin molecules present on the surface of the leukocytes (Luscinskas et al. 1992; Tanaka et al. 1993).

The integrins are comprised of two distinct chains, the α and β chains. There are 14 α chains and 8 β chains which have been isolated to date, and these associate with each other to produce the distinct integrin molecules (Smyth et al. 1993; Hynes, 1992). The integrins which are important in the diapedesis of leukocytes across the endothelial cell surface are members which contain the β 1 (CD29) and β 2 (CD18) chains. There are three members of the β 2 integrins, which are distinguished by association with distinct CD11 α chains; CD11a (α^L), CD11b (α^M) and CD11c (α^X). Association of CD11a or CD11b with the CD18 (β 2) chain on the surface of leukocytes leads to the expression of the adhesion molecules leukocyte function antigen 1 (LFA-1) and Mac-1, respectively (Smyth et al. 1993; Hynes, 1992). Both of

these integrins recognise a member of the immunoglobulin superfamily, termed intercellular adhesion molecule 1 (ICAM-1, CD54), whereas LFA-1 additionally recognises other members of this superfamily, termed ICAM-2 (CD102) and ICAM-3 (CD50) (de Fougères, Springer, 1992; Springer, 1990). Endothelial cells constitutively express ICAM-1 and ICAM-2, but only the expression of ICAM-1 is increased by the inflammatory mediators IL-1 α , TNF- α , and interferon (IFN) gamma approximately 24 hours after exposure (Nortamo et al. 1991; Denning et al. 1988). In addition, the integrin very late antigen 4 (VLA-4), which is comprised of the α 4 (CD49d) integrin chain in association with the β 1 integrin chain (CD29), is expressed on the surface of the antigenically activated lymphocytes, and at the surface of unactivated natural killer cells and monocytes (Issekutz et al. 1995; Allavena et al. 1991; Janeway, Travers, 1996). One counter-receptor for VLA-4 is also a member of the immunoglobulin superfamily and is termed vascular cell adhesion molecule 1 (VCAM-1, CD106) (Smyth et al. 1993; Rice et al. 1990). The expression of the latter adhesion molecule is induced on the surface of endothelial cells by the cytokines IL-1 β , TNF- α , and IL-4 approximately 16-24 hours after exposure (Briscoe et al. 1992; Shimizu et al. 1991). The consequence of this integrin/immunoglobulin interaction is that the leukocyte attaches firmly to the endothelium and the rolling is arrested. This firm adhesion process is thus essentially the same in both the non-inflammatory and inflammatory reactions.

The leukocyte is now poised to cross the endothelial cell surface. The leukocyte flattens and extravasates into the underlying tissues by moving between the junction of two endothelial cells in juxtaposition. This obviously requires that firm adhesion is reversed, but the mechanisms responsible for this are far from clear. However, the migration of the leukocyte through the tissues is known to be under the influence of chemoattractant molecules (Kunkel et al. 1997). The proteoglycans expressed on the extracellular matrix of the underlying tissues bind locally secreted chemokines and provide a solid substrate through which the leukocytes migrate. Leukocyte migration is thus directed across a gradient of the chemokine, which increases in concentration towards the site of infection (Baggiolini, 1998; Kunkel et al. 1997). Although the exact mechanisms involved in this final process are still

unknown, ICAM-1 (Oppenheimer Marks et al. 1990) and a fourth member of the immunoglobulin superfamily, platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) is thought to be important (Berman et al. 1996; Zocchi et al. 1996; Muller et al. 1993). PECAM-1 is expressed on granulocytes, platelets, monocytes, B-lymphocytes and endothelial cells, and adhesion is either homotypic (i.e. the ligand for PECAM-1 is PECAM-1), or possibly heterotypic (Newman, 1997). Regardless of the adhesion molecules involved, the final result is the controlled movement of the leukocyte from the blood and into the body tissues, where it may now exert its immune effect.

1.9. The innate and adaptive immune response mediated by the host.

The immune response to any infectious agent consists of non-specific (innate) and specific (adaptive) recognition of the invading organism. The former response is generally mediated by the non-specific recognition of pathogen by macrophages, and occurs quickly after infection (Janeway, Travers, 1996b). These cells are stationed at sentinel points throughout the body, and operate as 'housekeepers' by removing both endogenous and exogenous debris (Johnston, Jr. 1988). During the course of this debris removal, macrophages can recognise extracellular pathogens which are present in the vicinity. This recognition is aided by cell surface receptors for opsinins such as immunoglobulins (Ig) and the complement component C3, and results in the macrophage phagocytosing the invading organism (Johnston, Jr. 1988). The foreign material can then be broken down and presented to the cells of the adaptive immune system (see below). Furthermore, due to their high secretory potential (Wewers, 1997), macrophages can indirectly inhibit virus replication in neighbouring cells. One important set of products which achieve this aim are the Type I interferons (IFN- α and IFN- β), which are not only secreted by the macrophages, but also by other cell types in response to an infection (Sen, 1997).

Additional cytokines secreted by the macrophages, such as TNF- α and IL-8 (Wewers, 1997) can also indirectly affect viral replication, by attracting and activating two additional cell types, the neutrophils and the natural killer cells (Lanier et al. 1997; Fantone, 1997). The former cell type is quickly recruited to the site of inflammation, where it acts to clear both foreign pathogen, and damaged tissues in

preparation for a wound healing response. Moreover, neutrophils can secrete a number of cytokines, including TNF- α , IL-1 β , and members of the C-X-C and C-C chemokine family, which further drives the inflammatory reaction by recruiting additional leukocytes to the site of infection (Fantone, 1997). Natural killer cells are important in the early phases of immune responses against certain viruses, parasites and microbial pathogens (Herberman, Ortaldo, 1981). These cells are highly cytotoxic and rapidly eliminate the virally infected target, and because they become functional in the absence of major histocompatibility antigen (MHC) expression, have been termed 'non-MHC restricted' (Long et al. 1997). Furthermore, the ability of natural killer cells to produce IFN- γ rapidly after infection plays a critical role in the innate defence mechanisms (Lanier et al. 1997), by inducing both an antiviral state in surrounding cells, and by promoting the microbicidal 'respiratory burst' in activated macrophages (Ruby, 1997; Karupiah, Harris, 1997). Consequently, by lysing cells early in the course of an infection, the macrophages, neutrophils and natural killer cells can restrict the amount of pathogen which is produced. This, however, is not always successful, and many organisms can survive or replicate within the macrophages themselves.

Once the innate immune response has been breached by the invading organism, an adaptive immune response is generated by the lymphocytes. This first requires that antigen is specifically recognised by one of two highly variable receptor molecules that are present on the surface of the lymphocytes; the T cell receptor (TCR) on the T-lymphocytes and the membrane bound immunoglobulin molecules on the B-lymphocytes (Davis, Bjorkman, 1988; Reth, 1994). In both cases, firmer interactions between the antigen and its specific receptor are conferred by co-receptor molecules present at the surface of the lymphocytes. The co-receptor at the surface of the T-lymphocyte consists of either CD4 or CD8 (hence these T-lymphocytes are termed CD4⁺ or CD8⁺), whilst the co-receptor at the surface of B-lymphocytes is a complex of CD21, CD19 and CD81 (Fearon, 1993; Janeway, Jr. 1992). A further requirement for the generation of adaptive immunity is that the naïve (unexposed to specific antigen) lymphocytes are induced to proliferate and differentiate into the effector cells responsible for the cell-mediated (T-lymphocyte) and the humoral (B-

lymphocyte) 'arm' of the response. This occurs in specialised sites away from the focus of infection, and is dependant upon 'professional' antigen presenting cells that produce the necessary co-stimulatory signals; B7.1 (CD80) and B7.2 in the case of the T-lymphocytes, or the CD40 ligand in the case of the B-lymphocytes. These are recognised by the lymphocyte molecules CD28 and CD40, respectively (Janeway, Travers, 1996c; Janeway, Travers, 1996c). Additional requirements, such as cytokine production, adhesion molecule expression and consolidation of antigen at the surface of specialised cells, are necessary to promote the generation of highly specific effector lymphocytes, which can recognise and destroy the invading pathogen.

Effector T-lymphocytes, appropriately 'armed' with a TCR capable of recognising molecules to which it has previously been exposed, can now migrate to the site of infection to carry out their individual functions. Once recruited by the inflammatory mechanisms outlined in Section 1.7, the T-lymphocyte now 'samples' the tissues for foreign antigen which is displayed at the cell surface via molecules encoded by the major histocompatibility complex (MHC). Replication of infectious agents within the intracellular cytosol of the infected cell leads to the presentation of processed antigen in association with MHC Class I molecules, and this MHC:peptide complex is recognised by the TCR of CD8⁺ T- lymphocytes (Parham, 1992; Townsend et al. 1989). In contrast, endocytosis of the infectious organism from the extracellular fluid results in the degraded antigen associating with the MHC Class II molecules, and this MHC:peptide complex is recognised by the TCR of CD4⁺ T- lymphocytes (Parham, 1992; Neefjes et al. 1990). Subsequent recognition of the specific MHC Class I:peptide complex by the TCR of CD8⁺ lymphocytes generates signals within the lymphocyte which lead to the secretion of cytotoxins. These soluble proteins ultimately destroy the infected target cell, hence these effector CD8⁺ lymphocytes are also known as 'cytotoxic T cells' (CTL) (Janeway, Travers, 1996c). In contrast, recognition of the specific MHC Class II:peptide complex by the TCR of CD4⁺ lymphocytes generates one of two signals within the cell. The CD4⁺ lymphocytes either secrete cytokines which activate the cytotoxic or inflammatory properties of the macrophages, or else they secrete soluble factors which are necessary to promote antibody production and differentiation of B-lymphocytes. Thus effector CD4⁺ lymphocytes may be further divided into inflammatory T-lymphocytes

(Th1) or helper T-lymphocytes (Th2), depending on the cytokines they secrete (Janeway, Travers, 1996c).

B-lymphocytes recognise specific antigen, either on the pathogen itself or inserted into cells during the course of the infection, by virtue of the immunoglobulin molecules expressed on the cell surface. The bound antigen is then endocytosed, and the internalised antigen is degraded and represented at the cell surface via MHC Class II molecules (Janeway, Travers, 1996a). Activated Th2 CD4⁺ T-lymphocytes can now recognise this processed antigen in the context of MHC:peptide, and thus 'help' the B-lymphocyte to secrete antibody capable of recognising molecules to which it has previously been exposed. These antibodies have one of three functions; they either neutralise the pathogen, or else they mark the pathogen for destruction by phagocytosis or complement activation (Janeway, Travers, 1996b). These latter two functions are carried out by the cells of the innate immune response, and thus B-lymphocytes, via antibody, confer 'specificity' on the otherwise non-specific cells of the innate immune system.

Direct neutralisation of the pathogen by antibodies can result from either surface conformational changes caused by antibody binding, or by blocking the interactions of the pathogen with viral cell surface receptor, required for initial entry events (Section 1.4), through steric hindrance. IgG is probably the most important class of antibody involved in this process. In addition, coating the pathogen in antibody can increase the ability of mononuclear cells or polymorphonuclear leukocytes to phagocytose the organism (Burton, Woof, 1992). This function is mediated by the presence of receptors for the Fc portion of the antibody on the surface of these cells, and again, IgG Fc receptors (FcγR) are probably the most important. There are three human FcγR's (termed FcγRI, FcγRII and FcγRIII) which are distributed on a variety of cell types, most notably those with phagocytic capabilities (i.e. the macrophages and the neutrophils) (Hullett, Hogarth, 1998). Binding of the antibody-coated pathogen to the FcγR results in endocytosis of the antigen:antibody complex and thus brings it into contact with destructive enzymes within the phagocyte. Antibody binding also leads to the activation of the 'classical' pathway of the complement cascade, which assists in both the neutralisation and the

destruction of the pathogen (Burton, Woof, 1992). Finally, pathogens may insert glycoproteins into the surface membrane of the infected cells during their replicative cycle. These glycoproteins are recognised by specific antibody, which is in turn recognised by the Fc γ R at the surface of the activated natural killer cell. These leukocytes then destroy the infected cell in a similar manner to that of the CTL, and this is termed antibody-dependant cell mediated cytotoxicity (Janeway, Travers, 1996b).

Thus the cells and molecules of the immune system work as an integrated host defence system to eliminate the infectious agent and to provide long lasting protective immunity. The effector cells of the innate immune response act immediately after encountering the pathogen, whilst the cells of the adaptive immune response must be activated and clonally expanded before they can carry out their effector functions. This requirement means that there is a delay in the generation of the adaptive immune response in the face of primary infection. Following re-exposure to the original pathogen, however, circulating 'memory' lymphocytes can more quickly recognise the organism and generate a faster immune attack (Janeway, Travers, 1996b). Therefore, there is a difference in the timing of the adaptive immune response following primary or secondary infection. These responses are important in controlling all infectious agents, including CMV.

1.10. The role of the host immune response in limiting CMV infection.

Although the exact mechanism of the protective immune response to CMV is unknown, a number of inferences can be made by studying the data from susceptible individuals. Clinical symptoms associated with CMV infections almost exclusively involve individuals with impaired immunity, and this is usually associated with T-lymphocyte defects (see Section 1.1). Furthermore, seronegative transplant recipients are at risk from primary infection, and often develop more severe disease than an individual who is undergoing a reactivation response, as do neonates who are born to mothers with a primary infection (see Section 1.1). These data suggest that an adaptive immune response is important in protecting the infected individual from CMV disease, although there is some evidence to suggest that the innate immune response plays a role in limiting infection.

In healthy individuals, natural killer cells from the peripheral blood of both seronegative and seropositive individuals were shown to be capable of lysing CMV-infected cells, although there were significantly higher numbers of natural killer cells in the latter case (Kirmani et al. 1981). Conversely, in children with symptomatic congenital CMV infection, a reduced number of natural killer cells were detected in the peripheral blood than for children who experienced an asymptomatic infection (Cauda et al. 1987). There has also been a correlation between the lack of a natural killer response and lethal infection in mice infected with murine CMV (Bukowski et al. 1985; Bukowski et al. 1985) and a similar result has been confirmed in humans. In particular, a positive correlation between the ability of bone marrow transplant recipients to induce a natural cytotoxic response against CMV-infected target cells and an increase in survival rate has been reported (Quinnan, Jr. et al. 1982; Quinnan, Jr. et al. 1981), as has the ability of renal transplant patients to mount a strong natural killer response during primary CMV infection (van den Berg et al. 1993). Furthermore, one patient who had an insufficient natural killer response due to a congenital defect developed severe protracted CMV infection, and also developed CMV-associated pneumonitis (Biron et al. 1989). This patient had normal T-lymphocytes and antibody responses, suggesting it was the natural killer cell defect which allowed the CMV disease to occur.

With regards to the adaptive immune response, a protective role for antibody is likely in some patient populations. Pre-existing immunity has been shown to limit the severity of disease associated with CMV infection in solid organ transplant recipients (Smiley et al. 1985). This has been attributed to antibody mediated protection, since the cellular responses in these patients were depressed. Conversely, although the antibody response was delayed immediately after heart transplantation, severe disease occurred in those individuals with no or a reduced ability to mount an antibody response (Rasmussen et al. 1982), and a similar finding has been observed following bone marrow transplantation (Neiman et al. 1977). In congenitally infected infants, pre-existing immunity also seemed to prevent severe foetal infections and disease in the newborn (Fowler et al. 1992), as both transmission and severe clinical manifestations were associated with previously seronegative mothers, despite a similar incidence of CMV infection between these women (Britt, 1996). The passive

transfer of protective antibodies across the placenta was thought to prevent viral transmission because cell mediated responses in these individuals were depressed. In addition, passively transferred hyperimmune globulin appeared ^{to} result in a decrease of the severity of CMV infections in renal allograft recipients who underwent such prophylaxis (Snydman et al. 1993; Snydman et al. 1987). The data for hyperimmune globulin administration in BMT patients are more controversial, although there is a suggestion that early prophylaxis reduces the severity of disease in this patient group (Britt, 1994).

These above findings in man are supported by results from the murine model of CMV infection, where transfer of immune serum into B-lymphocyte deficient mice during viral reactivation decreased the virus titre in both the spleen and the lungs (Jonjic et al. 1994). Furthermore, the administration of polyclonal murine CMV-specific antibody, either prior to or after the viral infection, prevented systemic and ocular viral dissemination in infected mice, presumably by neutralising cell-free virus in the bloodstream (Hayashi et al. 1995; Shanley et al. 1981). Interestingly, high levels of neutralising antibody in HIV positive patients with CMV-associated retinitis correlated with a delay in the progression of invasive CMV disease, although the overall antibody titre did not. Thus the administration of passively-transferred antibody did not appear to prevent CMV infection *per se*, as the overall incidence of infection was not reduced (Boppana et al. 1995). This finding is also supported by the data from other patient populations. CMV can be reactivated and shed persistently in pregnant women and members of the general population, despite the presence of CMV specific antibodies. Moreover, the women who transmitted CMV to the foetus were found to contain an higher CMV antibody titre than women who did not transplacentally infect their offspring (Britt, 1996). This presumably reflects an higher rate of viral replication in the former group, and indicates that maternal CMV-specific antibodies might not be sufficient to prevent invasive disease in the foetus. Thus, although the presence of antibodies might limit viral spread in certain populations, it does not exclusively preclude viral excretion following infection with CMV.

A number of findings infer that the cell-mediated response to herpesvirus infections is more important in the resolution of infection than the humoral immune response. In bone marrow transplant recipients, the ability to reconstitute MHC-

restricted CMV-specific CTL activity to levels found in normal individuals correlated with a favourable outcome in the post-transplant period (Reusser et al. 1991). Moreover, a low or absent CTL response in this group of patients was also correlated with an increased mortality rate (Reusser et al. 1991). Similarly, in renal transplant patients, the most severe symptoms of disease were associated with both a primary infection and the failure of the host to mount a T-lymphocyte response to CMV (van den Berg et al. 1993), and a weaker immune response was recorded in the peripheral blood of heart transplant recipients with severe infections than with mild or asymptomatic CMV infections (Koskinen et al. 1994).

Support of a role for the CTL response being able to clear infections again comes from the murine model of CMV infection. Mice which were immunosuppressed through γ -irradiation showed enhanced virus growth and an increased mortality rate compared to their non-irradiated counterparts, but protection from murine CMV was restored by the adoptive transfer of spleen cells (Reddehase et al. 1985). The cells responsible for this protective response were found to be CD8⁺ T-lymphocytes (Reddehase et al. 1985). More recently, the adoptive transfer of CMV-specific CD8⁺ lymphocytes, but not CD4⁺ lymphocytes, modulated the course of infection in bone marrow depleted mice by reducing the viral load in the lung, adrenal and salivary glands (Steffens et al. 1998). This finding was important because it has previously been shown that the extent of virus dissemination in the acute phase of murine CMV infection determines the subsequent viral load during latency (Reddehase et al. 1994). Furthermore, an high viral load was associated with a greater frequency of reactivation in these latently infected mice (Reddehase et al. 1994). Thus, although the administration of the CD8⁺ lymphocytes did not prevent the establishment of latency in mice, a reduced risk of recurrent infection occurred because of this adoptive transfer procedure (Steffens et al. 1998). These findings are encouraging, as a similar adoptive transfer approach is being used in humans (Greenberg et al. 1991).

Although the majority of studies imply that the CD8⁺ lymphocyte response is the most important in providing protective immunity, some evidence suggests that CD4⁺ T-lymphocytes may also be protective. Congenitally infected infants who had a prolonged failure to generate a CMV-specific T-lymphocyte response also exhibited

diminished lymphokine production. These defects were related to low frequencies of virus-specific CD4⁺ T-lymphocytes (Hayward et al. 1984). Furthermore, significant association between the presence and absence of CMV-specific CD4⁺ responses and the development of CTL in bone marrow recipients was found, although it was a defect in the latter response which was associated with fatal infection (Reusser et al. 1991). This implied that the CD4⁺ lymphocyte may play an important role in the simultaneous generation of the CTL response. A separate study, however, demonstrated that lymphocytes from healthy seropositive volunteers could promote the lysis of CMV-infected monocytes in a Class II restricted manner, suggesting a direct antiviral effect of CD4⁺ lymphocytes (Lindsley et al. 1986). In the murine model, CD4⁺ depletion of infected B-lymphocyte deficient mice abrogated virus clearance from the salivary gland epithelial cells, whereas CD8⁺ depletion did not (Jonjic et al. 1989; Jonjic et al. 1990). However, the role for the CD4⁺ lymphocyte in viral clearance may be unique to the salivary glands.

1.11. CMV interaction with the immune system, and possible contribution to pathogenesis.

Although an immune response to CMV is generated during the course of an acute infection, the virus has evolved a number of mechanisms in order to limit the immune reaction and thus ensure its survival. However, in doing so, CMV may also provoke a pathogenic response from the host. As stated above (section 1.6), CMV can directly infect cells of leukocyte origin, and an acute infection with CMV is often associated with a sustained general dysfunction of the immune system. Infection of mononuclear cells with CMV has been shown to profoundly alter their function in both humans and mice. Virus-induced suppression of macrophage accessory activities can lead to decreased lymphocyte proliferation, diminished natural killer cell activity, and impaired CTL responses to CMV-infected cells (Schrier, Oldstone, 1986; Buchmeier, Cooper, 1989), thus dampening the immune response to the virus. In immunologically incompetent individuals, however, this may further perturb the ability of the host not only to control the dissemination of CMV, but also to control other infectious agents as well (Chatterjee et al. 1978). Similarly, whilst the ability of CMV to establish an infection in haematopoietic progenitor and stem cells without

lytic effect may elicit a replicative environment which is not subject to immune surveillance, decreased regeneration of bone marrow precursors may promote graft loss in bone marrow recipients (Mayer et al. 1997; Mutter et al. 1988). Hence, infection with CMV may further complicate the condition of already immunosuppressed patients.

CMV induces the expression of an FcγR on the surface of infected fibroblasts, in common with other members of the herpesviridae (Litwin et al. 1990; McTaggart et al. 1978; Furukawa et al. 1975). These FcγR's may protect the infected cells from antibody-mediated destruction by binding the Fc portion of nonvirus-specific antibody, thereby impeding the attachment of specific antiviral IgG to the cell surface (Adler et al. 1978). Moreover, the simultaneous binding of virus-specific antibody to the viral antigen and to the virus-induced FcγR could prevent the functional engagement of the Fc portion of the antibody, thus preventing its participation in antibody-dependent cell mediated cytotoxicity, or antibody plus complement-mediated lysis (Dubin et al. 1991). Alternatively, whilst the induction of FcγR's may protect the virus from antibody-mediated host immune defences, aberrant expression of FcγRs might also result in the binding of immune complexes to the infected cell, a phenomenon that could contribute to tissue injury (Oldstone, 1975) or to the initial lesions involved in the development of atherosclerotic plaques (Griffith et al. 1988; Raines, Ross, 1993). Furthermore, there is evidence to suggest that immune complex formation may occur during, or be responsible for, the initiation of chronic rejection in kidney allograft recipients (Hayry et al. 1992a; Hancock et al. 1993a), thus CMV might initiate the deposition of immune complexes via the induction of a FcγR, which could lead to the destruction of host tissues.

Another potential immune escape mechanism which is associated with CMV involves the modulation of MHC Class I and Class II molecules. It has been shown that a down-regulation of Class I molecules occurs at the surface of CMV-infected fibroblasts *in vitro* (Yamashita et al. 1993; Barnes, Grundy, 1992), and a similar phenomenon has been reported during murine CMV infection (Campbell, Slater, 1994). In both cases, the viral expression of IE and early genes was necessary to promote the Class I down-regulation (Campbell, Slater, 1994; Ahn et al. 1996). The

interaction of early viral glycoproteins with the assembly of Class I:peptide complexes rendered them unstable, and consequently incapable of being transported to the cell surface (Davis-Poynter, Farrell, 1998). Furthermore, transfection of a CMV-encoded MHC Class I homologue (pUL18) led to an inhibition of natural killer cell function in both mice and man (Farrell et al. 1997; Reyburn et al. 1997). However, the expression of pUL18 during a natural infection, either *in vitro* or *in vivo*, has yet to be reported. Analogously, it has been suggested that Class II molecules were prevented from being induced by IFN- γ at the surface of endothelial cells following infection with CMV *in vitro* (Scholz et al. 1992), and inducible expression of Class II at the surface of murine macrophages was also prevented following infection with murine CMV (Heise et al. 1998a). This modulation in Class II expression was thought to prevent the interaction of CD4⁺ effector lymphocytes with the CMV-infected target cell by blocking the antigen presenting functions of the latter cells.

In vivo, however, the data to support reduced MHC distribution at the surface of CMV-infected cells are scant. In contrast, there are several reports suggesting that CMV infection augments the expression of Class I molecules at the surface of epithelial (van Dorp et al. 1993), smooth muscle (Hosenpud et al. 1991), and endothelial cells *in vitro* (Tuder et al. 1994; van Dorp et al. 1989). Similarly, CMV infection of thyroid follicular cells *in vitro* resulted in the increased expression of Class II molecules (Khoury et al. 1991), as did the *in vitro* infection of rat endothelial cells with rat CMV (Ustinov et al. 1993). Furthermore, adult rats undergoing an acute CMV infection *in vivo* displayed increased Class II antigens on endothelial cells in the kidney, liver and heart (Ustinov et al. 1994b), and the expression of these molecules was similarly augmented on tubular and endothelial cells of human renal allograft recipients undergoing an acute CMV infection *in vivo* (von Willebrand et al. 1986). However, it is unclear whether these *in vivo* effects were a result of direct viral infection, or mediated through the secretion of soluble inflammatory mediators.

Regardless of the mechanism involved, the increased MHC expression observed during CMV infection is interesting because MHC antigens are thought to be an important target in transplant rejection (Shoskes, Wood, 1994). Increased expression of Class I and Class II molecules has been with associated with the

initiation of rejection episodes in rat cardiac allografts (Milton, Fabre, 1985). In humans, the increase in Class II molecules is similarly associated with acute rejection episodes in kidney transplant recipients (von Willebrand et al. 1993; Hall et al. 1984), and with the development of accelerated atherosclerosis in cardiac transplant recipients (Labarrere et al. 1995). Similarly, increased Class I expression was observed on hepatocytes, bile duct epithelium and sinusoidal endothelium, and increased Class II expression on Kupffer cells and sinusoidal endothelium during liver allograft failure (Gouw et al. 1988). The induced MHC expression on the allograft was thought to promote immune activation, by providing a greater concentration of target molecules on the donor cells (Shoskes, Wood, 1994). Thus, the increased expression of Class I and Class II molecules on various cell types during CMV infection may elicit CD8⁺ and CD4⁺ lymphocyte activation, leading to the generalised destruction of the allograft.

Graft rejection and accelerated atherosclerosis are also characterised by the infiltration of T-lymphocytes, monocytes (Hayry et al. 1992b) and neutrophils (Adams et al. 1990) into the blood vessels and tissues of the donor organ, and alterations to adhesion molecules have been associated with the extravasation of these leukocytes during both acute and chronic rejection. In cardiac transplant recipients, there was an increase of ICAM-1 and VCAM-1 expression on capillary venular endothelial cells associated with a T-lymphocyte infiltrate during acute allograft rejection (Briscoe et al. 1991), and an increased ICAM-1 induction on tubular cells during kidney rejection has been demonstrated in biopsy specimens (Brockmeyer et al. 1993). Interestingly, up-regulation of these molecules was also associated with CMV infection in solid organ transplant recipients (Koskinen, 1993; Lautenschlager et al. 1996). Increased expression of ICAM-1 and LFA-3 on hepatocytes, bile duct epithelium and endothelial cells of liver allografts undergoing severe rejection episodes has been demonstrated (Adams et al. 1989a; Steinhoff et al. 1993a), and these two adhesion molecules have also been shown to be up-regulated at the surface of fibroblasts following infection with CMV *in vitro* (Grundy, Downes, 1993).

The expression of E-selectin has been implicated, albeit to a lesser extent, as a predictor of rejection in both heart and renal allograft recipients (Ferran et al. 1993; Briscoe et al. 1995). This molecule has also been shown to be transiently up-regulated

during CMV infection in heart transplant recipients (Koskinen, 1993). The expression of E-selectin was also increased at the surface of endothelial cells following infection with CMV *in vitro*, and this has been implicated in promoting increased polymorphonuclear cell binding to the infected endothelium (Span et al. 1991a). Increased expression of ICAM-1 and VCAM-1 on CMV-infected endothelium was also implicated in promoting increased adhesiveness for T-lymphocytes and monocytes (Shahgasempour et al. 1997a). Thus, increased expression of adhesion molecules may result in a prolonged inflammatory reaction in the allograft following infection with CMV. However, *in vitro*, other authors have either failed to detect alterations in the expression of these molecules at the endothelial cell surface (Scholz et al. 1992), but rather have shown that infection with CMV prevents the cytokine inducible expression of VCAM-1 and E-selectin (Sedmak et al. 1994b). Thus the effect of CMV infection on the expression of both adhesion and MHC molecules at the surface of the endothelial cell is still controversial, and further studies are necessary to resolve these issues.

1.12. Aims of the project

A number of alterations to immunologically important molecules occur during an acute CMV infection *in vivo*, but the overall contribution that the virus makes to these changes is uncertain. The general aim of the project, therefore, was to characterise CMV infection of endothelial cells *in vitro*, as a model to assess the potential role of CMV infection in the pathogenesis of the accelerated form of atherosclerosis associated with chronic transplant rejection *in vivo*.

The specific aims of the project were three-fold. The first of these was to establish the optimal conditions for the productive infection of endothelial cells, using both laboratory strains of CMV and recent clinical isolates of the virus. In addition, the effect of sodium butyrate treatment on the ability of all viral stocks to replicate in the endothelial cell cultures was assessed. These parameters were included because it has previously been reported that both *in vitro* culture of the virus and cell differentiation may influence the extent to which CMV replicates in a particular cell type.

The second aim of the project was to analyse the effect of CMV infection of endothelial cells on the expression of MHC Class I and Class II antigens, cellular FcγR's, and the adhesion molecules E-selectin, ICAM-1, ICAM-2, VCAM-1, PECAM-1 and LFA-3. All these molecules are associated with an inflammatory or adaptive immune response *in vivo*, and most have been associated with the development of vascular disease. However, contradictory reports exist as to whether CMV induces or decreases the expression of these molecules on the surface of endothelial cells. An additional objective was to determine whether any observed changes to the aforementioned molecules were due to a direct viral effect on CMV-infected cells, or due to indirect cytokine-mediated effects on bystander uninfected cells.

The final aim of the project was to investigate whether CMV infection of endothelial cells affects the binding of subsets of peripheral blood leukocytes to the endothelium. Increased binding of neutrophils and mononuclear cells have been associated with vascular disease *in vivo*, and with the dissemination of CMV infection in the host. Furthermore, because leukocytic cell infiltrates have also been associated with the promotion of chronic inflammatory diseases *in vivo*, the ability of leukocytes to transmigrate through infected endothelium was additionally investigated.

CHAPTER 2.
MATERIALS AND METHODS

2.1. Fibroblast and Endothelial Cell Culture.

2.1.1. Fibroblasts.

Human embryonic lung fibroblasts were isolated from foetal lung tissue (MRC TissueBank, London, U.K.) by trypsin digestion (500µg/ml) and maintained in fibroblast growth medium, of which the constituents were minimal essential medium, 10% foetal calf serum (FCS), 50 I.U./ml penicillin, 50 µg/ml streptomycin and 2mM L-glutamine (Gibco BRL, Scotland, U.K.). Fibroblasts were used between passage 9 and 17. Some cells were stored in 10% dimethylsulfoxide and 90% FCS at -180°C until use.

2.1.2. Endothelial cells.

Human umbilical vein endothelial cells were isolated from umbilical cords by collagenase digestion (200 I.U./ml, Boehringer-Mannheim, East Sussex, U.K.) and maintained in endothelial cell growth medium. This consisted of medium 199 containing 20% heat inactivated FCS, 100 I.U./ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 50µg/ml endothelial cell growth factor (Sigma Chemical Company, Dorset, U.K.) and 20 I.U./ml heparin (CP Pharmaceuticals Ltd., Wrexham, U.K.). In some instances, 10ng/ml endothelial cell growth factor containing 8µg/ml heparin (Boehringer-Mannheim) was used instead of the endothelial cell growth factor supplied by Sigma. For endothelial cell subculture, all flasks were pre-treated for 2-3 hours with 2% gelatine, and the cells were used between passage 2 and 5. For transmigration studies and for experiments involving 8-well slide chambers, the endothelial cells were treated as described above, except that 2µg/cm² fibronectin (Boehringer-Mannheim) was used to pre-coat all tissue culture flasks in the former case, and to pre-coat individual slide chambers in the latter case. Some cells were stored in 10% dimethylsulfoxide and 90% FCS at -180°C until use.

All cells were negative when tested for mycoplasma using a [³H]-labelled DNA probe homologous to *Mycoplasma* and *Acholeplasma* ribosomal RNA (Gen-probe, San Diego, CA).

2.2. Viral Stocks.

2.2.1. AD169, Towne and Davis.

These laboratory strains of human cytomegalovirus (CMV) were obtained from the American Type Culture Collection and were propagated by the serial inoculation of cell-free virus onto uninfected fibroblasts for 2 hours at 37°C in 5% CO₂, after which the supernatant was removed and the fibroblasts were maintained in medium containing 4% FCS. To prepare the cell-free viral inoculum, each laboratory strain was harvested from the supernatant fluids of infected fibroblasts at days 5-10 post inoculation, clarified by centrifugation at 800g for 20 minutes, and stored at -80°C until use. These laboratory strains had been highly passaged through fibroblasts in this manner for a number of years (passage 91,128 and 73, AD169, Towne and Davis, respectively), and were passaged a further 3-8 times after purchase.

2.2.2. Toledo.

Fibroblast cells harbouring this low passage (passage 5-8) clinical strain of CMV were a kind gift from Dr. Stuart Starr, Philadelphia, U.S.A.. The Toledo strain of CMV was serially propagated by mixing the cell-associated virus with uninfected fibroblasts, at an approximate ratio of 1:3, respectively. After each passage, the presence of cell-free virus was tested by inoculating the supernatant fluids from 'mixed' cells onto uninfected fibroblasts, after the former inoculated cells were displaying >80% late cytopathic effect. After four such cell passages, cell-free virus was detected and the Toledo strain of CMV was harvested from the infected cell supernatant fluids, as described above. This cell-free virus was subsequently propagated through fibroblasts a further 2-4 times, in the manner described for the laboratory strains of CMV.

2.2.3. Clinical isolates.

The buffycoat was removed from 10ml venous blood of an HIV positive patient suffering from cytomegalovirus disease. The leukocytes were washed three times in Hank's buffered saline solution (HBSS) to remove excess anticoagulant, and the cells, which had been resuspended in 2ml of medium containing 4% FCS, were inoculated onto fibroblasts contained in 6-well plates (Gibco). After an incubation period of 1 hour at 37°C in 5% CO₂, the leukocytes were removed, the cells were washed twice in HBSS and the media was replaced with fibroblast growth medium. These fibroblasts showed

evidence of CMV infection one week later, and the clinical isolate was serially passaged by mixing infected cells with uninfected fibroblasts, as described for the Toledo strain of CMV. The secretion of cell-free virus was determined as described above, and detectable amounts of virus were present in the supernatant fluids of the infected cells after five such passages through fibroblasts. The clinical isolate (C1F) was then harvested from the supernatant fluids of the infected cells, and the cell-free virus was propagated through fibroblasts a further 1-3 times, as described above. A second clinical isolate, C2, was obtained from the blood of renal transplant patient undergoing a primary CMV infection. The leukocytes were inoculated onto fibroblasts, and the cells treated in the manner described above for C1F. However, in this case, the isolate (C2F) was still largely cell-associated at passage 10, and little cell-free virus was recovered from the supernatant fluids of the infected fibroblasts.

2.2.4. Herpes simplex virus, type 1 (HSV-1).

The laboratory strain of HSV-1 (KOS) was obtained from the American Type Culture Collection and propagated by the serial inoculation of cell-free virus onto uninfected fibroblasts. The virus was harvested from the supernatant fluids of infected fibroblasts 24-48 hours after the initial inoculation, and clarified by centrifugation, as described for the laboratory strains of CMV.

All virus stocks were negative when tested for mycoplasma using a [³H]-labelled DNA probe homologous to *Mycoplasma* and *Acholeplasma* ribosomal RNA (Gen-probe, San Diego, CA).

2.3. Endothelial Cell Adaptation of Viral Stocks.

2.3.1. Clinical isolates.

C1F infected fibroblasts were removed by trypsin digestion when the cells were showing >90% late cytopathic effect. At this stage (passage 2, termed C1F₂), the virus was still cell-associated. The infected cells (approximately 3x10⁵ cells) were resuspended in 3ml of medium containing 4% FCS, and subjected to a single freeze-thaw cycle (-180°C). The total thawed cell suspension was then inoculated onto endothelial cells growing in a 75cm² tissue culture flask. After an incubation period of 2 hours, the cells

were removed and 10ml of endothelial growth medium was added. These endothelial cells were serially passaged until late cytopathic effect developed in >80% of the cells, after which the medium was replaced with endothelial growth medium lacking heparin, and the supernatant was harvested daily for a week. This stock was termed C1F₂E. In addition, 3ml of clarified supernatant from C1F (passage 5, termed C1F₅) infected fibroblasts, which contained approximately $1-5 \times 10^5$ pfu/ml of cell-free virus (see section 2.11), was inoculated onto the endothelial cells by centrifugation at 1,500g for 1 hour at 22°C. After a further 1 hour incubation period at 37°C in 5% CO₂, the cells were treated with endothelial cell growth media containing 10mM sodium butyrate (Sigma). The drug was removed 48 hours later and the inoculated endothelial cells were serially passaged until late cytopathic effect developed in >80% of the cells, after which the supernatants were harvested, as described for C1F₂E. The virus obtained through this latter method of adaptation was termed C1F₅E. The supernatant fluids were tested for the presence of cell-free virus by inoculation onto both uninfected fibroblasts or uninfected endothelial cells, and supernatants containing the endothelial cell adapted clinical strain (C1FE) were clarified as described above for the fibroblast grown strains of CMV. Aliquots were stored at -80°C until use. A second clinical isolate, C2E, was prepared by inoculating the leukocytes from a renal transplant patient directly onto endothelial cells, in the absence of prior fibroblast isolation. The endothelial cells were subsequently passaged as required, although in this case, no sodium butyrate was added at any stage. The cells were observed for characteristic late cytopathic effect, and the supernatant tested for the presence of virus, as described above. However, this isolate (C2E), like the fibroblast grown counterpart C2F (see section 2.2.3), was also largely cell-associated at passage 10.

2.3.2. Toledo.

3ml of clarified supernatant from Toledo-infected fibroblasts (passage 12), which contained approximately $0.5-1 \times 10^5$ pfu/ml of cell-free virus (see section 2.11) was inoculated onto the endothelial cells by centrifugation at 1,500g for 1 hour at 22°C. After a further 1 hour incubation period at 37°C in 5% CO₂, the cells were treated with 10mM sodium butyrate. The drug was removed after 48 hours, and the inoculated endothelial cells were treated as for the clinical isolate, C1F₅E. This endothelial adapted strain was termed Toledo.E.

Supernatants from C1F- or Toledo- inoculated endothelial cells contained virus after 4-6 passages, and the cell-free virus was passaged a further 2-5 times through endothelial cells, in the manner previously described. In addition, cells containing the appropriate viral strain were stored at -180°C in 10% dimethylsulfoxide and 90% FCS.

2.4. Viral Infection of Cell Monolayers.

2.4.1. Fibroblasts.

Cells were seeded at a concentration of 3×10^5 per well in a 6- well plate, or 2.5×10^4 per well in an 8-well chamber slide, and inoculated for 1 hour with 1ml or 100 μl of viral supernatant, respectively. Cell monolayers were inoculated 24 hours after initial plating with a multiplicity of infection of 2 for CMV and a multiplicity of infection of 2 for HSV-1 (see section 2.11), unless otherwise stated.

2.4.2. Endothelial cells.

Cells were seeded at a concentration of 2.5×10^5 per well in a 6-well plate, or 2×10^4 per well in an 8-well chamber slide. For adhesion assays, confluent monolayers were prepared by seeding the endothelial cells at 5×10^4 /well in a 48 well plate. The volumes of viral supernatants used were 1ml per well for cells seeded in a 6-well plate, and 100 μl per well for cells growing in both 48-well plates, or 8-well chamber slides. Cell monolayers were inoculated 24 hours after initial plating with a multiplicity of infection of 2-5, unless otherwise stated. No difference in the ability of C1F₂E or C1F₅E to infect endothelial cells by this method was observed. For infection of endothelial cells with the CMV strains AD169, Towne or Davis, 10mM sodium butyrate was added to the endothelial growth medium for 48 hours after the virus had been inoculated. In some experiments, the same concentration of sodium butyrate was added to the initial endothelial cell plating medium for 18-24 hours prior to viral inoculation. During viral inoculation of CMV onto endothelial cells, the plates were centrifuged at 1,500g for 1 hour at 22°C . After a further 1 hour incubation at 37°C in 5% CO_2 , the endothelial cells were washed twice in HBSS and the medium was replaced with endothelial cell growth medium lacking sodium butyrate. For infection of endothelial cells with the clinical

isolate, C1FE, the virus was inoculated as above, with the omission of the sodium butyrate treatment, unless otherwise stated.

2.5. Cytoplasmic Immunofluorescent Staining.

2.5.1. Cell monolayers.

CMV infected endothelial cells or fibroblasts were grown in 8-well chamber slides (Gibco BRL) for 1-5 days post infection and HSV-1 infected fibroblasts were grown for 18-24 hours post infection. Cell monolayers were fixed in acetone for 20 minutes at 4°C and stored at -20°C until use. 50µl of each antibody or Fc fragment (Section 2.10) was added per well and incubated for 60 minutes at room temperature in a humid chamber. Cell monolayers were washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), followed by incubation for 60 minutes at room temperature with the appropriate secondary antibody. For blocking studies, 10µg/ml of human Fc fragment was pre-incubated with the cell monolayer for 60 minutes at room temperature in a humid chamber, before the addition of the primary reagent.

2.5.2. Cytocentrifuge preparations.

CMV infected endothelial cells or fibroblasts were grown in 6-well plates for 1-5 days post infection, after which the cells were removed by trypsin digestion. Cytocentrifuge preparations of the cells ($2-5 \times 10^4$ cells/spot) were made and the slides were fixed in acetone for 20 minutes at 4°C. The slides were stored at -20°C until use. 30µl of human Fc fragment was pre-incubated with the cell spots for 60 minutes at room temperature before the addition of the primary reagent. After two washes in PBS, 10-30µl of the appropriate antibody was added per cell spot and incubated for 60 minutes at room temperature in a humid chamber. The cells were washed twice in PBS, and then incubated for 60 minutes at room temperature with 15µl of the appropriate secondary antibody.

2.5.3. Neutrophil staining.

Cytocentrifuge preparations of double density gradient purified neutrophils (see section 2.12.3) were made ($1-2 \times 10^5$ /spot), and the cells fixed for 10 minutes in 5% paraformaldehyde containing 2% sucrose. The cells were washed twice in PBS containing 1% FCS and then permeabilised for 5 minutes with 0.5% Nonidet P40, 10% sucrose and

1% FCS. The cells were washed a further two times and allowed to dry in air for a minimum of 1 hour. Cells prepared in this way were stained immediately after the drying procedure. Each cell spot was incubated with 10µl of antibody for 60 minutes at room temperature in a humid chamber, after which the cells were washed twice in PBS and incubated for a further 60 minutes with 10µl of the appropriate secondary antibody.

After a further two washes, all slides were dried in air and mounted using citifluor (Citifluor, City University, London, U.K.). The immunofluorescent staining was observed under an ultraviolet light using an epi-fluorescent microscope or was analysed using a BioRad MRC600 confocal scanning laser microscope (Bio-Rad, Hettfordshire, U.K.).

2.6. Surface Immunofluorescent Staining.

2.6.1. Fc gamma receptor (Fc γ R) analysis.

CMV infected endothelial cells or fibroblasts were grown in 6-well plates for 1-5 days and non-enzymatically removed using cell dissociation fluid (Sigma). Live cells were kept on ice during the entire analysis, and all proteins were spun at 13,000g for 20 minutes in a microcentrifuge before use in order to remove aggregated protein. Cells were washed twice in PBS containing 1% BSA, and resuspended at a concentration of 5×10^6 cells/ml. 50µl of each primary reagent was added to 50µl of cell suspension and incubated for 30 minutes on ice. The cells were washed twice, incubated for 30 minutes on ice with 50µl of the appropriate secondary antibody and, following a further two washes, resuspended in 500µl of PBS.

2.6.2. Adhesion molecule and major histocompatibility (MHC) analysis.

CMV infected endothelial cells or fibroblasts were grown in 6 well culture dishes for 1-5 days and removed by trypsin digestion. After 3-5 minutes, the cells were washed twice in PBS and resuspended at a concentration of 2×10^6 cells/ml. 10-50µl of each primary reagent was added to 50µl of cell suspension, and incubated for 45 minutes at room temperature. The cells were washed twice in PBS and incubated for 45 minutes at room temperature with 50µl of the appropriate secondary antibody. The cells were washed a further two times and resuspended in 500µl of PBS containing 2%

formaldehyde. The cells were stored at +4°C for a maximum of 7 days until analysis by flow cytometry (see section 2.9).

In some cases, specific surface staining was confirmed after analysis by attaching 100µl of excess sample to a poly-D-lysine coated slide and viewing the cells using a BioRad MRC600 confocal scanning laser microscope.

2.7. Trypsin Sensitivity of the FcγR.

CMV infected endothelial cells and fibroblasts were grown in 6-well plates for 4 days and non-enzymatically removed, as described in Section 2.6.1.. Aliquots of 1×10^6 cells were washed twice in PBS and excess fluid removed. The cell pellet was incubated with 1ml of either 500µg/ml trypsin (1:250) and 200µg/ml EDTA (trypsin-EDTA, Gibco BRL, Scotland, U.K.) or 50-500µg/ml of trypsin (1,000-1,500 BAEE units/mg) without EDTA (Sigma). At various intervals, the reaction was neutralised with 500µl of 1mg/ml soybean trypsin inhibitor (Type I-S, Sigma) dissolved in fibroblast growth medium. Cells were washed twice in cold PBS containing 1% BSA and stained as described above in section 2.6.1.

2.8. Detection of CMV Immediate Early Antigen by Flow Cytometry.

CMV infected endothelial cells or fibroblasts were grown in 6-well plates and the cells were removed by trypsin digestion. The cells were washed twice in PBS containing 0.5% gelatine (PBS/gelatine) for 5 minutes at 500g, and resuspended at a cell concentration of 5×10^6 /ml. 200µl of acetone was added to 100µl of cell suspension, and the cells fixed for 20 minutes at 4°C. The cells were washed once, spun at 800g for 5 minutes and resuspended in 50µl PBS/gelatine per 100µl original cell suspension. 50µl of the E13 antibody (Section 2.10) was added to each sample, incubated for 60 minutes at room temperature, and washed twice, as above. After a further 60 minute incubation with 50µl of the goat anti-mouse FITC-conjugated secondary antibody, the cells were washed twice, resuspended in 500µl of PBS and stored at 4°C until analysis.

2.9. Flow Cytometric Acquisition and Analysis.

All cells were analysed using a Facscan IV flow cytometer (Beckton-Dickinson, Oxford, U.K.) and the data processed using the Consort 30 and Lysis II software

programmes (Beckton-Dickinson), or WinList version 1.04 (Verity Software House, Maine, U.S.A). Dead cells were excluded from the original population using the forward and side scatter dot plot profiles. The data from the viable cells were obtained using a logarithmic scale, and presented as either histogram profiles, the percentage of cells positive for the appropriate antigen, or the median fluorescent intensity in arbitrary units. Where stated in the text, the logarithmically acquired fluorescent data were converted to median linear channel values using the Lysis II software programme, and the values of the irrelevant control antibody were directly subtracted from the experimental sample value. This was in order to test the statistical relevance of the data (see section 2.17).

2.10. Monoclonal Antibodies and Related Proteins.

2.10.1. Fc γ R antibodies.

A panel of murine monoclonal antibodies specific for human cellular Fc γ Rs was obtained from various commercial sources: GRM1 from Cymbus Bioscience, Southampton, U.K.; 214.1 from Oncogene Science, Cambridge Bioscience, Cambridge, U.K.; 10.1, 2E1 and B-E16 from Serotec, Oxford, U.K.; 32.2 and IV.3 from Mederex, West Lebanon, NH; and 3G8 from both Mederex and The Binding Site, Birmingham, U.K. The antibody KUFC79 was a kind gift from Professor Peter Lydyard, University College London, U.K.

2.10.2. Fc fragments and IgG molecules.

Purified rabbit and mouse Fc fragments were obtained from Jackson Products, Stratech Scientific, Bedfordshire, U.K. and purified human Fc fragments were obtained from Calbiochem C-N, Nottingham, U.K. All lyophilised Fc fragments were resuspended at a concentration of 1mg/ml and run on a 12% sodium dodecylsulfate polyacrylamide gel to confirm purity. Mouse myeloma IgG1, IgG2a, IgG2b and IgG3 proteins (Sigma) were reconstituted to a concentration of 1mg/ml. All proteins concentrations were confirmed by protein assay (Bio-Rad), and were centrifuged at 13,000g for 20 minutes in a microcentrifuge before use to remove any aggregated protein.

2.10.3. CMV antigens.

The antibody E13, which recognises CMV immediate early antigens 1 and 2, was obtained from Biosoft, Tissue Culture Services, U.K. The antibodies p63.27 and 7.17,

which recognise CMV p72 immediate early antigen and gB late antigen, respectively, were a kind gift from Dr. W. Britt, University of Alabama, U.S.A. . The detection of CMV pp65, a phosphoprotein associated with the antigenemia assay, was carried out using either the monofluor kit, Pasteur diagnostics, France, or the antibody clone 14 was used. This latter antibody was a kind gift from the late Dr. Jim Booth, St. George's Hospital, London, U.K.

2.10.4. Adhesion molecule antibodies.

The antibodies BB1G-11, 9G11 and BB1G-E4, which recognise human ICAM-1, human PECAM-1 and human E-selectin, respectively, were obtained from R&D Systems, Abingdon, U.K. The antibody BT-1, which recognises human ICAM-2, was obtained from Serotec, whilst the anti-human LFA-3 antibody, A1CD58.9 and HB205, was obtained from Boehringer-Mannheim. A second LFA-3 antibody was purified by ammonium sulphate precipitation from the supernatant fluids of the hybridoma HB205, obtained from the American Type Culture Collection.

2.10.5. MHC antibodies.

The MHC Class I specific antibodies, W6-32 and BBM.1, which recognise the Class I heavy chain and the β_2m light chain, respectively, were purified by ammonium sulphate precipitation from supernatant fluids of hybridomas obtained from the American Type Culture Collection. The MHC Class II specific antibody, DR7 was similarly purified from supernatant fluids of hybridomas obtained from the Royal Free Hospital, London, U.K., whilst the antibody BU25 was obtained from The Binding Site, Cambridge, U.K. Both these antibodies recognise non-polymorphic Class II epitopes.

2.10.6. Secondary conjugated antibodies.

Affinity purified $F(ab)_2$ fractions of FITC-conjugated sheep anti-mouse, goat anti-rabbit and goat anti-human secondary antibodies were obtained from Sigma, as was the goat-anti-mouse TRITC conjugate. The affinity purified $F(ab)_2$ fraction of Texas Red-conjugated sheep anti-mouse antibody was obtained from Jackson Products. All FITC-conjugated antibodies were used at a dilution of 1:80-1:100, the TRITC-conjugated antibody at a dilution of 1:200 and the Texas-Red conjugated antibody at a dilution of 1:100.

A summary of the properties of the respective antibodies, together with the optimal working dilutions for these antibodies, is shown in Table 2.1.

2.11. Determination of CMV and HSV-1 Titre in Supernatant Fluids.

Fibroblasts were seeded in a 48 well plate at a concentration of 5×10^4 cells/well and allowed to grow for one day. CMV or HSV-1 was titrated from 10^{-1} to 10^{-7} , in half log dilutions, in minimal essential medium containing 2% foetal calf serum and 10mM HEPES. 100 μ l of each dilution of virus was transferred to replicate wells and incubated for 1 hour at 37°C in 5% CO₂. After two washes to remove unbound virus, 0.5ml of minimal essential medium containing 1% methylcellulose (Sigma Chemical Company, U.K.), 30% Leibovitz L-15 medium (Gibco BRL, U.K.), 5% heat inactivated foetal calf serum, 2mM L-glutamine, 100 I.U./ml penicillin, 100 μ g/ml streptomycin and 0.2% sodium bicarbonate, was added to each well. The cells were incubated at 37°C in 5% CO₂ for 10-14 days (CMV), or 24-48 hours (HSV-1). After these times, 0.5ml of 4% formal saline was added to each well and left for 1 hour at room temperature. The cells were then washed ten times with tap water, stained with 0.5ml of 0.03% methylene blue (Sigma Chemical Company, U.K.) for 1 hour at room temperature, and washed a further ten times, as above. After the plates had been thoroughly dried in air, the number of plaques per well were counted using an inverted light microscope. Wells containing 10-50 plaques were used to determine virus titre, and the results expressed as plaque forming units(PFU)/ml, using the equation: mean number of plaques from triplicate wells x virus dilution factor x 10. A multiplicity of infection (MOI) of 1 indicated a PFU to cell ratio of 1:1. Where stated in the text, serial twofold dilutions of virus were made, and 100 μ l of each dilution was inoculated onto the cells by low speed centrifugation at 1,500g for 60 minutes at 22°C. After a further 60 minute incubation at 37°C in 5% CO₂, the cells were washed twice, and overlayed with 1% methylcellulose, as described above.

2.12. Leukocyte Purification.

All leukocytes were purified from venous blood which had been treated with preservative-free heparin (10 I.U./ml).

2.12.1. Peripheral blood lymphocytes (PBLs).

Peripheral blood mononuclear cells were isolated by density gradient sedimentation, using ficoll hyperpaque 1077 (Sigma). Briefly, venous blood was diluted 1:1 in HBSS and layered onto ficoll hyperpaque 1077, at a 2:1 ratio of blood to gradient, respectively. The cells were centrifuged at 800g for 30 minutes, after which the buffycoat was collected from the interface, and the cells were washed a further two times in HBSS. Cells were resuspended at 1×10^7 /ml in RPMI containing 20% FCS, transferred to a tissue culture flask and left for 60 minutes in 5% CO₂ to allow the monocyte population to adhere to the plastic surface. After this period of time, the non-adherent PBLs were removed, washed twice in HBSS containing 2% FCS and resuspended at a concentration of 2.5×10^7 cells/ml in RPMI containing 10% FCS.

2.12.2. T-Lymphocytes.

T-lymphocytes were prepared from gradient purified PBMC by negative depletion of 'unwanted' cell phenotypes, using an indirect magnetic separation technique. Briefly, the peripheral blood mononuclear cells were purified using density gradient sedimentation, as described above. The buffycoat was serially depleted of monocytes, natural killer cells and B-lymphocytes using the mouse monoclonal antibodies against CD14 (RM052; Immunotech, Marseille, France), CD16 (RG8; Immunotech) and CD19 (RFB9; Royal Free Hospital, London, U.K.), respectively. After each antibody incubation (15 minutes, room temperature), 100µl of goat anti-mouse coated Dynobeads (supplied at 4×10^8 /ml; Dynal Ltd., Bromborough, Wirral) were added, the antibody bound cells were magnetically separated from the target cells and discarded. After the final separation, non-bound cells (representing the T-lymphocyte population) were washed twice in HBSS and the cells were resuspended at a concentration of 2.5×10^7 cells/ml in RPMI containing 10% FCS.

2.12.3. Neutrophils.

Neutrophils were prepared from venous blood by double density sedimentation. Briefly, venous blood was diluted 1:1 in HBSS and layered onto a gradient which consisted of an 1:1 ratio of ficoll hyperpaque 1077 and ficoll hyperpaque 1119, for the top layer and bottom layer, respectively. Diluted blood was added at a ratio of 1:2, blood to gradient, respectively. The cells were centrifuged at 700g for 30 minutes, after which

the buffycoat was collected from the top layer and discarded. The cells which were present in the second interface were harvested, then washed twice in HBSS containing 2% FCS and 5mM glucose. The cells were confirmed by morphology as the neutrophil population by staining with 2% acetic acid/trypan blue, and were found to be >90% pure and >90% viable by this method. The cells were resuspended at $0.5-1 \times 10^7$ cells/ml in HBSS containing 2% FCS and 5mM glucose.

2.13. Endothelial Cell-Neutrophil Co-Cultures.

Endothelial cells or fibroblasts, which had been seeded in 12-well plates, were inoculated with 500 μ l of cell-free CMV and processed as previously described in section 2.4. The cells were maintained in the appropriate growth medium until 70-80% of the cells showed characteristic late cytopathic effect (see text), after which the medium was removed and the cells were washed 3 times in HBSS. Neutrophil suspensions, which had been purified as described above, were incubated at a ratio of 50:1 to 100:1 and incubated for 16-18 hours at 37°C in 5% CO₂. All cell types which were in suspension after this period of time were removed by aspiration, whilst adherent cells were removed using trypsin digestion. Both fractions were combined, and the cells collected by centrifugation at 500g for 5 minutes. The cell pellet was resuspended in HBSS and the neutrophil fraction was harvested as described previously in section 2.11.3. After washing twice in HBSS, cytocentrifuge preparations were prepared and analysed as described in 2.5.3.

2.14. Radioisotope Labelling of Cells.

PBL's suspended at 2.5×10^7 /ml, T-lymphocytes suspended at 2.5×10^7 /ml or neutrophils suspended at $0.5-1 \times 10^7$ /ml, were labelled with 2mCi ⁵¹Cr/ 10^6 cells at 37°C for 60 minutes, with gentle agitation every 20 minutes. All cells were washed four times in large volumes of the appropriate wash buffer (HBSS containing 2% FCS, with the addition of 5mM glucose for neutrophils) and then resuspended in RPMI containing 10% FCS. The final concentration of the different cell types was $0.5-1 \times 10^7$ /ml for PBL and T-lymphocytes, and $1-4 \times 10^6$ /ml for neutrophils.

2.15. Transmigration Assays.

2.15.1. Preparation of Transwell filters.

Endothelial cells ($4-5 \times 10^4/\text{ml}$) were seeded onto 6.5mm diameter millipore ($3\mu\text{m}$ pore size) Transwell filters (Costar Ltd., Buckinghamshire, U.K.) which had been pre-treated with $2\mu\text{g}/\text{cm}^2$ fibronectin (Boehringer-Mannheim, U.K.), one day before the assay was carried out. The formation of confluent monolayers was verified the following day by visual inspection using an inverted light microscope, and the barrier function of these monolayers was tested by measuring the ability of ^{125}I -labelled BSA to cross the monolayer. Only those monolayers which were completely confluent and which showed an equilibrium of $<5\%$ for ^{125}I -BSA permeability, were used in the assays. Equilibration of ^{125}I -BSA across filters alone was $>95\%$ during the same period of time.

2.15.2. Transmigration assay.

Transwell filters containing confluent endothelial cell monolayers were washed three times in warm medium and the filters transferred to wells containing fresh 10% FCS in RPMI. $100\mu\text{l}$ of either $5 \times 10^6/\text{ml}$ radiolabelled T-lymphocytes or $2-4 \times 10^6/\text{ml}$ radiolabelled neutrophils were placed on top of the filter and incubated at 37°C in $5\% \text{CO}_2$. The incubation times were 18-24 hours for T-lymphocytes or 2 hours for neutrophils, and duplicate or triplicate wells were used for each data point. At the end of the incubation period, the Transwell filters were removed and the lower surface was swabbed with a cotton wool tip. The cells present in the bottom chamber were lysed with $2\% \text{NP-40}$ for 2-12 hours before being counted, along with the appropriate cotton wool tip, using a gamma counter. The percentage of migrated cells was calculated by dividing the radioactivity harvested from each well by the counts obtained from $100\mu\text{l}$ of the original radiolabelled leukocyte suspension.

2.16. Adhesion Assays.

Confluent endothelial cell monolayers which had been seeded in 48-well plates were washed twice in HBSS, and $200\mu\text{l}$ of either $1 \times 10^7/\text{ml}$ radiolabelled PBLs or $1 \times 10^6/\text{ml}$ radiolabelled neutrophils were added to each well. After a 1 hour incubation period at 37°C in $5\% \text{CO}_2$, the monolayers were gently washed in HBSS containing $1\% \text{FCS}$, the plates inverted and centrifuged for 1 minute at $500g$. The adherent cells were

lysed with 2% NP-40 for 2-12 hours, and counted using a gamma counter. The percentage of adherent cells was calculated by dividing the radioactivity harvested from each well by the counts obtained from 100 μ l of the original radiolabelled leukocyte suspension. In some instances, the cells were photographed before lysis.

2.17. Statistical analysis.

The Wilcoxon rank signed paired comparison test was used to determine the significance of the data obtained. For flow cytometric analysis, linear median channel values (see Section 2.9) obtained from a single uninfected or infected sample of $3-5 \times 10^3$ cells were compared. For leukocyte binding/migration assays, percentages (see Sections 2.15 and 2.16) obtained from separate wells containing either uninfected or infected cells were compared. The data were regarded as significant when $p \leq 0.05$.

Table 2.1. Characteristics of the murine monoclonal antibodies used in this study.

Specificity	Clone	Murine IgG Subclass	Original Concentration	Source	Optimal Concentration /Dilution ¹
<u>Human cellular FcYR antibodies</u>					
Human FcYRI	32.2	IgG1	1mg/ml	not known	10-15µg/ml ²
	10.1	IgG1	not known	culture s/n ³	1:2
Human FcYRII	IV.3	IgG2b	1mg/ml	not known	5-10µg/ml ²
	2E1	IgG2a	1mg/ml	ascitic fluid	10µg/ml
	KUFC79	IgG1	0.5mg/ml	not known	10µg/ml
Human FcYRIII	3G8	IgG1	0.2mg/ml	ascitic fluid	5-10µg/ml ²
	GRM1	IgG2a	0.05mg/ml	culture s/n	10-15µg/ml ²
	214.1	IgG1	0.1mg/ml	ascitic fluid	10-15µg/ml ²
	B-E16	IgG2a	not known	ascitic fluid	1:10
<u>CMV specific antibodies</u>					
CMV p72 IEA (1)	63.27	IgG1	not known	ascitic fluid	1:100
CMV p76 IEA (1&2)	E-13	IgG1	0.35mg/ml	ascitic fluid	3.5µg/ml
CMV pp65	Clone 14	IgG1	not known	ascitic fluid	1:500
CMV gB	7.17	IgG3	not known	ascitic fluid	1:100
<u>Adhesion molecule antibodies</u>					
Human ICAM-1	BB1G-11	IgG1	1mg/ml	culture s/n ³	5µ/ml
Human ICAM-2	BT-1	IgG1	0.2mg/ml	culture s/n ³	2µg/10 ⁵ cells
Human PECAM-1	9G11	IgG1	1mg/ml	culture s/n ³	2-5µg/ml ²
Human E-selectin	BB1G-E4	IgG1	1mg/ml	culture s/n ³	10µg/10 ⁵ cells
Human LFA-3	HB205	IgG1	not known	culture s/n ³	1:25
	A1CD58.9	IgG1	0.25mg/ml	culture s/n ³	5µg/ml
<u>Human HLA antibodies</u>					
Human HLA Class I	W6.32	IgG2a	not known	ascitic fluid	1:200
	BBM.1	IgG1	not known	ascitic fluid	1:200
Human HLA Class II	BU25	IgG1	0.2mg/ml	ascitic fluid	10µg/10 ⁵ cells
	DR7	IgG1	not known	culture s/n ³	10µl/10 ⁵ cell
<u>IgG control antibodies⁴</u>					
Unknown ⁴	MOPC31C	IgG1	1mg/ml	ascitic fluid	As appropriate ⁵
Unknown ⁴	UPC10	IgG2a	1mg/ml	ascitic fluid	As appropriate ⁵
Unknown ⁴	MOPC141	IgG2b	1mg/ml	ascitic fluid	As appropriate ⁵
Unknown ⁴	FLOPC21	IgG3	1mg/ml	ascitic fluid	As appropriate ⁵

¹Optimal working dilution determined by flow cytometry or microscopic observation of the appropriate cell type. ² Different batches of antibody gave different optimal working concentration. ³s/n = culture supernatant. ⁴Obtained from murine myelomas of unknown specificity. ⁵Concentration varied according to the specific antibody used in the different studies

CHAPTER 3.
INFECTION OF ENDOTHELIAL CELLS WITH
CMV.

3.1. Introduction.

CMV causes widespread infection in the general population, with overt symptoms of disease usually confined to immunosuppressed individuals (Jacobson, Mills, 1988; Grattan et al. 1989). Following acute CMV infection, the virus has been detected in every major organ of the body (Wu et al. 1994; Furukawa et al. 1994; Egbert et al. 1980; Bronsther et al. 1988; Betts et al. 1975). Principal targets of CMV infection in the tissues and supplying blood vessels include smooth muscle cells, endothelial cells, epithelial cells and fibroblasts (Toorkey, Carrigan, 1989; Sinzger et al. 1995). Endothelial cell involvement, in particular, has been considered a feature of more severe infections (Goodman, Porter, 1973; Foucar et al. 1981), and to contribute both to viral dissemination (Waldman et al. 1995b; Percivalle et al. 1993b; Grefte et al. 1995b) and to the disease process *per se* (McDonald et al. 1989; Loebe et al. 1990; Grattan et al. 1989). Thus, it is important to determine the effects of CMV infection on this particular cell type.

It has proved difficult, however, to infect cultured endothelial cells with the virus, and therefore the following studies were carried out in order to optimise the conditions under which CMV may infect this cell type *in vitro*. Initial experiments which were carried out using highly passaged, fibroblast-grown strains of CMV indicated that AD169, Towne and Davis had a limited capacity to infect the endothelial cell type (<3% at a multiplicity of infection (MOI) of 5, data not shown). However, a number of methods have been reported to improve the percentage of cells which can be infected with CMV *in vitro* (Radsak et al. 1989; Weinshenker et al. 1988; Gonczol et al. 1984; Ho et al. 1993; Ho et al. 1984). Two such methods, treatment of cultured cells with short chain fatty acids, and inoculation of CMV using low speed centrifugal force, were tested for their ability to enhance the infection of endothelial cells with the laboratory CMV strain AD169. For these studies, the expression of CMV immediate early antigen (IEA) in the nucleus of acetone fixed endothelial cells was used to detect infection, and the number of positive cells was determined using flow cytometric techniques. In addition, the use of recent clinical isolates of CMV, which had been passaged a limited number of times through fibroblasts, were assessed for their ability to infect endothelial cells in a manner

similar to that described for AD169. The results obtained for the low passaged clinical strains were compared with the laboratory strains, in order to determine the most efficient method to infect endothelial cells with CMV.

3.2. Determination of the optimal conditions under which the laboratory strain of CMV, AD169, leads to the infection of endothelial cells.

As a poor rate of infection (<3%) was achieved when the laboratory strain of CMV (AD169) was used to inoculate endothelial cells, the ability of the short chain fatty acid, sodium butyrate, to improve the infection rate was studied. Preliminary experiments were carried out to determine the maximum concentration of sodium butyrate, with regards to efficacy and toxicity, to use in these studies. As this drug has previously been shown to increase the infection rate of CMV when administered to endothelial or epithelial cells prior to viral inoculation (Tanaka et al. 1991; Radsak et al. 1989), the endothelial cells were treated for 1 day with various concentrations (5-20mM) of sodium butyrate, before the addition of the virus. Furthermore, because centrifugal inoculation has also been shown to improve the infection rate of CMV in monocytes (Ho et al. 1993), CMV strain AD169 was inoculated onto the drug-treated endothelial cells by centrifugation, and the percentage of cells which expressed CMV IEA was determined 4 days later. These early results indicated that drug concentrations of 5-20mM increased the percentage of infected cells from 4% to 14-26% (Table 3.2.1). However, as fewer numbers of viable cells could be recovered after concentrations of 15mM-20mM were used, the optimum concentration of sodium butyrate, with regards to maximum efficacy and minimum toxicity, was determined to be 10mM (Table 3.2.1).

The next experiments were performed in order to ascertain whether 1 day of sodium butyrate treatment prior to virus inoculation was the optimal time to use in these studies, and also to confirm that centrifugal inoculation of virus had an augmentative effect. Endothelial cells which had been inoculated with AD169, either with or without a low speed centrifugation step, were treated for various periods of time with 10mM of sodium butyrate, and the percentage of cells expressing CMV IEA was determined 4 days later (Figure 3.2.1). In the absence of centrifugal inoculation, endothelial cells which had been treated with sodium butyrate, both before (20 hours)

and after (10-48 hours) viral inoculation, showed a marginal increase in the percentage of IEA positive cells, with the maximal effect (15%) occurring when the cells had been treated with the drug for two days post inoculation (Figure 3.2.1). The addition of the drug to the endothelial cells for periods longer than two days resulted in substantial cell death (data not shown).

A more marked increase in the percentage of cells expressing CMV IEA occurred when the virus had been inoculated onto the endothelial cells by low speed centrifugation (Figure 3.2.1). At all time points studied, the percentage of CMV IEA positive cells was increased in relation to those cells in which the virus had not been inoculated by centrifugation, but which had been treated with sodium butyrate for the same length of time (Figure 3.2.1). In agreement with the results obtained in the absence of a centrifugation step, sodium butyrate treatment of the endothelial cells for two days post 'centrifugal' inoculation resulted in maximum expression of the viral antigen, although in this case, the additional centrifugation step increased the number of CMV IEA positive cells to 66% (Figure 3.2.1). Thus, the consistent increase in the number of cells which expressed CMV IEA after centrifugal inoculation validated the use of this process for all subsequent experiments which involved the use of endothelial cells.

Using these optimal conditions for CMV inoculation of endothelial cells, the addition of higher titres of AD169 (MOI of 1-16) only increased the percentage of cells expressing CMV IEA to a maximum of 80% (Table 3.2.2). However, in later experiments, the inoculation of AD169 using viral titres of 1×10^6 /ml (MOI of 4) only resulted in infection rates of 15-20%. Moreover, re-titration of sodium butyrate treatment, with respect to time and concentration, indicated that the maximal effects again occurred after two days of treatment with 10mM of sodium butyrate, but did not lead to the expression of CMV IEA in any more cells (data not shown). The reason for these reduced infection rates in later experiments is unclear because the use of different batches of sodium butyrate, endothelial cell growth factor, or indeed the endothelial cells themselves, produced similar results (data not shown).

3.3. Comparison of the ability of the laboratory strain of CMV, AD169, or a low passage clinical isolate, C1F, to be propagated in endothelial cells.

The use of low fibroblast passaged clinical isolates, as opposed to highly passaged laboratory strains of CMV, has previously been shown to improve the percentage of endothelial cells which can be infected with the virus. Therefore, the ability of AD169 to be propagated in endothelial cells was compared with that of a clinical strain which had recently been isolated *in vitro*. This clinical strain was obtained by the inoculation of a blood specimen containing CMV onto fibroblasts, and the CMV strain thus isolated was designated C1F. The virus at this initial stage was still cell associated, and was therefore sequentially passaged by plating the C1F-infected cells onto fresh, uninfected fibroblasts. The number of infected fibroblasts was expanded in this way until the virus could be detected in the supernatant fluids of the infected cells (which occurred after five such passages), and the cell-free virus stock was termed C1F₅. Endothelial cells were then inoculated with C1F₅, and either treated with sodium butyrate for two days, or left untreated. CMV strain AD169 was inoculated onto the endothelial cells in parallel, using the same conditions as for C1F₅. When cytocentrifuge preparations of these endothelial cells were analysed five days after the removal of sodium butyrate, approximately 20% of the sodium butyrate-treated cells inoculated with either C1F₅ or AD169 were positive for IEA expression, whilst approximately 15% were positive for gB (cytoplasmic late antigen) expression (data not shown). Neither antigen could be detected in cells which had been inoculated with either virus strain, but which had not been treated with sodium butyrate (data not shown). Moreover, when the endothelial cells were examined using a light microscope at this stage, individual cells within the drug treated cultures which had been inoculated with either AD169 or C1F₅ were displaying a large, rounded morphology, whereas the similarly treated but uninfected cultures were not (Figure 3.3.1).

All inoculated cells were subsequently maintained by serial propagation (in the absence of sodium butyrate treatment), and an aliquot of these cells was analysed for the expression of CMV IEA after each passage. This process was continued for

either two months (approximately 6-8 passages), or until all cells showed characteristic late cytopathic effect. Treatment with sodium butyrate after each cell passage was toxic to the endothelial cells, therefore this treatment was only used directly after the initial viral inoculation. The results are shown in Table 3.3.1. Continual passage of endothelial cells which had been inoculated with the clinical isolate C1F₅ led to a progressive increase, after each passage, in the percentage of cells which were expressing CMV IEA. This was true both for the endothelial cells which initially had been treated with sodium butyrate, and for those cells which had received no drug treatment (Table 3.3.1), although the percentage of positive cells was always greater in the former case. In contrast, attempts to serially propagate the AD169 strain of virus in the endothelial cells were unsuccessful. Even after six passages, CMV IEA was undetectable in endothelial cells inoculated with AD169, whilst virtually 100% of the cells inoculated with C1F₅ were antigen positive by this stage (Table 3.3.1). The cells inoculated with AD169 were passaged for a further month, but remained negative for CMV IEA expression (data not shown). Thus, despite the use of sodium butyrate treatment to initiate a cycle of replication of CMV strain AD169 in a proportion of endothelial cells, this virus strain failed to spread to surrounding uninfected endothelial cells and could not be propagated in this cell type. In contrast, the clinical strain C1F₅ was able to spread progressively and eventually to infect all available endothelial cells, even though the initial titre of C1F₅ used was lower than that of AD169 (4×10^5 and 1×10^6 PFU/ml when titrated on fibroblasts, respectively).

3.4. Comparison of the ability of CMV strains AD169 and C1F to produce infectious virus in endothelial cells.

In order to see whether the difference in the ability of CMV strains C1F and AD169 to spread to uninfected endothelial cells was due to an abortive cycle of replication in the case of the AD169 strain, the amount of infectious virus secreted into the supernatant fluids by endothelial cells which had been inoculated with the two virus strains was compared. As the original inoculum of C1F, which had been propagated five times through fibroblasts (C1F₅), contained less virus than did the AD169 stocks of CMV (4×10^5 and 1×10^6 PFU/ml respectively), the clinical isolate

was propagated a further three times through fibroblasts in order to increase the viral titre to that of the AD169 stock. This stock was thus termed C1F₈. Endothelial cells were then infected with AD169, C1F₅ or C1F₈ and the cells were subsequently treated with sodium butyrate for two days. The supernatant fluids were removed at various times after inoculation, and the titre of infectious virus present in these cell-free fluids was estimated using a standard plaque assay on fibroblasts.

The results indicated that infectious virus was secreted into the supernatant fluids of the inoculated endothelial cells for both strain AD169 and strain C1F (stocks C1F₅ and C1F₈) for at least two weeks following viral inoculation (Table 3.4.1). Furthermore, comparable titres of virus were recovered from the supernatant fluids of endothelial cells infected with AD169, C1F₅ or C1F₈ (Table 3.4.1). Sodium butyrate treatment increased the titre of extracellular virus for all three virus preparations (Table 3.4.1). In accordance with the findings in the previous section, immunofluorescence staining of the endothelial cells five days after the removal of sodium butyrate revealed no marked differences in the percentages of cells expressing CMV IEA when strain AD169 was compared to C1F (stocks C1F₅ or C1F₈). In all cases, approximately 15-20% of the cells stained for the viral antigen after sodium butyrate treatment. However, after these cells had been propagated once, then left for a further three week incubation period (total incubation period of 5 weeks), only sodium butyrate treated endothelial cells which had been inoculated with the clinical isolate C1F, but not AD169, expressed CMV IEA (94% and <2%, respectively). This confirms that, during this experiment, CMV strain AD169 was unable to spread to the surrounding uninfected endothelial cells, as was found previously. The results, however, did show that sodium butyrate treatment of the endothelial cells allowed at least one complete cycle of CMV replication to occur in a proportion of cells infected with AD169 and C1F, but that only virus secreted from C1F infected cells could subsequently spread to uninfected endothelial cells. It was concluded that the inability of strain AD169 to spread from infected to uninfected endothelial cells was not due to the lack of production of infectious extracellular virus in this cell type.

Interestingly, although there was again no difference between the viral strains in the percentage of sodium butyrate-treated infected endothelial cells which initially

expressed CMV IEA, when the inoculated endothelial cells were propagated once and left for a further three week incubation period, substantially more cells inoculated with the lower fibroblast-passaged clinical isolate (C1F₅) were antigen positive when compared to the higher fibroblast-passaged (C1F₈) virus stock. Thus, 94% of C1F₅-inoculated cells which had initially been treated with sodium butyrate were CMV IEA positive after a total incubation period of five weeks, when compared to 26% of C1F₈-inoculated cells, even though the former stock was of a lower viral titre than the latter (4×10^5 and 1×10^6 PFU/ml, respectively). This result suggests that passage of clinical isolates in fibroblasts might decrease their ability to spread from infected to uninfected endothelial cells.

3.5. Comparison of the propagation of a clinical isolate of CMV, through either endothelial cells or through fibroblasts, on the ability of the virus to infect these two cell types.

The results presented above suggested that continual fibroblast propagation of the clinical isolate of CMV, strain C1F, reduced its capacity to infect endothelial cells. Conversely, continual endothelial cell propagation of this viral strain appeared to enhance its capacity to infect endothelial cells. To study this effect further, two 'stocks' of the clinical isolate C1F were produced. The first was propagated solely in fibroblasts for seven passages, to give the stock C1F₇. The second 'stock' was adapted for growth in endothelial cells, and termed C1FE. Briefly, the clinical isolate was propagated twice through fibroblasts, the infected cells lysed by two freeze-thaw cycles, and the resultant supernatant used to infect endothelial cells. After two days of sodium butyrate treatment, fresh medium was added to the cells and the number of infected endothelial cells was amplified by continual passage, as described above. Five such passages through endothelial cells were necessary to yield extracellular viral titres which were comparable to those of the fibroblast grown stock (C1F₂E₅ and C1F₇, 1.5×10^6 PFU/ml). Thus, both C1F₇ and C1F₂E₅ had been propagated a total of seven times. Serial dilutions of these viral stocks were then added to both fibroblasts and endothelial cells, and the percentage of cells expressing IEA was determined by flow cytometry, five days after the sodium butyrate had been removed.

The endothelial 'adapted' clinical isolate C1F₂E₅ showed a greater ability to

infect the endothelial cells when compared to the matched fibroblast passaged clinical isolate C1F₇, at all dilutions tested (Table 3.5.1). Sodium butyrate treatment did not further increase the ability of C1F₂E₅ to replicate in the endothelial cells, whereas minimal infection occurred when C1F₇ was added to the endothelial cells in the absence of sodium butyrate treatment (Table 3.5.1). In contrast, there was little difference in the ability of C1F₇ or C1F₂E₅ to infect fibroblasts (Table 3.5.1). Both virus stocks were much more permissive for fibroblasts than endothelial cells. It was concluded that although both the fibroblast propagated and endothelial cell propagated clinical isolate was equally able to infect fibroblasts, only virus which had been serially propagated in endothelial cells showed a propensity to infect the latter cell type.

3.6. The ability of three other fibroblast-propagated strains of CMV to infect endothelial cells.

The above results suggested that continual passage of CMV through fibroblasts resulted in a reduction in the efficacy of the virus to infect endothelial cells, but not fibroblasts. The ability of three other fibroblast propagated strains of CMV to infect endothelial cells was therefore tested. These were the two high passaged laboratory strains of CMV, Towne and Davis, and a low passage clinical isolate, Toledo. Extracellular virus stocks from these fibroblast-passaged viral strains (1×10^6 PFU/ml) were inoculated onto the endothelial cells in the presence or absence of sodium butyrate treatment. The endothelial cells were then serially passaged for two months (approximately 6-8 passages), and the percentage of cells expressing CMV IEA was determined by flow cytometry after each passage.

Inoculation of the fibroblast-passaged Towne and Davis strains of virus onto endothelial cells resulted in the initial expression (five days after the removal of sodium butyrate) of CMV IEA in 20% and 17% of the sodium butyrate treated cells respectively (Table 3.6.1), in contrast to <1% of untreated infected cells. Endothelial cells which had been inoculated with fibroblast-passaged Toledo under identical conditions showed a similar number of positive cells at this stage (Table 3.6.1). However, serial propagation of the endothelial cells which had been inoculated with either the Towne or the Davis strain of CMV failed to promote spread of the virus to

the uninfected cells, even after two months of continual passage (data not shown). This was true both for those cells which had initially received sodium butyrate treatment for the first two days after viral inoculation, and for those cells which had been left untreated. In contrast, when endothelial cells infected with the Toledo strain of virus were serially propagated, there was a progressive increase in the number of cells which were displaying cytomegaly (CPE) after each passage (data not shown). When the supernatant fluids were recovered from these Toledo-infected endothelial cells after five passages, the cell-free virus was able to infect 24% of the endothelial cells without sodium butyrate treatment (Table 3.6.1). This endothelial cell 'adapted' strain of virus was termed Toledo.E₅. Sodium butyrate treatment of the Toledo-infected cells enhanced the number of cells which were positive for CMV IEA to 64% (Table 3.6.1). After further propagation of Toledo.E in endothelial cells (Toledo.E₈), 60-70% of inoculated endothelial cells were antigen positive in the absence of sodium butyrate treatment, and this treatment did not further increase the percentage of antigen positive cells (Figure 3.6.1). These findings are similar to those described above for the clinical isolate C1F.

When the supernatant fluids harvested up to two weeks after the initial endothelial cell inoculation of the fibroblast passaged virus stocks (strains Towne, Davis and Toledo) were analysed by plaque assays in fibroblasts, sodium butyrate treated endothelial cells inoculated with all three strains were found to excrete titres of approximately 10^4 PFU/ml, whereas untreated cells secreted viral titres which were reduced by one order of magnitude (10^3 PFU/ml). These results were similar to those obtained above for the fibroblast propagated laboratory strain of CMV, AD169. Thus, although sodium butyrate treatment had a similar affect on promoting a first round replication cycle in endothelial cells initially infected with Towne, Davis or Toledo, only Toledo was able to subsequently spread to uninfected cells. Taken together, the results suggested that highly fibroblast passaged strains of virus (AD169, Towne and Davis) were inherently non-permissive for endothelial cells, whereas strains of virus which had been passaged fewer times through fibroblasts (C1F and Toledo) had an increased propensity to infect this cell type. Moreover, continual propagation of these low fibroblast passaged viral strains through endothelial cells eventually led to the production of virus (C1FE and Toledo.E) which was no longer influenced by sodium

butyrate treatment of the cells (Figure 3.6.1). In addition, once continuous CMV replication through endothelial cells had been established, it was the amount of virus excreted into the culture supernatants, rather than the number of times the virus had been propagated through fibroblasts, which had an effect on the subsequent ability of the endothelial cell-derived virus to infect endothelial cells. Thus, inoculation of equivalent titres either C1F₂E or C1F₅E onto endothelial cells resulted in a similar percentage of CMV IEA positive cells (Figure 3.6.1). Due to this fact, in all subsequent chapters, this endothelial cell propagated strain will be referred to as C1FE.

3.7. The ability of non-in vitro-passaged CMV to infect endothelial cells.

The studies above used strains of CMV which had first been isolated in fibroblasts, and then 'adapted' for growth in endothelial cells. Therefore, in order to investigate whether endothelial cell tropic virus occurs naturally *in vivo*, leukocytes from a renal transplant patient undergoing a primary CMV infection were directly inoculated onto endothelial cells. After a one hour incubation period, the leukocytes were removed and fresh medium, which did not contain sodium butyrate, was added. The endothelial cells were observed for any morphological changes associated with late cytopathic effect, and passaged as required. At the same time, an aliquot of the leukocytes were directly inoculated onto fibroblasts, and the cells treated in a similar manner. Cytopathic effect was observed in the fibroblasts one week after the original inoculation (4 or 5 foci after one passage), with 50% of the cells involved after four more passages (data not shown). The endothelial cells, however, did not show cytopathic effect until two months after inoculation (a single focus of infection appearing after five passages), with a continued increase in the number of cells involved at each consecutive passage (data not shown). A further three passages were required in order to produce cytopathic effect in approximately 50% of the endothelial cells. An example of this cytopathic effect is shown in Figure 3.7.1. When the supernatant fluids were tested for the presence of CMV, both the fibroblast grown isolate (C2F) and the endothelial cell grown isolate (C2E) appeared to be largely cell-associated, even after a total of 10 passages (data not shown). Thus, it was concluded that an *ex vivo* source of CMV replicated much more efficiently in fibroblasts than

endothelial cells, and that endothelial cell tropic virus could be selected for *in vitro* by continual amplification through endothelial cells.

Table 3.2.1. The effect of increasing concentrations of sodium butyrate on the ability of CMV strain AD169 to infect endothelial cells¹.

Sodium Butyrate Concentration	Viability	Percentage CMV IEA Expression (%)
0	97%	4%
5	96%	13%
10	97%	17%
15	83%	20%
20	72%	26%

¹Endothelial cells were pre-treated with various concentrations of sodium butyrate for 1 day and then inoculated by low speed centrifugation with CMV strain AD169 at a MOI of 5. The cells were analysed at 4 days post inoculation by flow cytometry. 5,000 cells were analysed, and a gate was set using the forward (size) and side (granularity) scatter histogram profiles, to determine the number of viable cells within the total cell population. The percentage of cells expressing CMV IEA within the viable cell population was then determined, as indicated. The data represent the mean percentage of cells for a representative experiment performed in triplicate.

Table 3.2.2. The effect of increasing virus to cell ratio on the ability of CMV strain AD169 to infect endothelial cells¹.

Plaque Forming Units/ml (PFU/ml)	Multiplicity of Infection (MOI)	Percentage CMV IEA Expression (%)
4×10^6	16	83%
2×10^6	8	77%
1×10^6	4	68%
5×10^5	2	50%
2.5×10^5	1	44%

¹Endothelial cells were inoculated with CMV strain AD169 at various MOI's, using centrifugal enhancement, and then treated for 2 days with 10mM sodium butyrate. The percentage of CMV IEA expression was determined by flow cytometry 4 days later. The data represent the mean percentage of cells for a representative experiment performed in triplicate.

Table 3.3.1. Comparison of the ability of the low passage clinical isolate C1F, or the high passage laboratory strain of CMV, AD169, to infect endothelial cells¹.

Passage Number	No Sodium Butyrate		Plus Sodium Butyrate	
	AD169	C1F5	AD169	C1F5
0	1% ²	1% ²	20% ²	20% ²
1 ³	nd ³	nd ³	nd ³	nd ³
2	1%	5%	2%	9%
3	1%	8%	2%	23%
4	1%	18%	1%	56%
5	1%	61%	1%	99%
6	1%	99%	1%	n.d.

¹Endothelial cells were inoculated with the laboratory strain AD169 (MOI of 4), or the clinical isolate C1F₅ (MOI of 1.6), which had been passaged through fibroblasts five times. The cells were treated with sodium butyrate for 2 days post inoculation, or left untreated. The percentage of cells expressing CMV IEA was determined by flow cytometry at each sequential passage, and the data represent the mean percentage of cells for a representative experiment performed in triplicate.

²Determination of CMV IEA expression, using immunofluorescence techniques, on cytocentrifuged preparations of the cells, 5 days post inoculation.

³Too few cells to determine CMV antigen expression using flow cytometry, (n.d. = not done).

Table 3.4.1. Comparison of the ability of the low passage clinical isolate C1F, or the high passage laboratory strain of CMV, AD169, to secrete virus into the supernatant fluids of infected endothelial cells¹.

Day	Plaque Forming Units (PFU)/ml					
	No Sodium Butyrate			Plus Sodium Butyrate		
	AD169	C1F ₅	C1F ₈	AD169	C1F ₅	C1F ₈
8	2.4 ±0.1 x10 ³	1.8 ±0.1 x10 ³	0.7 ±0.1 x10 ³	8.0 ±0.5 x10 ³	5.0 ±0.5 x10 ³	5.0 ±0.5 x10 ³
10	2.4 ±0.1 x10 ³	2.5 ±0.3 x10 ³	2.7 ±0.2 x10 ³	1.4 ±0.1 x10 ⁴	9 ±0.4 x10 ³	1.2 ±0.1x10 ⁴
12	3.3 ±0.2 x10 ³	1.9 ±0.3 x10 ³	2.7 ±0.5 x10 ³	1.8 ±0.1 x10 ⁴	1.0 ±0.1 x10 ⁴	1.0 ±0.1 x10 ⁴
15	2.9 ±0.6 x10 ³	1.4 ±0.5 x10 ³	4.3 ±0.5 x10 ³	3.4 ±0.3 x10 ⁴	3.2 ±0.1 x10 ⁴	3.9 ±0.2 x10 ⁴

¹Endothelial cells were inoculated with the laboratory strain AD169, or the clinical isolate of CMV which had been passaged either five (C1F₅) or eight (C1F₈) times through fibroblasts. The cells were inoculated at either a MOI of 4 (AD169 & C1F₈), or a MOI of 1.6 (C1F₅), and either treated with sodium butyrate for 2 days, or left untreated. The supernatants from the infected cells were harvested at various time points, and the cell-free viral titre was determined using a standard fibroblast plaque assay. The data represent the mean PFU/ml (± standard error) of three separate experiments performed in triplicate.

Table 3.5.1. The ability of a clinical isolate grown through two different cell types to infect either fibroblasts or endothelial cells¹.

Dilution of	Virus Inoculated Onto:					
	Fibroblasts		Endothelial cells			
Viral	No Sodium Butyrate		No Sodium Butyrate		Plus Sodium Butyrate	
Stock	C1F ₇	C1F ₂ E ₅	C1F ₇	C1F ₂ E ₅	C1F ₇	C1F ₂ E ₅
neat	nd ²	nd ²	5%	76%	31%	72%
1/5	nd ²	nd ²	4%	77%	12%	71%
1/10	nd ²	nd ²	3%	61%	6%	45%
1/20	nd ²	nd ²	3%	32%	3%	31%
1/40	nd ²	nd ²	2%	29%	2%	21%
1/80	94%	87%	nd ²	nd ²	nd ²	nd ²
1/160	92%	85%	nd ²	nd ²	nd ²	nd ²

¹Serial dilutions of a clinical isolate of CMV (starting concentration of 5×10^5 PFU/ml, MOI of 2) which had been propagated in fibroblasts (C1F) or endothelial cells (C1FE) were inoculated onto fibroblasts or endothelial cells, and either treated with sodium butyrate for 2 days, or left untreated. The percentage of cells which were positive for CMV IEA was determined at 4 days post infection by flow cytometry. The data represent the mean of triplicate samples for a single representative experiment.

²nd = not done.

Table 3.6.1. The ability of high and low passage fibroblast-propagated strains of CMV to infect endothelial cells¹.

Passage	No Sodium Butyrate			Plus Sodium Butyrate		
	Towne	Davis	Toledo	Towne	Davis	Toledo
0 ²	<1%	<1%	<1%	20%	17%	16%
3	<1%	<1%	6%	<1%	<1%	13%
5	<1%	<1%	24%	<1%	<1%	64%

¹Endothelial cells were inoculated at a MOI of 4 with the high fibroblast passage laboratory strains Towne or Davis, or the low fibroblast passage strain Toledo, and either treated with sodium butyrate for 2 days, or left untreated. The percentage of cells expressing CMV IEA was determined by flow cytometry at each sequential passage. The data represent the mean of triplicate samples for a single representative experiment.

²CMV IEA expression was determined visually on cytocentrifuged preparations of the cells, five days post inoculation, and an estimate of the cells positive for CMV IEA expression is indicated.

A. No Centrifugation

B. Plus Centrifugation

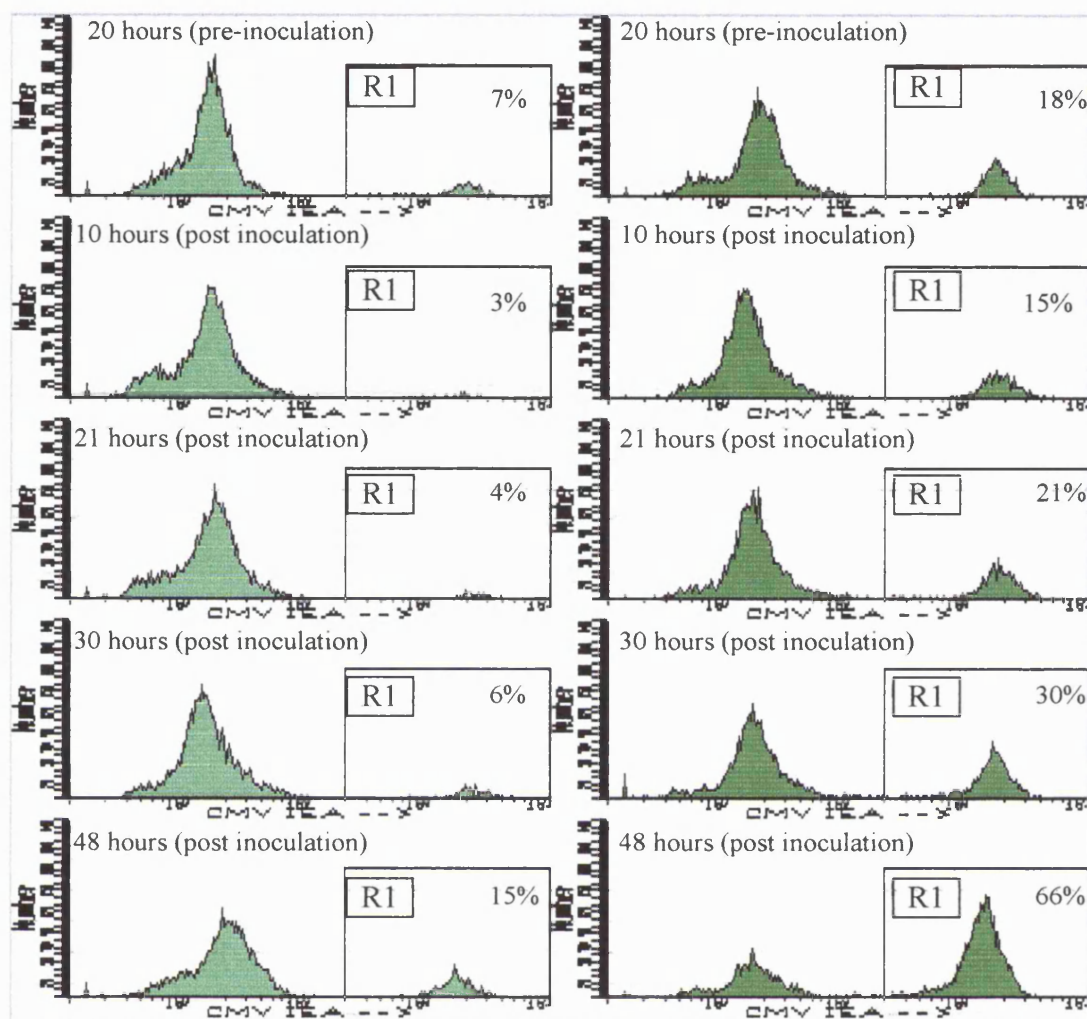


Figure 3.2.1. The effect of sodium butyrate treatment and centrifugal enhancement on CMV IEA expression in endothelial cells.

Endothelial cells were inoculated with CMV strain AD169 at a MOI of 5, and treated for various periods of time (either pre- or post- viral inoculation) with 10mM sodium butyrate. The percentage of cells which were positive for CMV IEA expression (R1) was determined at 4 days post inoculation by flow cytometry. In panel A, representative histogram profiles of the data for virus inoculated in the absence of a low speed centrifugation step are shown, whilst in panel B, the data show representative histogram profiles of the virus inoculated for 1 hour at 1,500g. The ordinate represents the number of fluorescence events (scale 0-250), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). The mean percentage of CMV IEA expression from a single experiment performed in triplicate is shown for each time point.

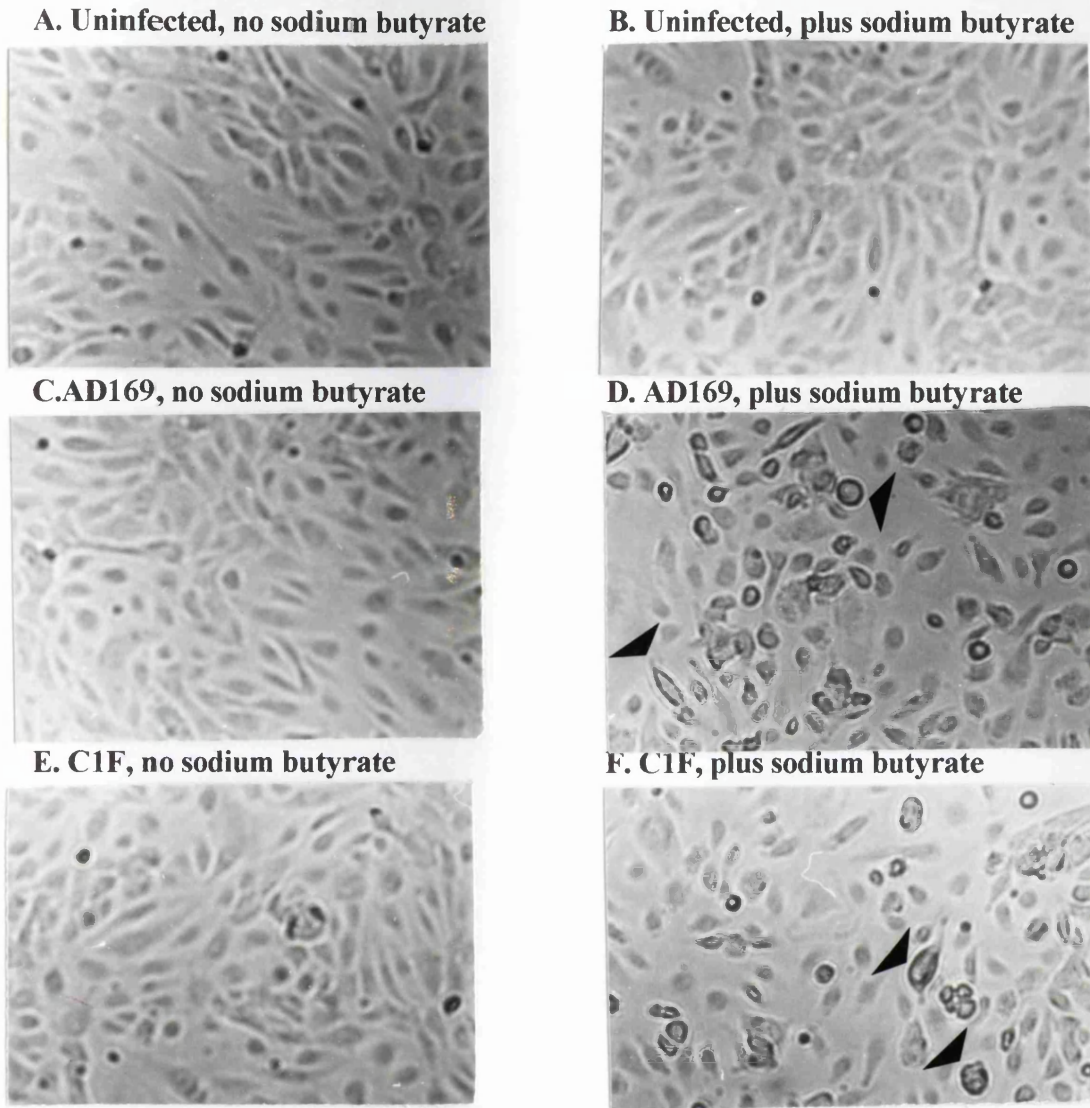


Figure 3.3.1. The ability of different strains of CMV to produce cytopathic effect after sodium butyrate treatment.

Endothelial cells were inoculated at a MOI of 4 with the high fibroblast-passaged laboratory strain of CMV, AD169, or the low fibroblast-passaged clinical strain C1F. Uninfected or CMV-inoculated endothelial cells were either treated with sodium butyrate for two days, or left untreated, and all cells were observed five days later, using a light microscope. In parts A, C & E, untreated cells are shown, whilst in parts B, D & F, sodium butyrate treated cells are shown, uninfected, AD169- and C1F-inoculated, respectively. Note individual cells displaying characteristic CPE (examples marked) in parts D & F. All photomicrographs were taken at x40 magnification.

A. No Sodium Butyrate

B. Plus Sodium Butyrate

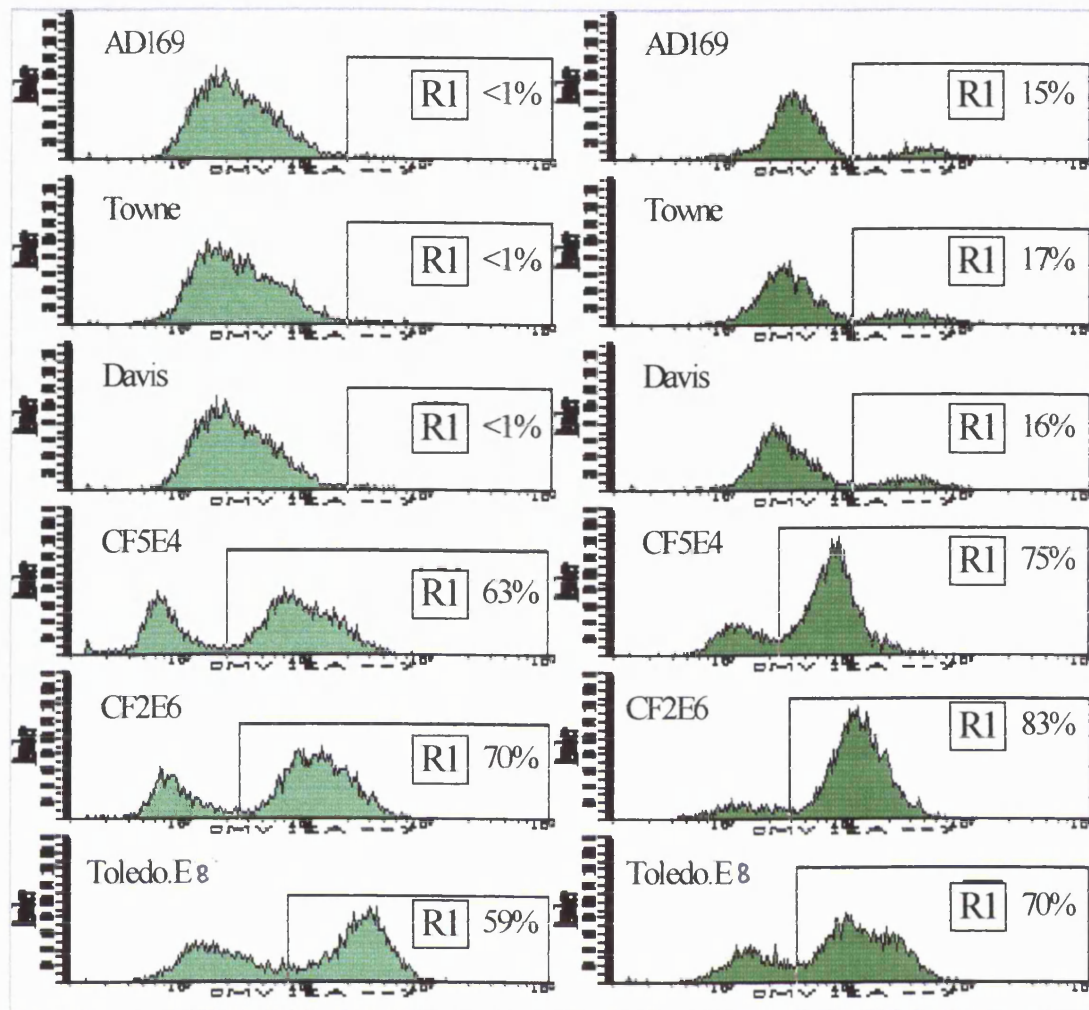
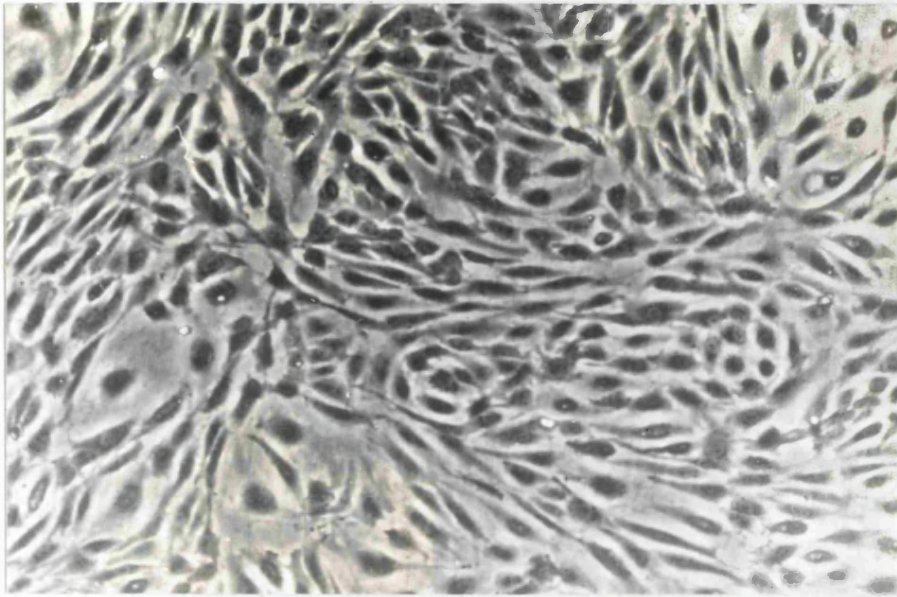


Figure 3.6.1. The ability of various strains of CMV to infect endothelial cells in the presence or absence of sodium butyrate treatment.

Endothelial cells were inoculated at a MOI of 4 with the high (>100) fibroblast passaged strains of CMV (AD169, Towne or Davis), or the low (2-8) fibroblast passaged clinical strains which had also been passaged a number of times (4-10) through endothelial cells (as indicated by the subscripts, C1F₅E₄, C1F₂E₆, and Toledo.E₈). In Part A, representative histogram profiles of cells which had been inoculated without sodium butyrate treatment are shown, whilst in part B, the data show representative histogram profiles of inoculated cells treated with 10mM of sodium butyrate for two days. The percentage of CMV IEA positive cells (R1) was determined by flow cytometry at 5 days post inoculation. The ordinate represents the number of fluorescence events (scale 0-180), and the abscissa represents log₁₀ fluorescence intensity in arbitrary units (scale 10⁰-10⁴). The mean percentage of CMV IEA expression from a single experiment performed in triplicate is shown for each viral strain.

A. Uninfected endothelial cells



B. C2E-infected endothelial cells

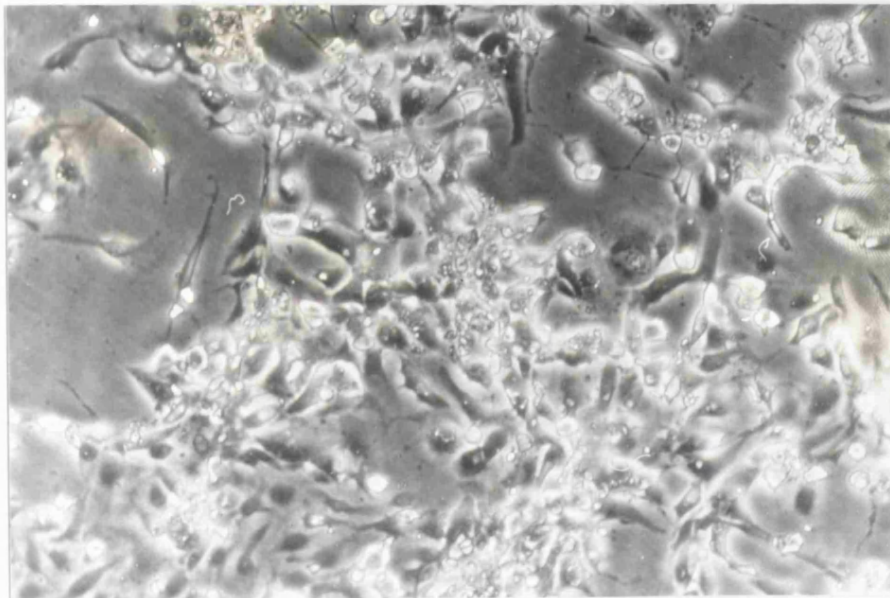


Figure 3.7.1. The ability of an ex vivo source of CMV to infect endothelial cells.

Leukocytes from a patient with a primary CMV infection were directly inoculated onto endothelial cells and removed after 1 hour. The endothelial cells were then propagated until characteristic CPE developed. In part B a phase contrast image of C2E-infected endothelial cells is shown, with extensive cell necrosis involving the whole monolayer. In part A, a phase contrast image of uninfected endothelial cells is shown, for comparison. All photomicrographs were taken at x100 magnification.

3.8. Discussion.

This study demonstrated that laboratory strains of CMV which had been highly passaged in fibroblasts, such as AD169, Towne and Davis, have a restricted capacity to infect endothelial cells *in vitro*, with <5% of inoculated cells expressing CMV IEA. Similar findings have been reported previously (Smiley et al. 1988; Friedman et al. 1981), and thus it was assumed from these early studies that CMV could not efficiently infect endothelial cells *in vitro*. However, two groups later suggested that sodium butyrate treatment of the endothelial cells improved the infection rate (Wu et al. 1994; Radsak et al. 1989), although in the earliest report, treatment of the cells prior to infection failed to have an augmentative effect. The data presented here supports the findings in the later report (Wu et al. 1994), as the percentage of CMV IEA positive endothelial cells could be increased by sodium butyrate treatment, either administered to the culture medium before or after virus inoculation, for all three virus strains. In addition, the percentage of CMV-infected cells could be further increased by low speed centrifugal inoculation of the virus onto the endothelial cells, a phenomenon which has also been found to increase CMV infection of fibroblasts and monocytes (Ho et al. 1993; Hodgkin et al. 1988).

The maximal percentage of infected cells found in this study was obtained with the combination of centrifugal inoculation plus sodium butyrate treatment for two days, for all three laboratory strains of CMV. The latter protocol resulted in a fully productive infection in a proportion of the cells, since infectious virus was detected in the endothelial cell supernatants five days after the viral inoculum had been removed. The fact that sodium butyrate could enhance CMV replication in endothelial cells after virus inoculation suggested that it affected events subsequent to viral entry into the cells, such as the induction of a protein necessary for viral replication, or the removal of an inhibitor of viral replication. Interestingly, a recent report showed that sodium butyrate stimulates Ras and the serine/threonine kinase Raf, and that these cellular proteins interacted with herpes simplex virus proteins to promote transcription of the viral genome (Frazier et al. 1996a; Frazier et al. 1996b). Induction of these cellular proteins have also been shown to be responsible for the increase in permissivity of certain neuroblastoma cells for CMV after stimulation with

substances such as retinoic acid (Angulo et al. 1995).

When compared to a low passage clinical isolate, C1F, inoculation of endothelial cells with the AD169 strain under the optimal conditions described above resulted in a similar percentage of cells which initially expressed CMV IEA, or CMV late (gB) antigen. In addition, these endothelial cells secreted similar titres of infectious virus into the extracellular fluid during the first two weeks following inoculation with either virus strain. Despite this, only the C1F strain could be serially propagated in endothelial cells, with an increasing number of the inoculated-cells displaying CMV IEA after each passage. Thus, although it was possible to artificially initiate one cycle of viral replication in endothelial cells using the AD169 strain by centrifugal inoculation and sodium butyrate treatment, the virus produced did not appear to be infectious for endothelial cells, and failed to spread to neighbouring uninfected cells. Similar results to those seen with AD169 were found for the other two highly fibroblast-passaged strains, Towne and Davis.

In contrast to the results obtained for the highly fibroblast-passaged laboratory strains of CMV, the low fibroblast-passaged clinical isolate, Toledo, could be propagated in endothelial cells in the same way as the C1F isolate. This data thus suggested that endothelial cell processing of CMV does not lead to the production of endothelial cell tropic virus per se, as all strains initially replicated in the endothelial cells to the same extent. Only the clinical isolate (C1F) and the Toledo strain of CMV, however, could be amplified through endothelial cells to produce cell-free virus which could replicate in endothelial cells without further treatment, although initial sodium butyrate treatment did appear to enhance this process. This latter fact may indicate that a critical level of some factor is required before full viral replication may take place; sodium butyrate treatment may accelerates this event. Thus it was more likely that continual passage of CMV through fibroblasts decreased its ability to infect endothelial cells. The latter proposal is supported by the finding here, as the C1F strain which had been passaged eight times in fibroblasts was less infectious for endothelial cells than was the same strain which had only been passaged in fibroblasts five times.

The clinical isolate C1 which had been passaged seven times in fibroblasts (C1F₇) was compared with the same strain which had been passaged twice in

fibroblasts and then five times in endothelial cells (C1F₂E₅). Cell-free viral supernatants of C1F₂E₇ showed increased infectivity for endothelial cells when compared to C1F₇, whereas both virus preparations were equally infectious for fibroblasts. This indicated that propagation through endothelial cells produced virus which was increasingly endothelial cell tropic, but which retained the capacity to infect fibroblasts. There were two possible explanations for these findings. First, that all particles in the virus population contained in the original blood specimen had the ability to grow in endothelial cells, but that this property was lost after *in vitro* passage through fibroblasts. Alternatively, the population of virus particles originally isolated from the infected patient contained endothelial cell-tropic variants, which were lost on passage through fibroblasts, but retained on passage through endothelial cells. Although it has not been formally demonstrated in this study, the latter proposal is supported by the fact that both C1F₇ and C1F₂E₅ replicated better in fibroblasts than in endothelial cells, suggesting that a larger proportion of fibroblast tropic virus than endothelial tropic virus was present in these preparations.

It was also possible, from the information stated above, that the original isolation of CMV from the clinical sample in fibroblasts selected out fibroblast-tropic virus at the initial isolation stage. However, the fact that direct isolation of virus present in the second clinical specimen, C2, was much more efficient on fibroblasts than endothelial cells suggested that CMV, as it exists *in vivo*, might be less endothelial cell-tropic than fibroblast-tropic. One difference between the endothelial cell and fibroblast cultures was the presence in the endothelial cell media of heparin, a substance known to inhibit the entry of extracellular virus (Neyts et al. 1992). However, as heparin was removed from the media during the initial inoculation of endothelial cells, and does not affect the cell to cell spread of CMV (Compton et al. 1993; Kari, Gehrz, 1992), it was unlikely that different culture conditions affected these results. Interestingly, despite a similar number of passages through endothelial cells, the two clinical isolates C1FE and C2E differed in the ability to produce extracellular virus. Thus, in contrast to previous reports, propagation of CMV through endothelial cells does not necessarily lead to the retention of cell-associated virus (Waldman et al. 1991; Waldman et al. 1989).

It therefore remains possible that fresh clinical specimens, and low fibroblast

passaged clinical isolates of CMV, contain a minor population of endothelial cell tropic variants which can be selected for *in vitro* by passage in endothelial cells. High fibroblast passaged strains of CMV such as AD169, Towne and Davis have lost the ability to be propagated in endothelial cells. Others have shown that high and low passaged strains of CMV have different abilities to replicate in epithelial cells (Mocarski et al. 1993). When the growth of Toledo, Towne and AD169 was compared in the SCID-hu mouse, the Toledo strain replicated to levels at least three orders of magnitude higher than did either of the laboratory strains, suggesting that cell tropism might also be important *in vivo* (Brown et al. 1995). Furthermore, the laboratory strains Towne and AD169 have been found to have large scale deletions of the viral genome when compared to low passage CMV isolates (Cha et al. 1996), and it should be emphasised that they were both developed as prototype vaccine strains, with the strategy of attenuating wild type virulence by high passage *in vitro*. Indeed *in vivo*, inoculation of the AD169 and Towne strains of CMV into healthy volunteers led to an asymptomatic seroconversion, whilst inoculation of the Toledo strain was associated with clinically apparent disease (Quinnan, Jr. et al. 1984). However, it has not been demonstrated that the deletions mentioned above were responsible for this altered virulence, and thus determination of the factors which govern both endothelial cell tropism and altered virulence warrants further investigation.

In summary, infection of endothelial cells with CMV occurs *in vitro*, and thus can provide a model by which *in vivo* alterations to this cell type during a natural infection may be elucidated. However, the ability of different strains of CMV to infect endothelial cells *in vitro* are influenced by the culture conditions. Highly fibroblast-passaged strains of the virus, such as AD169, Towne and Davis, may have lost their ability to replicate in endothelial cells *per se*, and thus require additional stimulation (e.g. by sodium butyrate treatment) in order to produce a limited infection in this cell type. Recent clinical isolates, however, are more endothelial cell tropic, and can productively infect a large number of endothelial cells without any treatment. The effect on endothelial cell surface molecule expression following infection with different strains of CMV is reported in the following chapters.

**CHAPTER 4. THE EFFECT OF CMV INFECTION
ON THE EXPRESSION OF CLASS I AND CLASS
II MHC MOLECULES.**

4.1. Introduction.

Class I and Class II MHC molecules are crucial in directing the adaptive immune response to foreign organisms. MHC-mediated presentation of peptide fragments obtained from these agents at the cell surface during an inflammatory reaction allows the T-lymphocyte to recognise infected tissues, and thus helps eliminate the invading organism (Janeway, Travers, 1996b). Decreased expression of either Class I or Class II MHC molecules during the course of an infection can therefore interfere with this process, and hence confer a potential mechanism by which the adaptive immune response may be evaded (Rinaldo, Jr. 1994). Conversely, the increased expression of Class I and Class II MHC has been implicated in promoting graft rejection in transplant recipients (Shoskes, Wood, 1994). Therefore, altered expression of these molecules on the blood vessel wall in the allograft could potentially enhance tissue destruction by alloreactive MHC-restricted T-lymphocyte effector cells (Shoskes, Wood, 1994).

Infection with CMV has been shown to alter MHC antigen expression *in vitro*, although the data obtained for cell surface MHC expression in different cell types are conflicting (Ibrahim et al. 1993; Hosenpud et al. 1991; Tudor et al. 1994; Barnes, Grundy, 1992). Possible explanations for these discrepancies include the fact that different viral strains have been used in the different studies, or that the effect of CMV infection on MHC expression may be dependent upon cell type. The following study was therefore undertaken in order to examine these possibilities. Endothelial cells or fibroblasts were inoculated with different viral strains (AD169, C1FE or Toledo.E for endothelial cells, and AD169, C1F or Toledo for fibroblasts), and the cell surface expression of Class I or Class II antigens was subsequently determined at various times post inoculation, using flow cytometric techniques. For endothelial cells which had been inoculated with CMV strain AD169, the effect of sodium butyrate treatment on the MHC expression at the surface of the uninfected and infected cells was also determined, for reasons discussed in the previous chapter. In addition, the effect of cell-free supernatants obtained from infected cells on the ability to alter Class I or Class II expression was also investigated, in order to determine whether any

changes observed were a direct viral effect, or due to the release of soluble mediators from infected cells.

4.2. The expression of Class I MHC molecules at the surface of endothelial cells following CMV replication.

The laboratory strain of CMV, AD169, was initially used to determine the effect of viral replication on the expression of Class I at the surface of endothelial cells. In the absence of sodium butyrate treatment, little replication of AD169 was observed in endothelial cells, but following such treatment the virus was induced to replicate in a proportion of the inoculated cells (see Section 3.2). Endothelial cells were thus inoculated with AD169 at a MOI of 4, and either treated with sodium butyrate for two days, or left untreated. All endothelial cells were then monitored for Class I expression 3 days later by flow cytometry. In addition, the ability of sodium butyrate to initiate viral replication in the AD169-inoculated endothelial cells was confirmed by CMV IEA detection of all treated or untreated cells in parallel. As expected, uninfected endothelial cells did not express this antigen, and the level of expression of CMV IEA in the inoculated but untreated cells was below the limit of detection (<1%). However, CMV IEA expression was detected in 15% of the inoculated and drug treated endothelial cells. Thus, in these latter cells, a mixed population of uninfected and CMV-infected endothelial cells existed. Consequently, the data obtained from subsequent experiments were recorded for all the cells included in the primary analysis (total population), and then the histogram profiles were observed for any subpopulations expressing different levels of Class I.

When the cells were monitored for the expression of Class I, using the total cell population, uninfected endothelial cells displayed a uniform level of Class I antigen expression at the cell surface, regardless of drug treatment (Figure 4.2.1). Furthermore, conversion of the logarithmic data, which had been acquired from three separate experiments performed in triplicate (n=9), to a linear form for statistical analysis (Sections 2.9 and 2.17), confirmed that there was no significant difference in the levels of Class I expression between the uninfected, sodium butyrate treated or untreated endothelial cells. In contrast, a significant increase in the level of expression of Class I was observed following inoculation of endothelial cells with the

laboratory strain AD169, regardless of drug treatment ($p=0.01$, $n=9$), suggesting that CMV increases the expression of MHC Class I on this cell type.

The above data was then re-analysed on the basis of the level of Class I expression observed for the histogram profiles. Only one level of Class I expression was observed for the uninfected cells which had been treated with sodium butyrate, and also for the untreated cells which had either been inoculated with CMV, or not (Figure 4.2.1). Thus the significance of these findings remained as described above. In contrast, when the histogram profiles of CMV-inoculated sodium butyrate treated cells were examined, two distinct 'peaks' of Class I expression were observed. The major 'peak' expressed higher Class I levels than those observed on the uninfected cells, whereas the minor 'peak' expressed lower Class I levels (Figure 4.2.1). When the percentage of cells contained within the 'high' or the 'low' Class I peaks was determined from the histogram profiles (84% and 16%, respectively), the percentage of cells in the latter peak was found to be similar to the percentage of cells expressing CMV IEA (15%). This suggested that CMV IEA positive cells were in fact the population which displayed decreased Class I expression at the endothelial cell surface, whilst the CMV IEA negative cells displayed increased expression of the same molecule.

4.3. The effect of CMV infection on the expression of Class I at the surface of endothelial cells at different times post inoculation.

The experiments presented above suggested that three days after sodium butyrate treatment of AD169-inoculated endothelial cells, down-regulation of Class I expression occurred in a minority of the cells. To verify that this was an effect of the virus, and was not due to drug treatment of the endothelial cells, the clinical CMV strain C1FE, which had been 'adapted' for growth in this cell type, was used to inoculate endothelial cells. Furthermore, because no sodium butyrate treatment was required after the original viral inoculation, it was possible to determine the effect of CMV infection on Class I expression, with respect to time. Endothelial cells were thus inoculated with C1FE at a MOI of 4 and the expression of Class I was analysed at various days post inoculation by flow cytometry, using the methods described above.

In agreement with the results obtained in section 4.2, two 'peaks' of Class I expression were observed following inoculation of the endothelial cells with CMV strain C1FE, whereas a uniform level of expression was observed at the surface of the uninfected cells. The median values of either the 'low', 'high' or 'total' Class I peaks obtained from three separate experiments performed in triplicate (n=9) are shown in Table 4.3.1. Characteristic 'high' and 'low' Class I peaks occurred at the surface of C1FE-inoculated for all time points tested, beginning at day 1 post inoculation and continuing through to day 5 (Table 4.3.1). Furthermore, the expression of Class I at the surface of the inoculated endothelial cells was significantly increased in the 'high' peak, and significantly decreased in the 'low' peak, with respect to the levels of this molecule on uninfected cells for all time points tested, when the logarithmically acquired data were converted to a linear form for statistical analysis ($p < 0.05$, $n = 9$, Table 4.3.1). Once again, the percentage of cells contained within the 'low' Class I peak (44%-56%, as determined from the histogram profiles) was found to be similar to the percentage of cells expressing CMV IEA at 3 days post inoculation (40-45%).

Interestingly, however, when the Class I values were taken from the 'total' population of inoculated endothelial cells, no significant difference between the expression of Class I was observed with respect to the uninfected cells at any time point (Table 4.3.1). This was in contrast to the obvious increase in Class I expression observed following inoculation of endothelial cells with AD169 in the presence of sodium butyrate (Section 4.2). One explanation for this apparent discrepancy was that, as stated above, a higher proportion of cells were initially expressing CMV IEA following inoculation with C1FE (40-45%), than was observed following inoculation with AD169 (15%, Section 4.2). Accordingly, the proportion of cells contained within the 'low' Class I peak (and presumably CMV IEA positive) at day 3 days post inoculation was also greater (44%-56%) than that obtained for AD169-inoculated and sodium butyrate treated cells (16%, Section 4.2). Thus it appears that the respective number of cells contained within the either the 'low' or the 'high' Class I peak influenced the results, when total Class I expression was determined for CMV-inoculated endothelial cells.

4.4. The effect of ganciclovir on the ability of CMV to alter the expression of Class I at the surface of endothelial cells.

Finally, the ability of either CMV immediate early/early, or late genes, to alter the expression of Class I at the surface of endothelial cells was investigated. The endothelial adapted strain of CMV, Toledo.E, was used in these studies for two reasons. First of all, this strain was consistently found to be susceptible to the effects of the antiviral drug ganciclovir, whereas C1FE (Section 4.3) was initially resistant to the drug (data not shown), and secondly, the viral inoculum contained slightly higher titres of CMV, thus leading to a greater percentage of cells initially expressing CMV IEA. Initial experiments performed using Toledo.E-inoculated endothelial cells revealed that Class I expression could once again be separated into 'high' and 'low' Class I peaks, and that the proportion of cells contained within the latter peak was similar to the percentage of cells initially expressing CMV IEA (data not shown). Accordingly, Toledo.E was inoculated onto endothelial cells at a MOI of 5, and CMV late gene expression was prevented by the addition of ganciclovir, a drug which prevents CMV DNA replication and thus late (but not immediate early or early) antigen expression (see Section 1.6). The expression of Class I was then determined at 4 days post inoculation, and the results obtained from the ganciclovir treated cells were then compared with either similarly treated uninfected cells, or untreated cells. The fact that ganciclovir treatment was effective in these experiments was confirmed by immunofluorescence staining for CMV late (gB) antigen expression. Thus, whereas both untreated and ganciclovir treated and inoculated cells were expressing approximately 75% CMV IEA, only the untreated cells expressed approximately 60% late antigen (data not shown).

When the expression of Class I was analysed at the surface of the ganciclovir treated or untreated, uninfected cells, a similar uniform level of this molecule was observed in both cases, whereas both the untreated or the ganciclovir-treated, Toledo.E-inoculated endothelial cells could be separated into 'high' and 'low' Class I peaks, on the basis of the histogram profiles. The results of this separation are shown in Figure 4.4.1. Furthermore, when the logarithmic data, which had been taken from three separate experiments performed in triplicate ($n=9$), were converted to a linear

form for statistical analysis, it was found that drug treatment had no effect on the uniform level of Class I expression for the uninfected cells, or the proportion of cells contained within the 'high' or 'low' Class I populations. Thus, the addition of the antiviral drug ganciclovir failed to prevent the alterations to Class I expression observed following inoculation of endothelial cells with CMV, suggesting that CMV late gene expression was not required for these functions.

Interestingly, however, there was an obvious decrease in the expression of Class I for both ganciclovir treated or untreated, inoculated cells, when compared to similarly treated but uninfected cells, after the total cell population had been analysed. As both a high infection rate (75-80% CMV IEA expression at day 3 post inoculation), and a corresponding increase in the percentage of 'low' Class I expressing cells (Figure 4.4.1) had been achieved during these experiments, the relative number of either 'low' or 'high' Class I expressing cells within the inoculated cell population appears to affect the results obtained using 'total' Class I expression. Thus, analysis of Class I expression using methods which only take into account the total level of this molecule is not the best way in which to present data from cell types which contain both CMV IEA positive and negative populations.

Taken together, the results from the experiments shown in sections 4.2. to 4.4 indicate a proportion of endothelial cells display decreased Class I expression, following inoculation with CMV strains AD169, C1FE and Toledo.E. Moreover, the proportion of cells contained within this 'low' Class I peak is approximately equal to the proportion of cells initially expressing CMV IEA, suggesting that it is the infected population which express 'low' Class I. The remainder of the (presumably uninfected) cells within the inoculated endothelial cell cultures display increased Class I expression. Both the decreased and the increased expression of this molecule was not prevented by the addition of the antiviral drug ganciclovir, indicating that CMV late gene expression was not responsible for these effects.

4.5. The expression of HLA Class I molecules at the surface of fibroblast cells following infection with CMV.

The experiments described above suggested that when the original inoculum of CMV only infected a proportion of endothelial cells, a dichotomous effect on Class

I expression occurred for three different virus strains. In order to confirm that this effect was due the low level of initial infection achieved, and was not a peculiar manifestation of endothelial cells, or of the strains capable of infecting this cell type, fibroblasts were inoculated with strains of virus which were propagated solely in the latter cell type (AD169 and Toledo). Furthermore, the titre of virus that was used during these studies infected approximately the same number of cells (25-70%) as observed after CMV was inoculated onto endothelial cells. Thus, fibroblasts were inoculated with strains AD169 or C1F at a MOI of 0.5, and the subsequent expression of Class I was monitored (using the histogram profiles) at various times post inoculation. Class I expression was not determined for the 'total' inoculated fibroblast population, for the reasons described above. In addition, the expression of CMV IEA was monitored in parallel.

As previously reported (Barnes, Grundy, 1992), following inoculation of fibroblasts with a titre of AD169 (and C1F) which resulted in an initial infection rate of 100%, Class I expression was uniformly decreased at the cell surface from day 1 post inoculation onwards (data not shown). After the fibroblasts had been inoculated with a viral titre which resulted in the initial infection of 43% of the cells, a dichotomous staining pattern was observed for Class I expression, beginning at day 1 post inoculation and continuing through to day 5 (Figure 4.5.1). Thus, a proportion of the inoculated fibroblasts showed a reduced expression of Class I, with respect to the uninfected cells, whilst the remainder showed an increased expression of the molecule (Figure 4.5.1). Furthermore, the proportion of fibroblasts which were expressing low Class I expression at day 1-3 post inoculation was similar to the percentage of fibroblasts initially infected with CMV (Figure 4.5.1).

When C1F was used as to infect fibroblasts, the results were essentially the same as for the laboratory strain AD169. A uninform level of Class I expression was observed at the surface of uninfected fibroblasts, whereas a dichotomous Class I expression was evident after the histogram profiles of the C1F-inoculated fibroblasts were examined. The median values of Class I expression taken from two separate experiments performed in triplicate (n=6) are shown in Table 4.5.1. When the logarithmically acquired data were converted to a linear form for statistical analysis, the results indicated that a proportion of the inoculated cells was again displaying a

significant decrease in Class I expression for all time points tested ($p=0.05$, $n=6$, Table 4.5.1). Moreover, the proportion of cells displaying this 'low' Class I expression (Table 4.5.1) was once again similar to the percentage of cells initially expressing CMV IEA (day 1, 40%; day 3, 65%; day 5, 95%). The remainder of the inoculated cells were showing an increase in Class I expression at all time points tested ($p=0.05$, $n=6$, Table 4.5.1), although the extent of this increase appeared to be diminishing by day 5 post inoculation (Table 4.5.1).

Thus, following inoculation of fibroblasts with either a laboratory strain of CMV, or a clinical isolate, a dual effect on Class I expression was observed at low viral doses. This indicated that CMV infection of both fibroblasts and endothelial cells affected Class I expression in a similar manner, and was not dependant on either the type of cell which was infected, nor on the CMV strain used. Furthermore, decreased Class I expression at early time points appeared to be related to the percentage of cells which were initially expressing CMV IEA, whilst the remaining cells increased the expression of this antigen. Although this correlation was not consistently observed at later time points, this was probably due to the *de novo* infection of previously uninfected cells in some experiments. Thus, both the timing and the methods used to examine Class I expression are important.

4.6. The effects of supernatants from CMV infected cells on the expression of Class I molecules at the surface of endothelial cells.

Finally, because CMV inoculation of endothelial cells always resulted in a mixed population of uninfected and infected cells, it was possible that soluble factors were produced by the infected cells which then acted on the 'bystander' uninfected cells to alter the expression of Class I MHC molecules. In order to test this possibility, supernatants were collected from C1FE-inoculated or uninfected endothelial cells at day 2 post inoculation, passed through a 0.2μ filter, and incubated with fresh, uninfected endothelial cells. The expression of Class I was then determined four days later, as described previously. Supernatants collected in this way failed to infect fresh endothelial cells, or the more permissive fibroblasts, indicating that infectious virus was not present in the supernatants at this time (data not shown).

The results from a representative experiment is shown in Figure 4.6.1. When the logarithmic data, which had been acquired from three experiments performed in triplicate (n=9), were converted to a linear form for statistical analysis, it was found that the expression of Class I at the uninfected cell surface was significantly enhanced by exposure to supernatants taken from C1FE-inoculated endothelial cells ($p=0.01$, $n=9$). Thus, an increase in Class I expression was observed when supernatants taken from these C1FE-inoculated cells were transferred onto fresh, uninfected endothelial cells, when compared to those cells which had received the supernatants from uninfected cells (Figure, 4.6.1). Furthermore, Class I expression was uniformly increased at the cell surface in the former case, with no decrease in Class I expression observed in any of the cells (data not shown). These results indicated that the presence of stable soluble factors in the supernatants taken from inoculated endothelial cells were responsible for the increased Class I expression seen previously (Sections 4.2 - 4.4), but were not responsible for the decrease in Class I expression seen during these same studies. Thus it can be inferred that Class I was up-regulated at the surface of bystander uninfected endothelial cells by soluble factors secreted during the course of CMV infection. Conversely, it was the CMV-infected cells within a mixed population of uninfected and infected cells which down-regulated the expression of this molecule.

4.7. The effect of CMV on the expression of Class II at the surface of endothelial cells.

The following set of experiments were undertaken in order to determine whether CMV infection of endothelial cells had any effect on the expression of Class II MHC molecules. Previous data have suggested that this molecule is not constitutively expressed at the surface of endothelial cells, but can be induced after interferon gamma (IFN- γ) stimulation of the cells. Initial experiments which were carried out during these investigations confirmed these findings, and showed that the maximum induction of Class II on the surface of endothelial cells occurred after 4 days of stimulation with 500 I.U. of IFN- γ (data not shown). As discrepancies concerning the ability of CMV to alter Class II expression may depend upon whether the endothelial cells had first been induced to express this molecule, the endothelial

cells were inoculated with C1FE (MOI of 4), and were either stimulated with IFN- γ immediately after inoculation, or left unstimulated. The expression of Class II was monitored at the surface of endothelial cells 4 days later by flow cytometry.

The results showed that neither uninfected nor C1FE-inoculated endothelial cells expressed Class II at the cell surface in the absence of IFN- γ stimulation, although after treatment with IFN- γ , both uninfected and C1FE-inoculated cells expressed this molecule on a proportion of the cells (Figure 4.7.1). In four separate experiments performed in triplicate ($n=12$), CMV-inoculated endothelial_{cells} did not increase the expression of Class II at the endothelial cell surface in the absence of IFN- γ treatment (uninfected cells, median 11, range 8-17; infected cells, median 10, range 9-17). In contrast, when the endothelial cells had been incubated with IFN- γ for 4 days, the inducible expression of Class II was markedly reduced at the surface of the inoculated endothelial cells (median 18, range 9-29) when compared with the induction of this molecule at the surface of the uninfected cells (median 71, range 26-124). Conversion of this logarithmically acquired data to a linear form, for statistical analysis, confirmed that a significant difference ($p=0.01$, $n=12$) occurred between the inoculated endothelial cells, and the similarly treated, but uninfected cells. In addition, the percentage of cells which were positive for the expression of this molecule, as determined by setting a marker on the basis of the profiles of the negative IgG1 control antibody, was also lower following CMV-inoculation of endothelial cells (Figure 4.7.1). Thus, whereas 65% of the uninfected, IFN- γ treated cells expressed this molecule, only 22% of the inoculated, IFN- γ treated cells were positive for Class II (data taken from the experiment shown in Figure 4.6.1). No direct correlation could be established between the percentage of cells expressing CMV IEA and the percentage of cells which could no longer be induced to express Class II (data not shown), and this may partly be due to the fact that not all the uninfected cells originally expressed Class II following IFN- γ stimulation. However, the results did show that Class II was not induced at the surface of unstimulated endothelial cells following infection with CMV, and that CMV infection resulted in a reduced ability to induce Class II expression following stimulation with IFN- γ .

4.8. The expression of Class II molecules at the surface of endothelial cells following inoculation of CMV, in the absence or presence of sodium butyrate treatment.

The above results indicated that infection of endothelial cells with CMV altered the expression of Class II MHC molecules only after the cells had been stimulated with IFN- γ . To determine whether viral replication was required for this process, the effect of inoculation with CMV strain AD169 on the inducible expression of Class II at the surface of endothelial cells was investigated. As mentioned previously, this strain requires sodium butyrate in order to replicate in a proportion of the inoculated cells. Thus, endothelial cells were inoculated with AD169 at a MOI of 4, and treated for two days with sodium butyrate, or left untreated. All the cells were then stimulated for a further 4 days with IFN- γ , and the expression of Class II determined as described previously. The ability of sodium butyrate to promote viral gene expression was confirmed by staining cytocentrifuged preparations of these cells for CMV IEA. Accordingly, it was found that only the sodium butyrate treated, inoculated cells were positive for this antigen in approximately 25-30% of the cells, whereas the similarly inoculated, untreated cells did not express this antigen.

When the expression of Class II was determined at the surface of the IFN- γ stimulated endothelial cells, a slight but insignificant increase in the expression of this molecule was observed when the uninfected, sodium butyrate treated cells were compared to the uninfected cells which had received no such treatment (Table 4.8.1). There was also no significant change in the expression of Class II when the untreated, AD169-inoculated endothelial cells were compared with either the sodium butyrate treated, or the untreated, uninfected cells (Table 4.8.1). After the inoculated cells had been treated with sodium butyrate, however, a significant decrease ($p=0.05$, $n=9$) in the level of expression of Class II occurred at the endothelial cell surface, when compared with any of the other cell populations (Table 4.8.1). In addition, there was also a marked reduction in the percentage of inoculated, sodium butyrate treated cells which were positive for Class II expression, when compared with any of the other groups (Table 4.8.1). This data suggested that active CMV replication was necessary to prevent effective induction of Class II following IFN- γ stimulation of endothelial

cells. Once again, however, no definite correlation was determined between the percentage of cells expressing CMV IEA and the percentage of cells in which Class II could no longer be induced.

4.9. The effect of ganciclovir on the ability of CMV to alter the expression of Class II at the surface of IFN- γ treated endothelial cells.

As stated above, it appeared that CMV replication was required in order to alter the inducible expression of Class II at the surface of inoculated endothelial cells. However, it was not clear whether immediate early, early or late gene expression was responsible for this effect. Thus the effect of CMV late gene expression on the IFN- γ inducible expression of Class II was investigated, using the ganciclovir-susceptible strain, Toledo.E. Endothelial cells were inoculated with Toledo.E at a MOI of 5, and the subsequent expression of late viral antigen was prevented by the addition of ganciclovir, as previously described. The results obtained from the ganciclovir-treated cells were then compared with similarly inoculated, but untreated cells, after all the cells had been stimulated with IFN- γ for 4 days. Ganciclovir treatment was effective in these experiments, as the Toledo.E inoculated endothelial cells which had been treated with the drug did not express CMV late (gB) antigen, whereas the untreated inoculated cells expressed this antigen in approximately 60% of the cells (data not shown).

In the absence of ganciclovir treatment, the results for both the uninfected (median 96, range 83-124) and the Toledo.E-inoculated (median 42, range 17-70) endothelial cells were essentially the same as described above for the C1FE and AD169 strains in four separate experiments performed in triplicate (n=12). Thus, when the logarithmically acquired data were converted to a linear form for statistical analysis, there was a significant decrease ($p=0.01$, $n=12$) in the expression of Class II at the surface of the inoculated cells in the absence of ganciclovir treatment, when compared to the similarly untreated uninfected cells. There was also a reduction in the percentage of cells positive for Class II expression, when the uninfected and Toledo.E-inoculated, untreated cells were compared (74% and 32%, respectively). The addition of ganciclovir to both the uninfected and Toledo.E-inoculated cells did not alter the trend in results, with a significant ($p=0.01$, $n=12$) reduction in the

expression of Class II occurring at the surface of the inoculated cells (median 49, range 35-79), with respect to the similarly treated, uninfected cells (median 111, range 85-154). Once again, the percentage of Class II positive cells was reduced in the former case (35%) with respect to the latter (65%). Thus, CMV late antigen expression was not required in order to down-regulate Class II expression at the surface of IFN- γ stimulated endothelial cells.

4.10. The ability of supernatants from CMV infected cells on the expression of Class II molecules at the surface of endothelial cells.

Finally, the ability of soluble factors produced by the infected cells to alter the expression of Class II MHC molecules was investigated. Supernatants were collected from C1FE-inoculated or uninfected endothelial cells at day 2 post inoculation, passed through a 0.2 μ filter, and incubated with fresh, uninfected endothelial cells. IFN- γ was added to these supernatants, prior to transfer, and the IFN- γ -inducible expression of Class II molecules was then determined four days later, as described previously. Supernatants collected in this way failed to infect fresh endothelial cells, or the more permissive fibroblasts, indicating that infectious virus was not present in the supernatants harvested at this time post inoculation (data not shown).

A representative experiment is shown in Figure 4.10.1. When the IFN- γ -inducible expression of Class II was investigated in this way, similar levels of IFN- γ induced Class II expression were observed after supernatants taken from C1FE-inoculated cells were transferred onto fresh, uninfected endothelial cells, when compared to those cells which had received the supernatants from uninfected cells (Figure 4.10.1). When the logarithmically acquired data, taken from three experiments performed in triplicate, were converted to a linear form for statistical analysis, it was found that no significant change ($n=9$) in the expression of Class II occurred at the surface of the incubated endothelial cells, regardless of the source of the supernatant. These results suggested that a soluble factor was not responsible for the reduced induction of Class II expression observed previously (Section 4.7 - 4.9) following inoculation of endothelial cells with CMV.

It was possible, however, that Class II expression was affected by soluble factors which were produced at times other than day 2 post inoculation. In order to

test this possibility, transwell filters containing C1FE-inoculated or uninfected endothelial cells were placed on top of fresh uninfected endothelial cells growing in a separate chamber, and incubated for 4 days. Thus the uninfected cells in the bottom chamber were continually exposed to factors released from the uninfected or infected cells present in the upper chamber. IFN- γ was added to the top chamber immediately after inoculation, in order to verify the ability of soluble factors to pass through the filter. The expression of Class II on the uninfected endothelial cells contained within the bottom chamber was then determined as described previously. A representative experiment is shown in Figure 4.10.1. When the logarithmically acquired data, taken from two experiments performed in triplicate ($n=6$), were converted to a linear form for statistical analysis, the results indicated that no significant change in the expression of Class II occurred on these latter cells, when exposed to soluble factors from either uninfected or C1FE-inoculated endothelial cells ($n=9$). The C1FE-inoculated cells contained within the top chamber, however, displayed a significant ($p=0.01$, $n=6$) decrease in Class II expression, when compared to the uninfected cells, confirming the ability of CMV to affect the IFN- γ inducible expression of Class II during these experiments. Thus, soluble factors were not responsible for the decrease in Class II expression observed following CMV inoculation of endothelial cells, suggesting it was mainly the infected cell population which altered the ability of Class II to be induced by IFN- γ .

Table 4.3.1. The effect of CMV on the expression of Class I at the surface of endothelial cells with respect to time.¹

	Uninfected	Infected (C1FE)		
		Total Class I	High Class I	Low Class I
DAY 1	70 (32-104)	65 (48-340)	237 (137-1000)** ²	36 (23-58)** ²
DAY 3	100 (67-153)	110 (58-160)	221 (149-965)** ²	41 (14-54)** ²
DAY 5	78 (56-133)	93 (38-160)	229 (78-506)* ²	32 (15-54)** ²

¹Endothelial cells were inoculated with the clinical isolate C1FE at a MOI of 4, and at various times post inoculation, the expression of Class I was determined by flow cytometry. The data represent the median values of three experiments performed in triplicate (n=9), plus the range of median values obtained. The values were either taken from the total population of uninfected or inoculated endothelial cells, or the C1FE-inoculated cells were separated into 'low' and 'high' Class I expression on the basis of the histogram profiles. Markers were set between the two 'peaks' observed, and the median fluorescent intensity values were determined for each peak separately.

²Logarithmically acquired data were converted to a linear form, and the values obtained for the C1FE-infected cells were compared with the values obtained for the uninfected cells. Significance was determined using the Wilcoxon paired comparison test. * p = 0.05, ** p = 0.01, n=9.

Table 4.5.1. The effect of infection with a clinical isolate of CMV on the expression of Class I at the surface of fibroblasts.¹

	Uninfected	Infected (C1F)			
	MDFI	MDFI		% Cells	
	Total Class I	High Class I	Low Class I	High Class I	Low Class I
Day 1	45 (32-344)	132 (117-566)* ²	25 (23-94)* ²	60%	40%
Day 3	49 (41-322)	116 (96-891)* ²	17 (16-41)* ²	57%	45%
Day 5	59 (53-433)	83 (78-227)* ²	15 (14-19)* ²	61%	39%

¹Fibroblasts were inoculated with the clinical isolate C1F at a MOI of 0.5, and at various times post inoculation, the expression of Class I was determined by flow cytometry. The data represent the median values of two experiments performed in triplicate (n=6), plus the range of median values obtained. The values were either taken from the total cell population of uninfected cells, or the C1F-inoculated cells were separated into 'low' and 'high' Class I expression on the basis of the histogram profiles. Markers were set between the two 'peaks' observed, and the median fluorescent intensity values (MDFI), or the respective number of cells contained within these peaks (% cells), determined for each peak separately.

²Logarithmically acquired data were converted to a linear form, and the values obtained for the C1FE-infected cells were compared with the values obtained for the uninfected cells. Significance was determined using the Wilcoxon paired comparison test. * p = 0.05, n=6.

Table 4.8.1. The effect of CMV infection on the expression of Class II following stimulation of endothelial cells with interferon gamma.¹

	MDFI		% Cells	
	Uninfected	Infected	Uninfected	Infected
No n-butyrate	69 (33-107)	69 (28-100)	65% (± 9)	61% (± 11)
+ n-butyrate	54 (24-75)	30 (25-31)* ²	63% (± 8)	27% (± 6)* ²

¹Endothelial cells were inoculated with the laboratory strain, AD169, at a MOI of 5, and either treated with sodium butyrate (+n-butyrate) for two days, or left untreated (no n-butyrate). IFN- γ was then added to all cells, and the expression of Class II was monitored at the endothelial cell surface 4 days later by flow cytometry. The data represent the median values (MDFI) of three experiments performed in triplicate (n=9), plus the range of median values obtained. In addition, the percentage of cells positive for Class II was determined by using the histogram profiles of an irrelevant control antibody as a marker for negative Class II expression.

²Logarithmically acquired data were converted to a linear form, and the values obtained for the C1FE-infected cells were compared with the values obtained for the uninfected cells. Significance was determined using the Wilcoxon paired comparison test. * p = 0.01, n=9.

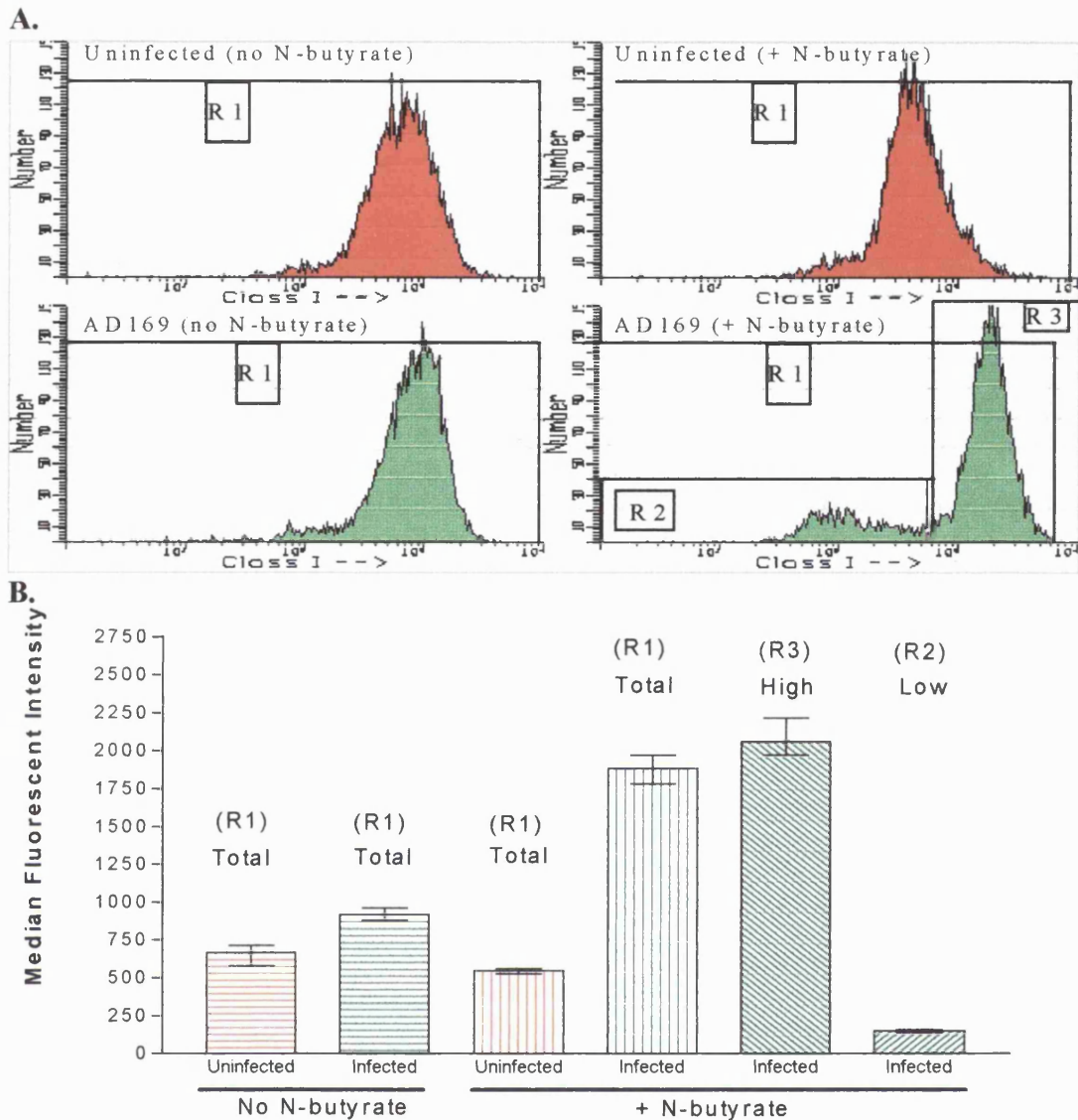


Figure 4.2.1. The effect of CMV infection on the expression of Class I MHC molecules at the surface of endothelial cells.

CMV strain AD169 was inoculated onto endothelial cells at a MOI of 4, and the cells were either treated with 10mM sodium butyrate (+ N-butyrate) or left untreated (no N-butyrate). Class I expression was determined 4 days later, using flow cytometric techniques. In part A, representative histogram profiles from a single experiment are shown. Markers were set using either the total population of cells (R1), or were separated on the basis of Class I expression (high or low, R3 and R2, respectively). The ordinate represents the number of fluorescence events (scale 0-150), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). In part B, the data represent the median fluorescent intensity of triplicate samples for a representative experiment. The values were either taken from the total cell population (total population, R1), or from the 'high', (R2) and 'low' (R3) Class I populations, where appropriate. The error bars show the range of values obtained.

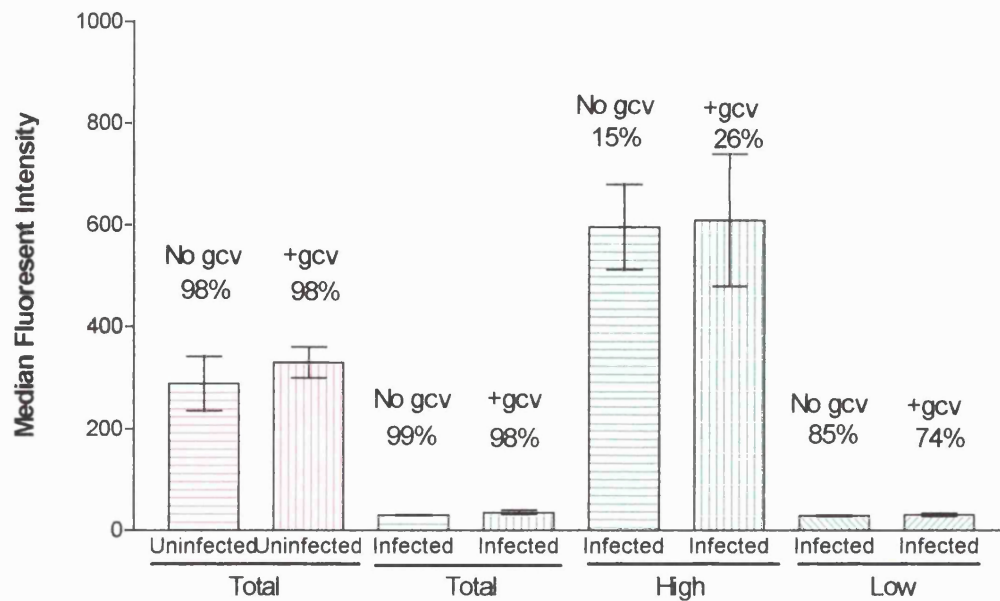


Figure 4.4.1. The effect of ganciclovir on the ability of CMV to alter the expression of Class I MHC molecules at the surface of endothelial cells.

Endothelial cells were inoculated with CMV strain Toledo at a MOI of 5, and either treated with 50 μ g/ml of ganciclovir (+gcv) or left untreated (No gcv). The expression of Class I MHC was determined four days later, using flow cytometric techniques. The data represent the median fluorescent intensity of triplicate samples for a representative experiment. The values were either taken from the total population of uninfected and CMV-inoculated cells, or the CMV-inoculated cells were separated into 'low' and 'high' Class I expression on the basis of the histogram profiles. The respective percentage of cells contained within each of these population is indicated. The error bars show the range of values obtained.

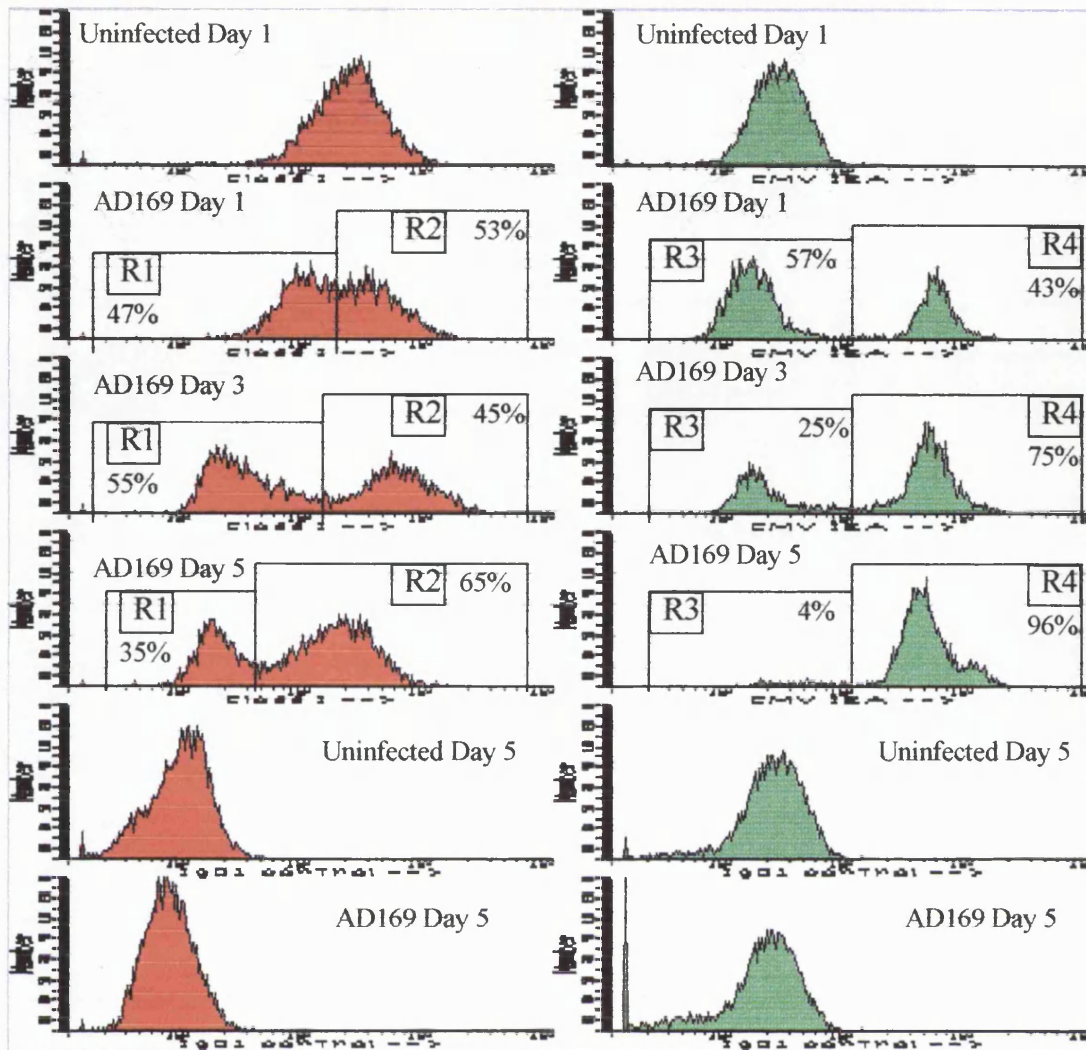


Figure 4.5.1. The effect of CMV infection on the expression of Class I MHC molecules at the surface of fibroblasts.

Fibroblasts were inoculated with strain AD169 at a MOI of 0.5 and left for various periods of time. The expression of Class I or CMV IEA was then monitored at the cell surface or in the nucleus of the fibroblasts, respectively, using flow cytometric techniques. Representative histogram profiles are shown for either unfixed fibroblasts (red) incubated with a Class I antibody, or acetone fixed cells (green) incubated with the CMV IEA antibody, E13 (top four panels). Class I expression was separated into 'low' or 'high' peaks (R1 and R2, respectively), whilst CMV IEA expression was separated into negative or positive populations (R3 and R4, respectively). The percentage of cells contained in each population is indicated. In addition, representative histogram profiles of uninfected or AD169-inoculated fibroblasts incubated with the IgG1 negative control antibody are shown at day 5 (last two panels). The ordinate represents the number of fluorescent events (scale 0-150), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4).

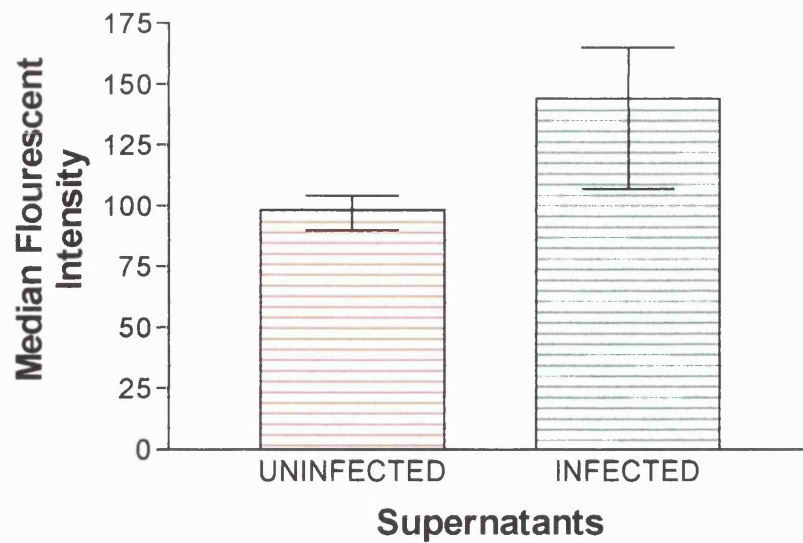


Figure 4.6.1. The effect of soluble factors on the expression of Class I MHC molecules at the surface of endothelial cells.

Uninfected endothelial cells were incubated with supernatants taken from either uninfected endothelial cells (uninfected), or from C1FE-inoculated endothelial cells which had been infected for 2 days (infected). The expression of Class I was determined 4 days later by flow cytometry. The data represent the median fluorescent intensity values of a representative experiment performed in triplicate. The error bars show the range of values obtained.

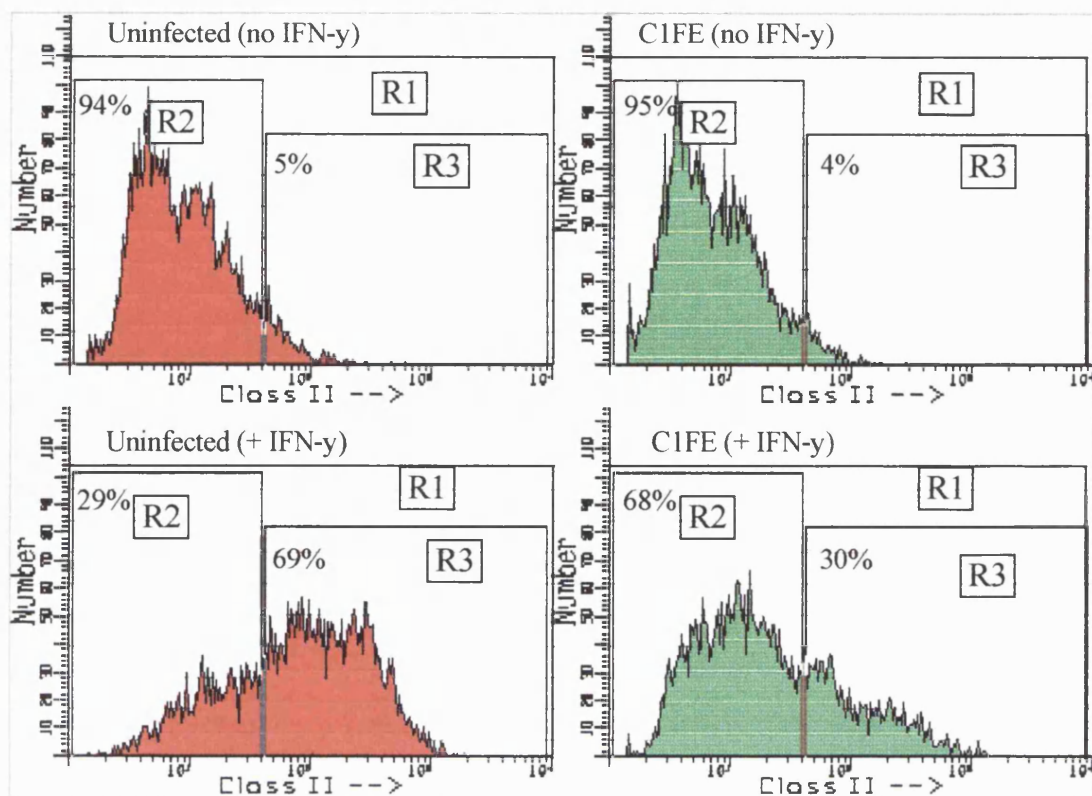


Figure 4.7.1. The effect of CMV infection on the expression of Class II MHC at the surface of endothelial cells.

CMV strain C1FE was inoculated onto endothelial cells at a MOI of 5, and either treated with 500 I.U. of interferon gamma (+ IFN-γ), or left untreated (no IFN-γ). Class II expression was determined four days later, using flow cytometric techniques. Representative histogram profiles are shown for uninfected or C1FE-inoculated cells. The median fluorescent intensity of Class II was determined for the total population (R1). In addition, both the negative population (R2) or the positive population (R3) was determined by using an IgG1 isotype control as a negative marker (not shown), and the percentage of cells contained within each population is indicated. The ordinate represents the number of fluorescence events (scale 0-130), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4).

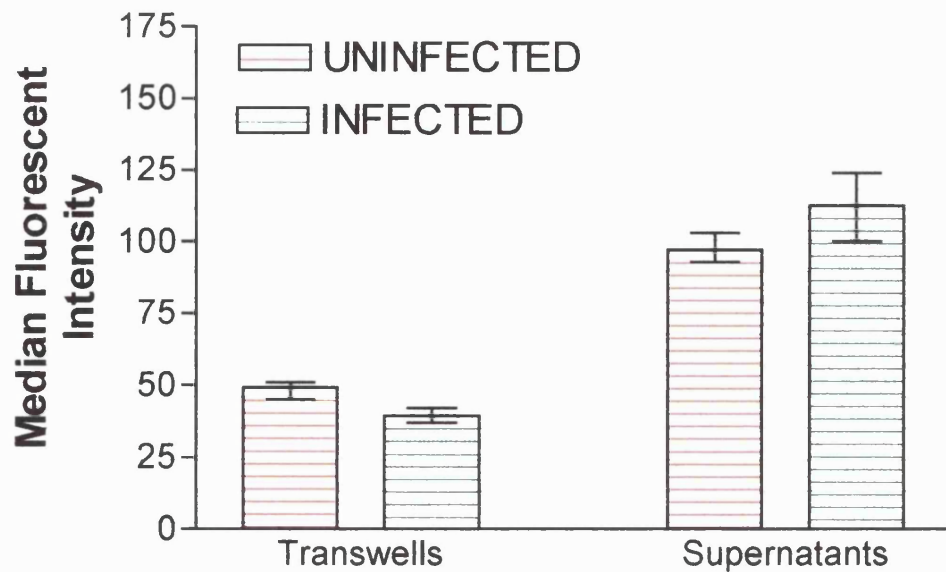


Figure 4.10.1. The effect of soluble factors on the expression of Class II MHC molecules at the surface of endothelial cells.

Uninfected endothelial cells were incubated with supernatants taken from either uninfected endothelial cells (uninfected), or from C1FE-inoculated endothelial cells which had been infected for 2 days (infected). IFN- γ was added to these supernatants, and Class II expression was determined 4 days later by flow cytometry. In addition, uninfected or C1FE-inoculated endothelial cells were grown on a transwell filter, and placed on top of fresh uninfected endothelial cells growing in a separate chamber (Transwells). IFN- γ was then added to the top (Transwell) chamber, and the expression of Class II on the surface of endothelial cells contained in the bottom chamber was determined 4 days later by flow cytometry. The data represent the median fluorescent intensity values of a representative experiment performed in triplicate. The error bars show the range of values obtained.

4.11. Discussion.

The results presented in this chapter indicated that a dual effect on the expression of Class I molecules occurred at the surface of endothelial cells following inoculation with CMV for all time points studied; A proportion of the inoculated endothelial cells displayed significantly decreased Class I expression in relation to the levels found on the uninfected cells, whilst the remainder showed a significant increase in the expression of this molecule. These effects were not strain dependant, as three different viral strains (AD169, C1FE and Toledo.E) produced essentially the same results. Active CMV replication to at least the IE stage was necessary in order to promote this dual effect, as only sodium butyrate treated endothelial cells which had been inoculated with strain AD169 showed evidence of both decreased and increased Class I expression; similarly inoculated but untreated cells failed to down-regulate Class I. Furthermore, the proportion of CMV-inoculated cells which displayed the decreased Class I expression at days 1-3 post inoculation was similar to the percentage of cells that were initially infected with CMV, for all three viral strains. Thus, whilst formal proof has not been demonstrated in this study, the most likely explanation was that the actively CMV-infected cell population down-regulated Class I expression, whilst the bystander 'uninfected' endothelial cells up-regulated the surface expression of this molecule.

Previous studies concerning the effect of CMV infection on the expression of Class I at the surface of various cell types have led to equivocal results. Thus it has been reported that CMV either induces the expression of Class I at the surface of endothelial cells (van Dorp et al. 1989; Tudor et al. 1994), smooth muscle cells (Hosenpud et al. 1991) and proximal tubular epithelial cells (van Dorp et al. 1993), or else had no effect (Ustinov et al. 1991; Hosenpud et al. 1991). In fibroblasts, however, the majority of reports conclude that surface Class I expression was decreased following infection with CMV (Warren et al. 1994a; Yamashita et al. 1993a; Barnes, Grundy, 1992a). Furthermore, it has been shown that at least three separate genes in the unique short region of the CMV genome were responsible for the down-regulation of Class I in fibroblasts and astrocytes. The first of these, US3, is expressed at the IE stage of infection, and prevents both the maturation and transport of Class I molecules

through the endoplasmic reticulum (Ahn et al. 1996; Jones et al. 1996). At the early stage of infection, two further gene products, US2 and US11, destabilise and increase the turnover of newly synthesised Class I molecules, and promote heavy chain transport back to the cytosol for degradation by proteosomal enzymes (Jones et al. 1995; Wiertz et al. 1996). The results presented here support the notion that, as seen in fibroblasts, Class I expression is in fact decreased in endothelial cells following infection with CMV. Moreover, the fact that the addition of ganciclovir (an inhibitor of late viral gene expression) to the culture medium had no effect on the alterations to Class I expression seen following inoculation with CMV suggested that either IE or early gene expression was responsible. However, it is unclear at present which of the above gene products were responsible for this effect because each mechanism may be independently operative in different cell types, as is the case for murine CMV (Slater, Campbell, 1997).

The fact that some studies have shown an increase in Class I expression following infection with CMV in cell types other than fibroblasts (including endothelial cells) is at first confusing, especially as altered Class I expression was attributed to a direct viral effect in some cases (van Dorp et al. 1993; van Dorp et al. 1989). One possible explanation for these discrepancies was that CMV infection produced a cell type-specific increased Class I response. This explanation, however, was considered unlikely because a proportion of endothelial cells in this study did decrease Class I expression following CMV replication. Furthermore, the dual effect on Class I expression was not cell type specific, as inoculation of fibroblasts with low doses of CMV (strains AD169, C1F and Toledo) which did not produce infection of all the cells, also produced decreased and increased expression in separate populations of the cells. This relationship has been reported previously, where it was shown that in mixed populations of infected and uninfected fibroblasts, the CMV-infected population down-regulated Class I expression, whilst the majority of cells which were up-regulated belonged to the uninfected population (Kobayashi et al. 1995; Steinmassl, Hamprecht, 1994). An alternative explanation for these conflicting data, therefore, may be due to differences in experimental conditions. In the majority of the studies cited above, only minimal infection of the cell type in question was achieved (<10%), and the methods used to monitor Class I expression focused on the

total inoculated cell population. Thus, it was likely that the measurements obtained were distorted by the presence of uninfected cells within the cultures expressing high levels of Class I, so masking the down-regulation of Class I at the surface of the CMV infected cells. The results presented here support this latter assumption, as an increase in Class I expression was observed at the surface of endothelial cells when the total cell population was analysed following infection of less than 50% of the cells, whereas at higher percentages of infection, an overall decrease in Class I expression was observed. It is therefore imperative to closely analyse the extent of the infection in order to distinguish between the effect on the infected cell itself, or the uninfected bystander cells within the culture.

The results presented here also showed that the inoculation of endothelial cells with CMV strain AD169 in the absence of sodium butyrate treatment (a condition which did not promote viral replication) led to a moderate increase in Class I expression, rather than the high levels seen on a proportion of the cells after the replication of AD169 was stimulated by drug treatment. Two possible explanations for these results could be suggested. One was that the increased Class I expression was mediated by a soluble factor which was both present in the initial viral inoculum, and which further accumulated in the culture medium after being released from actively infected cells. The alternative explanation was that CMV caused a direct enhancement of Class I expression on the surface of cells harbouring the virus in the absence of full replication. The transfer of supernatants from C1FE-inoculated endothelial cells onto fresh uninfected cells led to the increased expression of Class I on the surface of the latter. As these cell-free supernatants were taken from the C1FE-inoculated endothelial cells after two days, infectious virus was unlikely to have been present because it took at least three days before progeny virus was produced, and these supernatants did not infect the more permissive fibroblast cell type. Thus, it was more likely that the high increase in Class I expression seen on the 'uninfected' cells during an active viral replication was mediated, at least in part, by a soluble factor. Although the soluble factor responsible was not examined in the present study, other groups have suggested that the release of interferon- β from CMV-infected fibroblasts contributed to the increase in Class I expression on bystander uninfected cells,

because the addition of interferon- β antibodies abrogated this effect (Steinmassl, Hamprecht, 1994; Grundy et al. 1988). Endothelial cells are also capable of producing interferon- β in response to CMV infection (Sedmak et al. 1995), and thus may cause a similar effect on Class I expression on the bystander uninfected cells.

The addition of interferon- β antibodies in some studies, however, did not completely prevent the increase in Class I expression, and thus other components may be involved in this process (Steinmassl, Hamprecht, 1994; Grundy et al. 1988). Thus, some groups have attributed the increase in Class I expression to a direct viral effect, because no increase in Class I expression occurred at the surface of epithelial and smooth muscle cells following inoculation with ultraviolet irradiated virus-containing supernatants (Tuder et al. 1994; van Dorp et al. 1993; van Dorp et al. 1989). Thus CMV gene expression was necessary in order to increase Class I in these experiments (Tuder et al. 1994; van Dorp et al. 1993; van Dorp et al. 1989). In support of this proposal, it has been shown that CMV IE1 and IE2 gene expression was sufficient to activate Class I transcription through interactions with the minimal promoter regions in the HLA.A2 gene (Burns et al. 1993). Moreover, deletion of the US2-US11 region (the genes responsible for down-regulating Class I) also leads to the increase in expression of Class I (Jones et al. 1995). A similar mechanism (i.e. direct viral effect/partial gene expression) may be responsible for the moderate increase in surface Class I seen in the present study, after CMV strain AD169 was inoculated onto endothelial cells in the absence of sodium butyrate treatment. However, another study demonstrated that Class I expression was in fact increased at the surface of fibroblasts following inoculation with $\alpha\gamma$ inactivated virus, due to the presence of IFN- β in the supernatants (Yamashita et al. 1993). Thus, the effect of soluble factors, either present in the original virus inoculum, or induced following partial virus replication, could also explain the aforementioned moderate increase in Class I expression observed in the present study, and thus warrants further investigation.

With regards to the effect of CMV infection on the expression of Class II molecules, in the present study no increase occurred at the surface of CMV-inoculated endothelial cells in the absence of IFN- γ stimulation. It was only after the addition of this cytokine to the culture medium that a change in Class II expression

was detected. Thus, the percentage of cells expressing Class II antigen after four days of IFN- γ stimulation was severely reduced on the endothelial cells which had been inoculated with either AD169, C1FE or Toledo.E. This failure of the CMV-inoculated cells to express Class II after cytokine stimulation was dependant upon viral replication, as CMV strain AD169 did not prevent IFN- γ Class II induction in the absence of sodium butyrate treatment. The fact that the transfer of supernatants from C1FE-inoculated endothelial cells to fresh uninfected cells also failed to prevent the induction of Class II on the surface of the latter suggested that the actively infected cell population produced this effect, rather than it being mediated by a soluble factor acting on the uninfected cell population. However, no direct correlation between the percentage of cells lacking Class II and the percentage of cells initially infected with CMV could be established. This was possibly due to the fact that not all the uninfected endothelial cells expressed Class II following stimulation with IFN- γ , and so alterations in the CMV-inoculated endothelial cells, infection might have occurred in an already 'non-responding' population of cells.

It was not possible to study the timing of altered Class II expression in the present study because the maximum stimulation of this molecule occurred after 4 days of IFN- γ treatment, and so was the only time point studied. However, the fact that ganciclovir failed to prevent the down-regulation of Class II on CMV-inoculated endothelial cells suggested that IE or early genes were responsible. An identical conclusion was reached in a recent study by a separate group (Miller et al. 1998). This, however, was in contrast to the situation described following infection of rat endothelial cells with rat CMV, whereby induction of Class II expression was abrogated after the addition of this drug to the culture medium (Ustinov et al. 1994). Furthermore, the authors of the latter study suggested that ganciclovir directly promoted Class II expression following IFN- γ stimulation of uninfected endothelial cells (Ustinov et al. 1994). In the study presented here, however, no such induction was demonstrated.

Another difference between the data obtained following infection of rat endothelial cells with rat CMV, and the experiments concerning human CMV infection presented here, was that in the former case, the induction of Class II was

attributed to a direct viral effect which occurred in the absence of cytokine stimulation (Ustinov et al. 1994a; Ustinov et al. 1994). No such induction was observed following endothelial cell inoculation with any of the human CMV strains studied here, either with or without IFN- γ stimulation, which is in agreement with previous reports by others (van Dorp et al. 1989; Sedmak et al. 1990). In the one human study where CMV was purported to directly increase Class II expression, only one of four specific Class II antibodies reacted with the infected cells (Khoury et al. 1991). Furthermore, the effect in question was limited exclusively to thyroid follicular (epithelial) cells, and not to contaminating infected mononuclear or fibroblastoid cells contained within the cultures, and thus may have reflected a cell type specific response (Khoury et al. 1991). However, this latter proposal seems unlikely considering other studies have shown that lung alveolar or kidney tubular epithelial cells did not induce Class II expression following infection with CMV (van Dorp et al. 1993; Ng Bautista, Sedmak, 1995). One possible explanation was that the particular antibody in question was cross-reacting with a DR β chain (Class II) homologue encoded by the IE-2 region of the CMV genome (Fujinami et al. 1988), but whatever the reason, the report of Khoury remains unique in demonstrating a direct up-regulation of Class II by human CMV.

Following IFN- γ stimulation of CMV-inoculated endothelial cells, however, reduced Class II expression was observed when compared to IFN- γ stimulated uninfected cells (Sedmak et al. 1994a; Scholz et al. 1992a), regardless of the source of the endothelial cell cultures (Knight et al. 1997). The studies presented here support this view, as CMV-inoculated cells were refractory to IFN- γ stimulation of Class II expression. In one study this reduction was partly attributed to the production of IFN- β during the viral infection, because supernatants from CMV-infected cells also reduced Class II expression on uninfected cells (Sedmak et al. 1995). This cytokine has previously been shown to be a soluble inhibitor of IFN- γ -induced Class II up-regulation on endothelial cells (Lapierre et al. 1988). In the present study, however, transfer of cell-free supernatants from C1FE-inoculated endothelial cells did not produce this effect, suggesting that the down-regulation of Class II expression was occurring at the surface of the CMV-infected cells, rather than on the bystander

uninfected cells. In agreement with this assumption, the percentage of cells which were lacking Class II expression following IFN- γ stimulation were found by others to be proportional to the percentage of cells expressing CMV antigens, (Scholz et al. 1992), again suggesting that it was the infected cells which were refractory to Class II induction. The reasons for the differences in these separate studies are unclear, but may reflect different experimental procedures. In particular, CMV infection was allowed to progress to a greater extent in the former study (Sedmak et al. 1995) than in the present one, and this may have resulted in the accumulation of the inhibitory factor in the former case. Indeed, IFN- β -mediated Class II down-regulation on macrophages during a murine CMV infection was only observed after an high viral dose was administered (Heise et al. 1998b). Interestingly, an alternative mechanism responsible for the Class II defect in CMV infected cells has recently been elucidated. Disruption to the Jak/Stat pathway of the INF- γ signal transduction pathway following inoculation with CMV prevented this molecule being expressed at the surface of the infected cell (Miller et al. 1998), and is likely to have been responsible for the lack of induction of Class II on the CMV infected endothelial cells observed here.

In summary, following inoculation of endothelial cells with CMV, Class I down-regulation occurred on a proportion of the cells. This appeared to be a direct viral effect, and was not cell type specific. However, when the CMV-infected population was less than 100%, a soluble factor was produced which up-regulated Class I expression on the remaining uninfected cells within the culture. Similarly, Class II expression was reduced following inoculation of endothelial cells with CMV. In this case, however, the effect was only evident following IFN- γ stimulation of the infected cells, and no increased expression was observed of the surrounding uninfected cells. The possible *in vivo* implications of these effects are discussed in Chapter 8.

CHAPTER 5.

THE EFFECT OF CMV INFECTION OF ENDOTHELIAL CELLS AND FIBROBLASTS ON THE INDUCTION OF A RECEPTOR FOR THE F_c PORTION OF IgG.

5.1. Introduction.

Receptors for the Fc portion of IgG (FcγR) are important in maintaining the function of both the innate and the adaptive immune response, and any alteration to the expression of these molecules may result in perturbation of immune function. It is known that infection with certain herpesviruses induces the expression of an FcγR (Litwin et al. 1990; Bruggeman et al. 1984; Furukawa et al. 1975; McTaggart et al. 1978). The α-herpesviruses, HSV-1, -2, and VZV, all encode homologous proteins (Litwin et al. 1992; Para et al. 1982; Para et al. 1980; Dubin et al. 1990). In contrast, the CMV genome does not contain genes with sequence homology to the FcγRs of the α-herpesviruses, nor to the FcγR gene identified in murine CMV (Thale et al. 1994), although CMV also induces a FcγR in the cytoplasm of fibroblasts (Westmoreland et al. 1976; Keller et al. 1976). However, the ability of the same virus to induce a functional fibroblast FcγR at the cell surface has been poorly documented, as has the ability of CMV to induce an FcγR in other cell types. Furthermore, the origin of the CMV-induced FcγR remains equivocal, as it is unclear whether a viral protein with FcγR properties is responsible for this effect, or whether a cellular FcγR is induced during viral replication

In view of these facts, the effect of CMV infection of both fibroblasts or endothelial cells on the cell surface expression of an FcγR was investigated using flow cytometry. This technique was employed here because, as indicated in Chapter 4, positive or negative antigen expressing subpopulations of cells can be distinguished in a mixed culture containing both uninfected and infected cells. In addition, confocal microscopy was used to monitor the expression of the CMV-induced FcγR in the cytoplasm of the inoculated cells. Finally, a panel of monoclonal antibodies specific for the three known cellular FcγR's were reacted with virally infected fibroblasts or endothelial cells, in order to establish whether the expression of an FcγR in these cells was due to the induction of pre-existing cellular receptors.

5.2. Determination of the optimum conditions necessary to detect the CMV-induced Fc γ R. 1. Observations in the cytoplasm of CMV-infected fibroblasts, with respect to time.

Initial experiments were performed in order to determine the optimum conditions required to detect the CMV-induced Fc γ R, with respect to time. Fibroblasts were used during these studies, as it has previously been reported that an Fc γ R is expressed in the cytoplasm of this cell type, following infection with the laboratory CMV strain, AD169 (Furukawa et al. 1975). Furthermore, an 100% infection rate can easily be achieved in this cell type. Thus, fibroblasts were inoculated with CMV strain AD169 at a MOI of 2, and the expression of Fc γ R activity was analysed by studying the binding of purified human Fc fragments to permeabilised cell monolayers, at various times post inoculation. In addition, the expression of CMV IEA and late (gB) antigen was monitored in parallel.

The CMV-induced Fc γ R activity was first detected as a diffuse pattern in the cytoplasmic region of the inoculated fibroblasts at 2 days post inoculation, and gradually condensed into a punctate, perinuclear pattern at the later time points (data not shown). The timing of the expression of the CMV-induced Fc γ R was similar to that seen for the expression of CMV late (gB) antigen (Table 5.2.1), whereas CMV IEA was present at all time points tested (Table 5.2.1). From these experiments, it was deduced that the maximum expression of the Fc γ R in the cytoplasm of CMV-inoculated fibroblasts occurred at days 4-5 post inoculation.

5.3. Determination of the optimum conditions necessary to detect the CMV-induced Fc γ R. 2. Conditions required to detect the CMV-induced Fc γ R at the surface of fibroblasts.

The next experiments were performed in order to determine the optimal conditions for detecting an Fc γ R at the cell surface following infection with CMV, using flow cytometric techniques. This required that the fibroblasts were first removed from the culture flask, and then placed in suspension. However, it has been suggested that one α -herpesvirus induced Fc γ R is sensitive to trypsin (Litwin et al. 1990; McTaggart et al. 1978). Thus, fibroblasts were inoculated with CMV strain AD169 at a MOI of 2, and the

cells were removed using dissociation fluid at day 4 post inoculation, the time when maximum FcγR expression occurred in the cytoplasm of the CMV-inoculated fibroblasts (see above). Following dissociation, various concentrations of trypsin were incubated with the CMV-inoculated cells for various periods of time. The ability of the fibroblasts to subsequently bind purified Fc fragments of human IgG at the surface of live, unfixed cells, was then analysed, using flow cytometric techniques.

The results demonstrated that inoculation of fibroblasts with CMV strain AD169 resulted in the induction of an FcγR at the cell surface (Figure 5.3.1). However, the addition of cell culture grade trypsin-EDTA (500μg/ml trypsin) to the CMV inoculated fibroblast suspensions for 5 minutes greatly reduced the ability of human Fc fragment to bind to the cells (Figure 5.3.1). Partial reduction of binding was observed when these cells had been exposed to trypsin-EDTA for as little as 30 seconds (Figure 5.3.1). In separate experiments, treatment of AD169-inoculated fibroblasts with trypsin (lacking EDTA and dissolved in phosphate buffered saline) for 2.5 minutes at concentrations as low as 50μg/ml had a similar effect (Figure 5.3.1), suggesting that EDTA was not responsible for this reduction in FcγR activity. It was therefore concluded that the reactivity of the CMV-induced FcγR with human Fc fragments at the surface of fibroblasts is specifically abrogated by trypsin proteolysis, and thus was not used to analyse fibroblast surface FcγR expression.

5.4. Determination of the optimum conditions necessary to detect the CMV-induced FcγR. 3. Observations at the surface of CMV-infected fibroblasts, with respect to time.

In order to confirm that the kinetics of the FcγR expression at the cell surface was similar to that observed in the cytoplasm of the inoculated-fibroblasts, the ability of CMV to induce an FcγR at the surface of fibroblasts, with respect to time, was next investigated. Fibroblasts were inoculated with CMV strain AD169 at a MOI of 2, and the expression of an FcγR was determined at various time points post inoculation, as described above.

The results indicated that uninfected fibroblasts did not bind these Fc fragments, at any time point tested (Figure 5.4.1). In contrast, an increased binding of these

molecules to the surface of AD169-inoculated fibroblasts occurred at the cell surface with respect to time (Figure 5.4.1). When the logarithmically acquired data from three separate experiments performed in triplicate were converted to a linear form for statistical analysis, significant binding ($p=0.01$, $n=9$) of the Fc fragments to the CMV-induced Fc γ R occurred at days 2-6 post inoculation. However, the maximum binding to the inoculated-fibroblasts occurred at day 4 (Figure 5.4.1). Thus, the cell surface expression of the CMV-induced Fc γ R was subsequently examined at day 4 post inoculation, unless otherwise stated.

5.5. The expression of an Fc γ R on the surface of endothelial cells.

The ability of CMV strain AD169 (MOI of 4) to induce an Fc γ R on the surface of live, unfixed endothelial cells was now investigated, using the techniques described above. However, as this cell type is not as permissive for the replication of AD169 as are fibroblasts, infectivity was increased by pre-treatment of the endothelial cells with 10mM sodium butyrate (described in Chapter 2), and the cells left for a further 4 days. The results from these experiments indicated that Fc γ R activity could be detected only on the surface of the sodium butyrate treated, infected endothelial cells, but not on the surface of the sodium butyrate treated, uninfected cells, nor at the surface of those endothelial cells inoculated with CMV in the absence of sodium butyrate (Figure 5.5.1). Cytocentrifuge preparations of these endothelial cells subsequently revealed that CMV IEA could be detected in the nucleus of 40% of the AD169-inoculated endothelial cells which had been treated with sodium butyrate, compared to less than 2% expression in infected endothelial cells which had not been exposed to this agent (data not shown). When the percentage of CMV inoculated, sodium butyrate treated endothelial cells which bound human Fc fragment (32%) was compared to the percentage of cells which were expressing the CMV IEA, it was found that the two percentages corresponded reasonably well.

To confirm that this observed Fc γ R expression was not in some way due to the sodium butyrate treatment of the cells, the endothelial cell adapted clinical isolate of CMV, C1FE (MOI of 4), was used as the inoculum for the endothelial cell monolayers. In this case, it was demonstrated that infection with C1FE also resulted in the binding of human Fc fragments to 49% of the inoculated cells (Figure 5.5.1). Moreover, addition of

trypsin to the endothelial cell suspension reduced this percentage to 13% (Figure 5.5.1). Cytocentrifuge preparations of these endothelial cells subsequently revealed the proportion of the C1FE-inoculated cells which stained positive for CMV IEA was 60% (data not shown). Thus, as has been shown with fibroblasts, CMV infection of endothelial cells results in the cell surface expression of a receptor for the Fc portion of human IgG, following infection with either a laboratory or clinical strain of CMV. Moreover, the endothelial cell surface Fc γ R which was induced following infection with CMV was also sensitive to trypsin proteolysis.

5.6. The effect of virus dose on the surface expression of the CMV induced Fc γ R.

The results presented above indicated that not all CMV-inoculated endothelial cells bound human Fc fragments. This suggested that viral dose could have affected the level of the cell surface Fc γ R expression. To confirm this, fibroblasts were infected with CMV (strain AD169) at multiplicity of infections of 0.25, 0.5 or 1, resulting in CMV IEA expression in approximately 30%, 65% and 95% of the cells at day 1, respectively (data not shown). At all viral doses, the percentage of cells expressing surface Fc γ R activity (Figure 5.6.1) was similar to the percentage of cells which were initially expressing CMV IEA. Little reduction in the median fluorescent intensity of staining was observed at the lower viral inoculum suggesting that if Fc γ R activity was present, it was always expressed at a similar level (Figure 5.6.1). The expression of a control cell surface marker, Class I HLA antigen, which is down-regulated on CMV infected fibroblasts (Chapter 4), showed the expected correlation in the opposite direction between virus dose and the percentage of cells positive for this cellular antigen (Figure 5.6.1). When a recent clinical isolate of CMV (C1F) was used to inoculate fibroblasts in the manner described above, the expression of either the Fc γ R or Class I was similar to that described for AD169 (data not shown). Thus it was concluded that the percentage of cells expressing Fc γ R activity (and low Class I expression) was dependent upon the percentage of cells which were initially infected with CMV, but that the level of Fc γ R expression remained constant, irrespective of virus dose.

5.7. The effect of ganciclovir on the expression of the CMV-induced FcγR at the surface of fibroblasts.

In order to determine whether the expression of the CMV-induced FcγR was affected by the addition of the anti-viral drug ganciclovir, fibroblasts were inoculated with CMV strain Toledo at a MOI of 2, and either treated with 50μg/ml of ganciclovir, or left untreated. The ability of the cells to bind human Fc fragment was then determined, as described above. The results indicated that, as for the other strains of CMV, infection of fibroblasts with the Toledo strain resulted in the induction of a cell surface FcγR. However, a reduced ability to bind human Fc fragments via the cell surface FcγR was evident after the inoculated cells had been treated with ganciclovir throughout the incubation period. Thus, the proportion of inoculated cells which bound Fc fragments was reduced from 65% to 25% in the presence of ganciclovir (data taken from two separate experiments performed in triplicate, $p=0.05$, $n=6$). The fact that the induction of the CMV-induced FcγR was affected by a drug which inhibited late viral gene expression indicated that a late viral protein was responsible for this effect.

5.8. The expression of the CMV induced FcγR in the cytoplasm of infected fibroblast and endothelial cells.

The results presented above indicated that a surface FcγR was induced following inoculation with CMV. However, it was unclear whether the FcγR was induced at the surface of the infected or the uninfected 'bystander' cells. In order to address this question, it was necessary to study the cytoplasmic form of the FcγR, as techniques were only available to localise internal CMV antigen expression at this time. Therefore, fibroblasts were inoculated with CMV strain AD169 at a MOI of 0.25, in order to produce a mixed population of uninfected and infected cells. In addition, endothelial cells were pre-treated with sodium butyrate for one day, and then inoculated with CMV strain AD169 at a MOI of 4. A double-labelling immunofluorescence technique was then used to analyse the simultaneous expression of CMV IEA and the FcγR in CMV-inoculated fibroblasts or endothelial cells, four days later. CMV IEA expression was used in this case because the distinct nuclear localisation of the antigen would be unlikely to mask the perinuclear FcγR expression observed previously (Section 5.2).

The results, shown in Figure 5.8.1, demonstrated that Fc binding to both cell types was observed only in cells which also expressed the CMV IEA, but not in the surrounding uninfected cells. The figure also shows the localisation of the Fc γ R activity to a distinct perinuclear region of both the infected fibroblast, and the infected endothelial cell. Thus, the CMV-induced Fc γ R expression was located exclusively to the infected cell, suggesting a direct virus effect, rather than a result of a soluble factor acting on the uninfected 'bystander' cell population.

5.9. Reactivity of monoclonal antibodies specific for cellular Fc γ Rs with the cytoplasm of CMV infected fibroblasts.

To determine whether the Fc γ R activity in the CMV infected cells was a result of the induction of a cellular protein, a panel of murine monoclonal antibodies specific for the three distinct subclasses of known human cellular Fc γ R (Fc γ RI, Fc γ RII and Fc γ RIII) were tested for their ability to react with permeabilised CMV (strain AD169) infected cells at 4 days post inoculation. The more permissive fibroblasts were used since a 100% infection rate could be more easily achieved in this cell type than in endothelial cells, and the distinct localisation of the Fc γ R in the perinuclear region of the cytoplasm favoured the identification of a positive reaction. At least two different antibodies were used for each of the Fc γ Rs and, where possible, these antibodies were of different murine IgG subclasses (Table 5.9.1). As antibodies 10.1, IV.3 and 3G8 were specific for the ligand binding region of Fc γ RI, Fc γ RII and Fc γ RIII respectively, epitope recognition would be adversely affected by any blocking conditions which involved pre-incubation with IgG from various species, and thus such steps were avoided. The correct reactivity pattern of each antibody with monocytes and B-lymphocytes (Fc γ RI & Fc γ RII), or neutrophils (Fc γ RIII) was verified by flow cytometry, and the optimal binding concentrations determined (data not shown).

Using these optimal antibody concentrations (10-15 μ g/ml), no reactivity with the CMV infected fibroblasts was observed with any of the antibodies in the panel (data not shown). At higher antibody concentrations (>20 μ g/ml), clear positive staining of the infected fibroblasts was seen with some, but not all, antibodies against each of the three cellular Fc γ Rs. However, the staining appeared to be isotype specific, with reactivity

being confined to antibodies of the murine IgG1 subclass, namely 32.2, KUFC79, 3G8 and 214.1 (Table 5.9.1). Only one IgG1 antibody, 10.1, failed to show strong reactivity with the infected cells, but the concentration of this antibody in the hybridoma supernatant supplied by the manufacturer was not known. The one IgG2b antibody used in this study (IV.3), and the remaining three IgG2a antibodies (2E1, GRM1 and B-E16), showed no reactivity with the CMV infected fibroblasts (Table 5.9.1). Thus for each of the cellular FcγRs, antibodies of the IgG1 subclass showed positive staining on the CMV infected cells, whilst antibodies specific for the same molecule, but of the IgG2a or IgG2b subclass, were negative. No binding to uninfected fibroblasts was observed with any of the panel of monoclonal antibodies, even at concentrations as high as 40μg/ml (data not shown).

The nature of the antibody binding to the CMV infected fibroblasts was addressed by blocking the FcγR with the FcγR ligand, namely the Fc portion of human IgG. In all cases, the strong positive staining pattern observed in the absence of pre-treatment with Fc fragment was completely abolished by its presence (Table 5.9.1), suggesting that competition for binding sites between the antibodies and the Fc ligand was occurring. However, this effect was true, not only as expected for those antibodies specific for the ligand binding regions of the FcγR molecules (KUFC79, 3G8), but also for those FcγR antibodies which recognised sites distinct from the ligand binding regions, (32.2, 214.1, Table 5.9.1). The latter should not have been affected if the FcγR antibody was binding to the CMV infected cells via its antigen combining site on the F(ab) portion of the molecule. The reactivity of antibodies specific for either the CMV IEA, or CMV glycoprotein B, was not blocked under these conditions (data not shown). This data thus suggested that the cellular FcγR antibodies of the murine IgG1 subclass may be specifically reacting with the CMV induced FcγR via their Fc portion, rather than recognising cellular FcγR antigen through the F(ab) portion of the IgG molecule.

5.10. Binding of IgG and Fc fragments of different species to CMV infected fibroblasts and endothelial cells.

The results presented above suggested that the Fc portion of some murine antibodies reacted with CMV-infected cells. To investigate the ability of IgG from different species to react with CMV infected fibroblasts, deaggregated whole IgG

molecules of a single murine subclass, or deaggregated Fc fragments purified from human, rabbit or murine sera were incubated with permeabilised CMV infected fibroblasts, and tested for reactivity by a semi-quantitative immunofluorescence technique. Two-fold serial dilutions (0.038 μ g-40 μ g/ml) of each IgG molecule or Fc fragment were reacted with the infected cells, and the end point of positive fluorescence was determined microscopically. HSV-1 infected fibroblasts were studied in parallel, as the reactivity pattern of the HSV-1 induced Fc γ R is known. An example of Fc γ R activity observed following inoculation with either CMV or HSV-1 is shown in Figure 5.10.1.

Both CMV and HSV-1 infected fibroblasts demonstrated distinct binding of Fc fragments of human or rabbit origin at concentrations from 1.25 μ g-40 μ g/ml, but only CMV infected fibroblasts reacted with the Fc fragments at concentrations below this level (Table 5.10.1). CMV infected cells consistently bound human Fc fragment at lower concentrations than rabbit Fc fragment, the end points being 0.15 μ g/ml and 0.31 μ g/ml respectively. No detectable signal was observed when murine Fc fragments were used as the probe for HSV-1 infected fibroblasts. In contrast, a clearly detectable signal was observed when murine Fc fragments were used to probe the CMV infected cells. This fluorescence was detectable when murine Fc fragments were used at a concentration of 20-40 μ g/ml (Table 5.10.1). Thus the pattern of reactivity of the CMV induced and the HSV-1 induced Fc γ Rs differed.

Further analysis was performed to determine which subclasses of murine IgG were responsible for the binding of murine Fc fragments to CMV infected fibroblasts. The results (Table 5.10.1) indicated that the binding of murine IgG1 paralleled the observed reaction pattern of murine Fc fragments, producing a detectable level of fluorescence in the concentration range of 20 μ g-40 μ g/ml. However, murine IgG3 reacted with the CMV induced Fc receptor at lower concentrations (1.25 μ g-40 μ g/ml) than that observed for either the murine Fc fragment, or murine IgG1. No staining was observed for the IgG2a nor the IgG2b subclass, even at concentrations as high as 40 μ g/ml (Table 5.10.1). Pre-incubation of CMV infected fibroblasts with human Fc fragments abolished the positive cytoplasmic staining pattern observed for both murine IgG1 and IgG3 molecules, as well as for the murine Fc fragment (data not shown).

Endothelial cells also showed binding of murine IgG1 and IgG3 molecules in the cytoplasm of the cells infected with CMV C1FE (Table 5.10.1). As shown for infected fibroblasts, this binding was abolished by pre-incubating the cells with human Fc fragment. However, as the percentage of infected cells was lower (approximately 50%) in this cell type than in the fibroblast cell type, no direct comparison of the strength of binding could be made. Nevertheless, it was concluded that murine IgG1, IgG3 and murine Fc fragment competed with human Fc fragments for the same binding sites in both fibroblasts and endothelial cells following infection with CMV.

5.11. Reactivity of the cell surface form of the CMV induced Fc γ R with antibodies specific for cellular Fc γ Rs.

The panel of monoclonal antibodies specific for cellular Fc γ Rs used previously (Table 5.9.1) were then tested for reactivity with the cell surface form of the CMV-induced Fc γ R. Fibroblasts or endothelial cells were inoculated at an MOI of 2 with AD169, or an MOI of 4 for C1FE, and the cells treated as previously described. Neither CMV infected, nor uninfected, endothelial cells or fibroblasts reacted with any of the cellular Fc γ R antibodies in the panel, even at antibody concentrations which had produced a positive reactivity pattern in the cytoplasm (data not shown). When the logarithmically acquired data from three separate experiments performed in triplicate were converted to a linear form for statistical analysis, no significant reaction with either the fibroblasts or the endothelial cells was demonstrated for any antibody in the panel. However, the binding of purified Fc fragment to control infected cells demonstrated that, as before, the CMV induced Fc γ R was expressed at the cell surface in these experiments. This suggested that the binding of human Fc fragment to the surface of CMV infected cells was not due to the expression of known cellular Fc γ Rs.

5.12. Reactivity of the cell surface form of the CMV induced Fc γ R with IgG and Fc fragments from different species.

As antibodies to the human cellular Fc γ Rs which were of the murine IgG1 subclass reacted with the cytoplasmic form, but not with the cell surface form of the CMV induced Fc γ R, the ability of the surface form of this receptor to bind murine or human Fc fragments, as well as the different subclasses of murine IgG, was investigated.

The results indicated that neither murine Fc fragment, nor murine IgG myeloma proteins of any isotype demonstrated binding to the surface of CMV infected fibroblasts (Figure 5.12.1), even when used at concentrations (40µg/ml) which had given positive staining in the cytoplasm of permeabilised infected cells. In contrast, a typical dose response curve was seen, with maximal binding occurring at 10µg/ml, when both rabbit and human Fc fragments were serially diluted and incubated with the surface of infected fibroblasts (Figure 5.12.1). The murine IgG myeloma proteins also failed to react with the surface of endothelial cells infected with the clinical isolate, C1FE (Figure 5.12.1). As found previously, human Fc fragments did bind to the surface of 55% of the infected endothelial cells, confirming that the CMV-induced FcγR was present in these experiments (data not shown). Thus, although similar reactivity was observed in the binding of human or rabbit Fc fragments to the FcγR expressed on the surface compared to the cytoplasm of CMV infected fibroblasts, murine IgG Fc fragment, or murine IgG3 and IgG1 molecules, reacted only with the cytoplasmic, and not with the cell surface form, of the CMV induced FcγR in both fibroblasts and endothelial cells.

Table 5.2.1. The expression of the CMV-induced FcγR in the cytoplasm of fibroblasts with respect to time¹.

CMV INOCULATED FIBROBLASTS EXPRESSING:			
DAY P.I.	CMV IEA	CMV Late (gB) antigen	FcγR
1	++	-	-
2	++	+/-	+/-
3	++	+	+
4	+	++	++
5	+	++	++
6	+	++	++

¹Fibroblasts were inoculated with CMV strain AD169 at a MOI of 2, and the expression of CMV IEA, late (gB) antigen, or CMV-induced FcγR activity in the cytoplasm of permeabilised, cytocentrifuged cell preparations was determined at 4 days post inoculation. Reactivity was scored as: ++ = strong positive; + = positive; +/- = weak positive; - = negative.

Table 5.9.1. The reactivity of a panel of murine monoclonal antibodies specific for human cellular FcγRI, II, and III with the CMV-induced FcγR in the cytoplasm of fibroblasts¹.

Human cellular FcγR recognised	Murine antibody clone	Epitope region recognised	Murine IgG subclass	Original antibody concentration	Antibody source:	Reactivity with the cytoplasm of CMV infected fibroblasts after pre-incubation with:		Optimal antibody concentration required to react with:	
						PBS ¹	human Fc fragment ¹	Cytoplasm of CMV infected fibroblasts ¹	Cellular FcγR on surface of leukocytes ²
FcγRI	32.2	non-ligand	IgG1	1mg/ml	not known	YES	NO	40μg/ml	10-15μg/ml ³
	10.1	ligand	IgG1	not known	culture sn	Weak	not applicable	NEAT	1/2
FcγRII	IV.3	ligand	IgG2b	1mg/ml	not known	NO	not applicable	not applicable	5-10μg/ml ³
	2E1	ligand	Ig2a	1mg/ml	ascites fluid	NO	not applicable	not applicable	10μg/ml
FcγRIII	KUFC79	ligand	IgG1	500μg/ml	not known	YES	NO	40μg/ml	10μg/ml
	3G8	ligand	IgG1	200μg/ml	ascites fluid	YES	NO	40μg/ml	5-10μg/ml ³
	GRM1	distinct from 3G8	IgG2a	50μg/ml	culture sn	NO	NO	not applicable	10-15μg/ml ³
	214.1	non-ligand	IgG1	100μg/ml	ascites fluid	YES	NO	40μg/ml	10-15μg/ml ³
	B-E16	not known	IgG2a	not known	ascites fluid	NO	not applicable	not applicable	1/10

¹Positive binding of the antibody to acetone-fixed fibroblasts was determined microscopically.² Optimal antibody concentrations were determined by flow cytometry on unfixed leukocytes.³ Different batches of antibody gave different optimal working concentration

Table 5.10.1. Reactivity pattern of IgG and Fc fragments from various species with the FcγR induced in the cytoplasm of HCMV or HSV-1 infected fibroblasts or endothelial cells¹.

μg/ml	HSV-1 infected fibroblasts incubated with:			CMV infected fibroblasts (or endothelial cells)* ² incubated with:						
	IgG Fc fragments from:			IgG Fc fragments from:			murine IgG subclass:			
	human	rabbit	mouse	human	rabbit	mouse	IgG1	IgG2a	IgG2b	IgG3
40	+	+	-	+	+	+	+	-	-	+
20	+	+	-	+	+	+	+	-	-	+
10	+	+	-	+	+	-	-	-	-	+
5	+	+	-	+	+	-	-	-	-	+
2.5	+	+	-	+	+	-	-	-	-	+
1.25	+	+	-	+	+	-	-	-	-	+
0.63	+	+	-	+	+	-	-	-	-	+
0.31	+	+	-	+	+	-	-	-	-	+
0.15	+	+	-	+	+	-	-	-	-	+
0.075	+	+	-	+	+	-	-	-	-	+
0.038	+	+	-	+	+	-	-	-	-	+

¹Positive binding to acetone-fixed cells was determined microscopically and the reactivity scored as: + = positive binding; +/- = weak binding; - = no detectable binding. Fibroblasts were infected with HSV-1 or CMV (strain AD169), and endothelial cells with CMV strain C1FE. ² The reactivity of human or murine Fc fragments, or the various subclasses of murine IgG, with CMV-inoculated endothelial cells was determined for certain concentrations, and the results are shown in parenthesis.

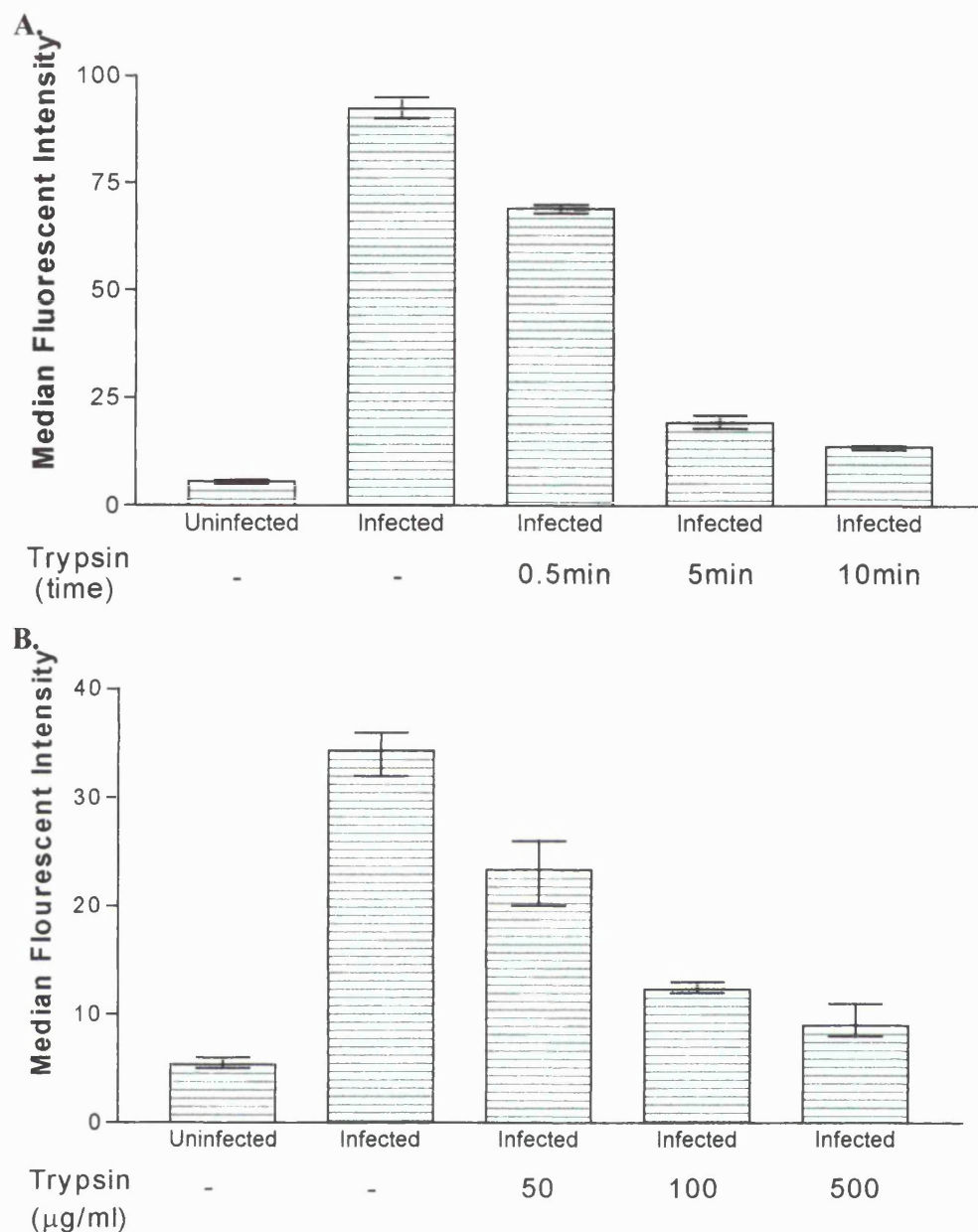


Figure 5.3.1. Trypsin sensitivity of the Fc γ R induced at the surface of fibroblasts following infection with CMV.

Fibroblasts were inoculated at a MOI of 2 with CMV strain AD169, and the cells removed using cell dissociation fluid, 4 days post inoculation. The cells were then incubated with trypsin-EDTA (500µg/ml) for various periods of time (Part A), or with various concentrations of trypsin for 2.5 minutes (Part B). The ability of Fc fragments of human IgG to bind to the fibroblast cell surface was determined by flow cytometry. The results are expressed as the median fluorescence intensity of a representative experiment, performed in triplicate. The error bars show the range of values obtained.

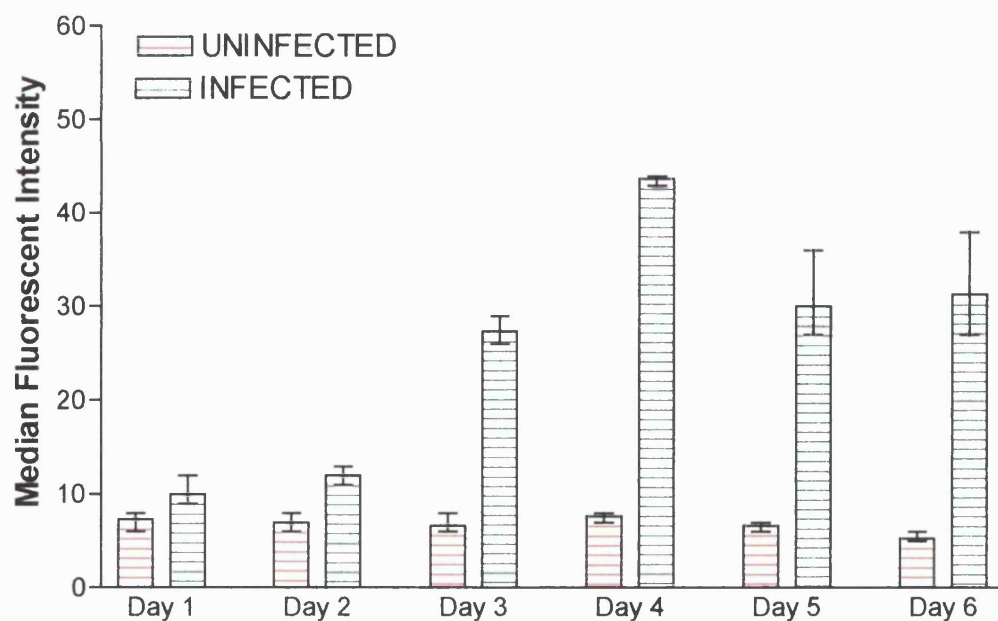


Figure 5.4.1. The induction of an Fc γ R at the surface of fibroblasts following infection with CMV, with respect to time.

Fibroblasts were inoculated at a MOI of 2 with CMV strain AD169, and at various times post inoculation, the cells were removed using cell dissociation fluid. The ability of Fc fragments of human IgG to bind to either the uninfected or AD169-inoculated fibroblast cell surface was determined separately at each time point, by flow cytometry. The data are represented as the median fluorescent intensity of a representative experiment, performed in triplicate. The error bars show the range of values obtained.

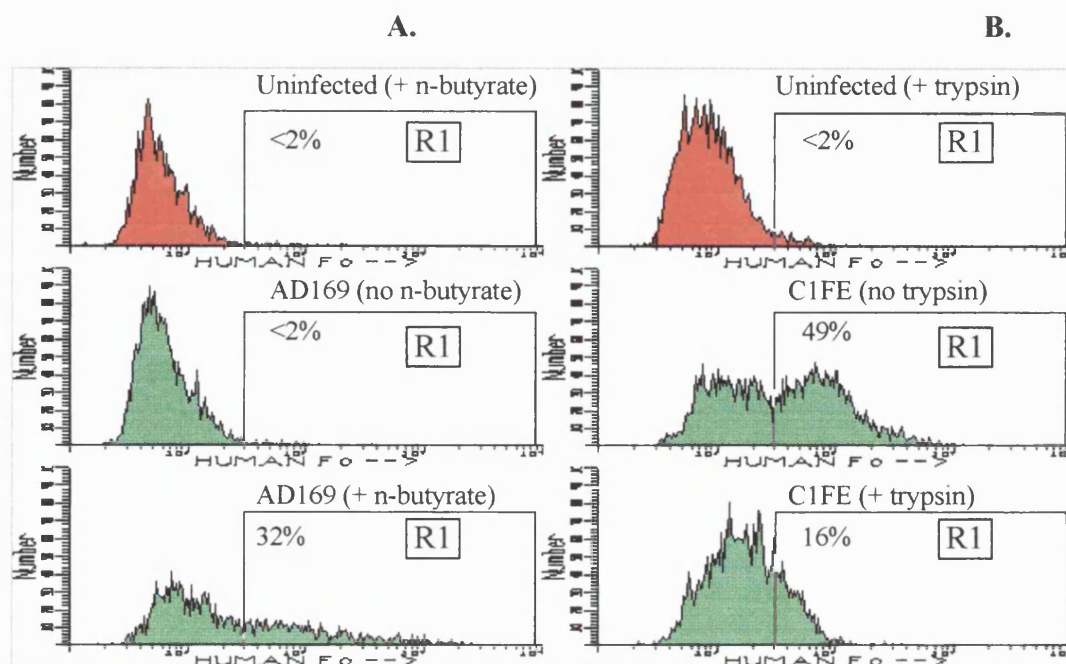


Figure 5.5.1. The effect of CMV infection on the expression of an Fc γ R at the surface of endothelial cells.

Endothelial cells were inoculated with CMV at a MOI of 4, and purified human Fc fragments were incubated with the live, unfixed endothelial cells 4 days later. In Part A, endothelial cells were either treated with sodium butyrate (+ n-butyrate), or left untreated (no n-butyrate), and CMV strain AD169 was inoculated onto the cells the following day. In Part B, the endothelial adapted clinical strain, C1FE, was used as the viral inoculum, and the cells were either treated with trypsin for 5 minutes (+ trypsin), or left untreated (no trypsin), prior to the addition of the Fc fragments. Representative histogram profiles of uninfected (red) or infected (green) cells are shown. In addition, the percentage of cells which bound human Fc fragments (R1) was determined by using the cells which had only been incubated with the secondary antibody as a negative control. The ordinate represents the number of fluorescence events (scale 0-100), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4).

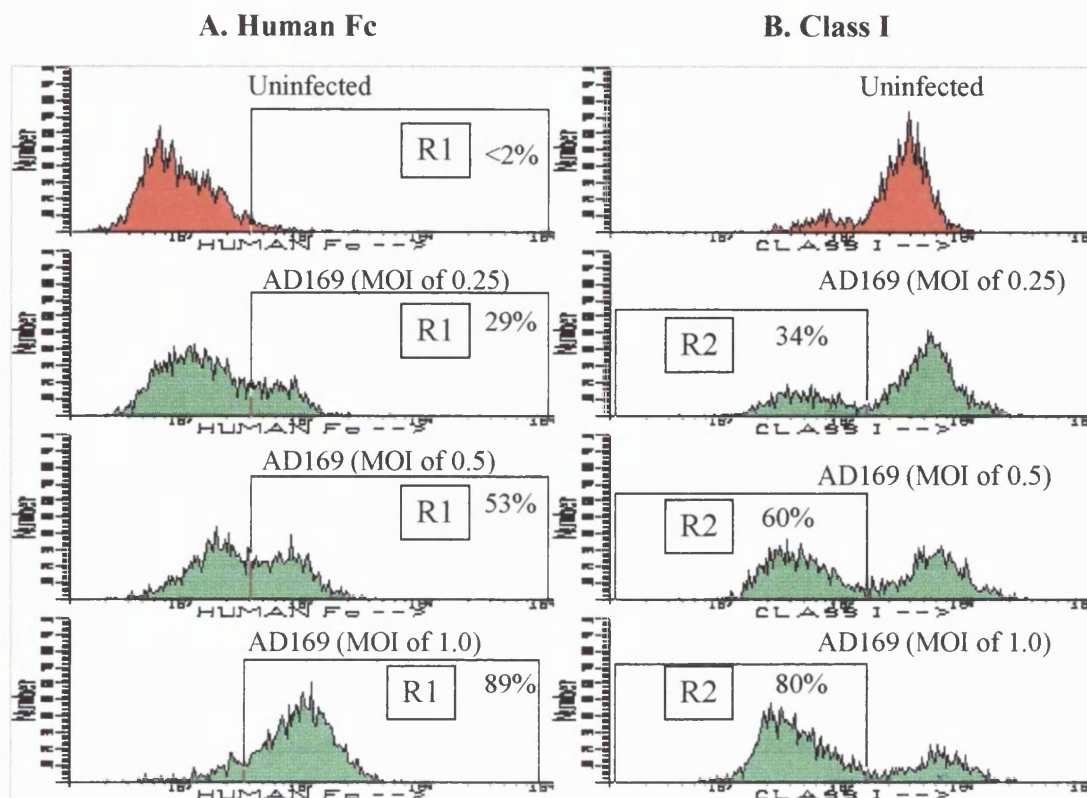


Figure 5.6.1. The effect of virus dose on the ability of CMV to induce an Fc γ R at the surface of fibroblasts.

Fibroblasts were inoculated with serial dilutions of CMV strain AD169 to achieve MOI's of 0.25, 0.5, and 1, and at 4 days post inoculation, the unfixed cells were either incubated with human Fc fragments (Part A), or a Class I specific antibody (Part B). Representative histogram profiles from a single experiment are shown for uninfected or AD169-inoculated cells. In part A, the percentage of uninfected cells (red) or infected cells (green) which bound human Fc fragments (R1) was determined by using the cells which had only been incubated with the secondary antibody as a negative control. In part B, the percentage of inoculated cells which displayed 'low' Class I expression (R2) is shown. The ordinate represents the number of fluorescence events (scale 0-100), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4).

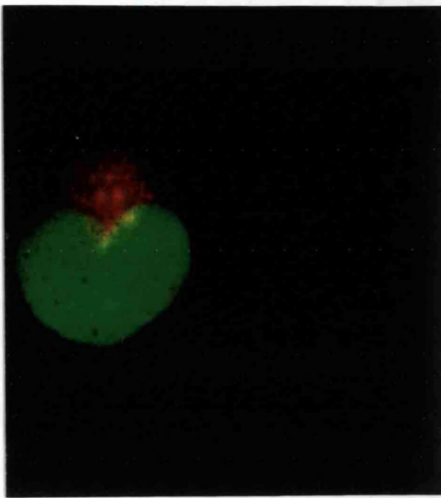
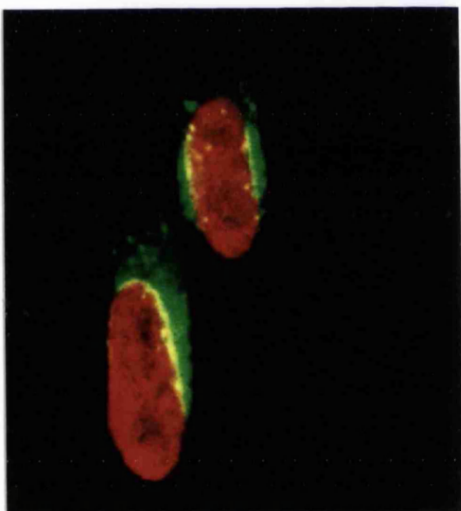
A.**CMV-inoculated endothelial cells****B.****C.****CMV-inoculated fibroblasts****D.**

Figure 5.8.1. The expression of the CMV-induced Fc γ R in the cytoplasm of endothelial cells or fibroblasts.

Endothelial cells were pre-treated with sodium butyrate and inoculated at a MOI of 4 with CMV strain AD169 (parts A & B). Fibroblasts were inoculated with CMV strain AD169 at an MOI of 0.25 (parts C & D). The CMV inoculated cells were double-labelled with a combination of CMV IEA antibody (red) and human Fc fragment (green) at 4 days post inoculation (parts A & C). The phase contrast images for both cell types are shown in parts B & D. Scale bar = 25 microns.

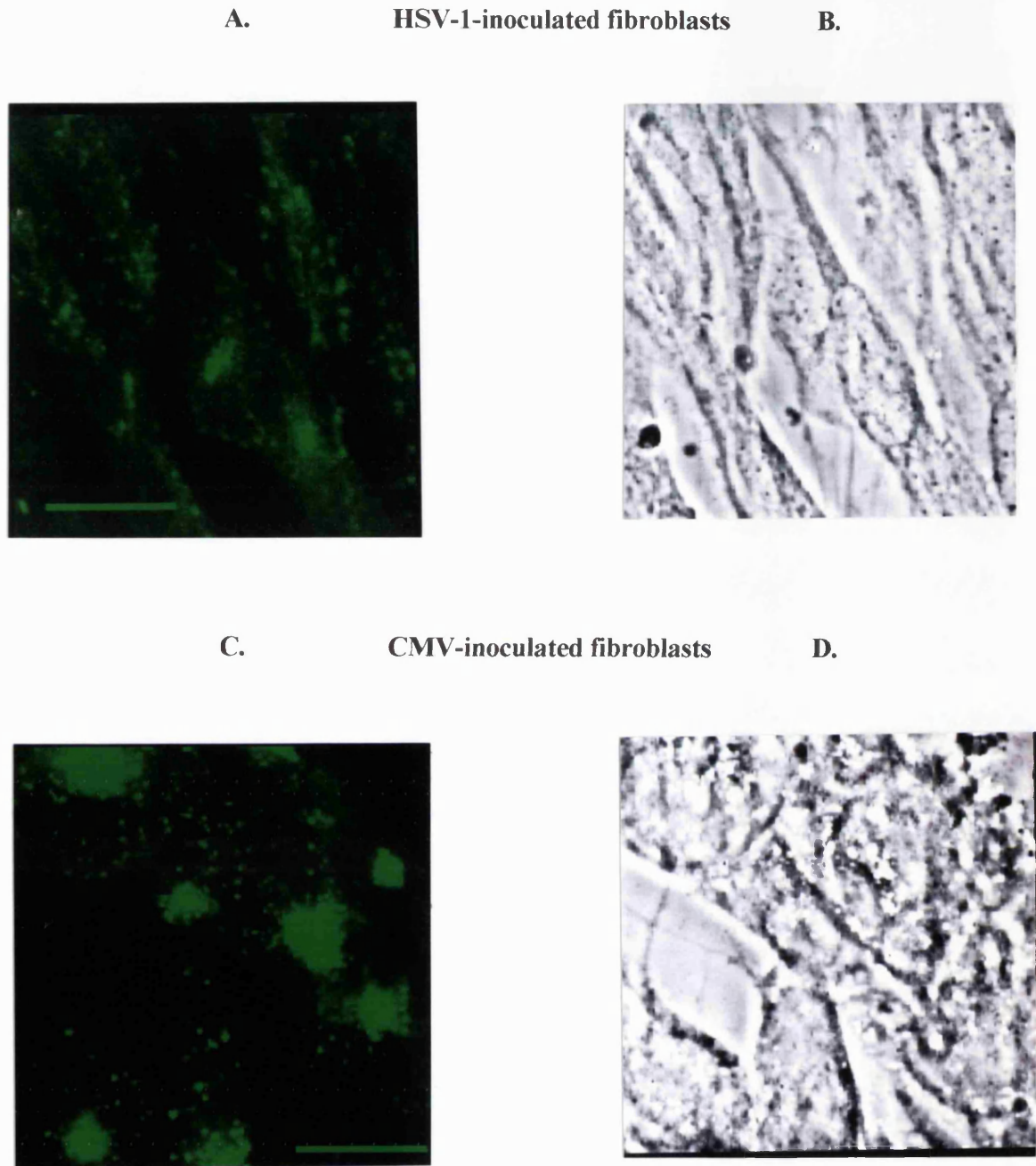
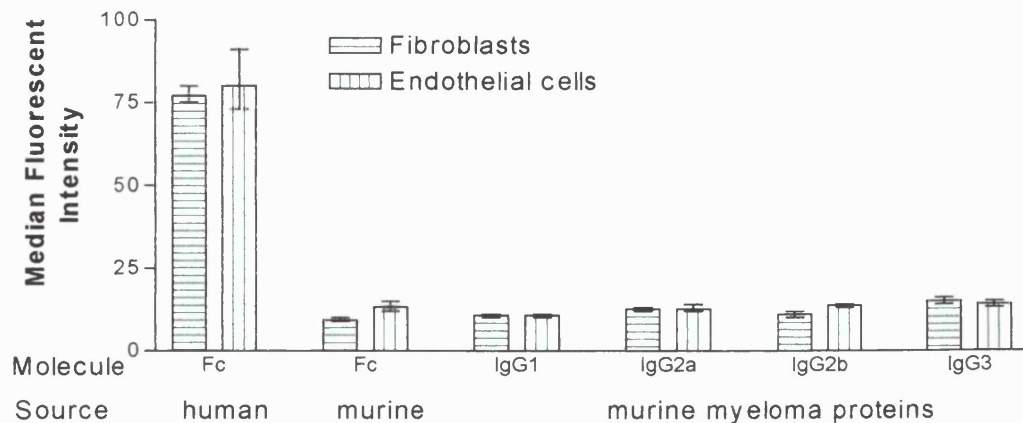


Figure 5.10.1. The expression of the HSV-1- or CMV-induced FcγR in the cytoplasm of fibroblasts.

Fibroblasts were inoculated (MOI of 2) with HSV-1 (parts A & B) or CMV strain AD169 (parts C & D), and the acetone fixed cells were incubated with human Fc fragments (parts A & C) at 18 hours or 4 days post inoculation, respectively. The phase contrast image of the cells are shown in parts B & D. Scale bar = 25 microns.

A.



B.

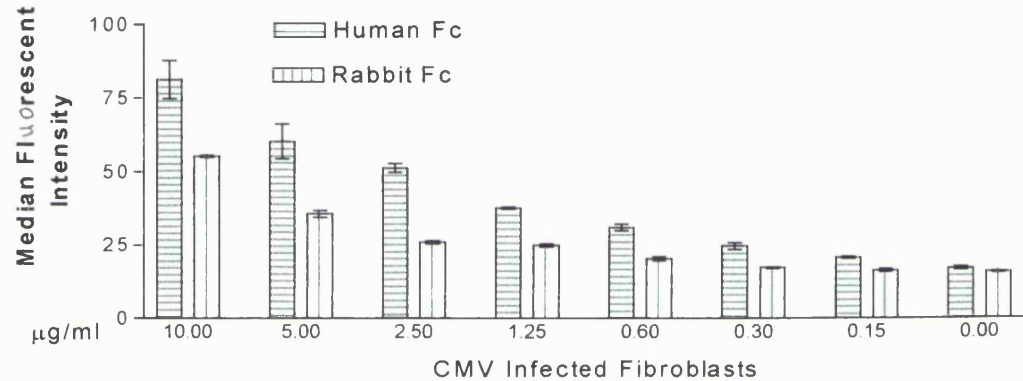


Figure 5.12.1. The reactivity pattern of Fc fragments and IgG molecules from different species with the surface of CMV infected fibroblasts and endothelial cells.

CMV strain AD169 was inoculated at a MOI of 2 to achieve a 100% infection rate in fibroblasts, and CMV strain C1FE was inoculated at an MOI of 4 to achieve an infection rate of 60% in endothelial cells. The reactivity of human, murine or rabbit Fc fragments, and murine IgG molecules with the infected cells was determined using flow cytometric techniques. A marker was set between the positive and negative populations, using the cells which had only been incubated with the secondary antibody as a negative control, and the median fluorescence intensity was determined for each population. In part A, the results illustrate either the positive binding of human Fc fragment, or the total binding of murine Fc fragment or IgG to fibroblasts and endothelial cells. In part B, the reactivity of decreasing concentrations of human or rabbit Fc fragments with infected fibroblasts was determined. A representative experiment performed in triplicate is shown for each. The error bars show the range of values obtained.

5.13. Discussion.

Although the ability of human cytomegalovirus (CMV) to induce a receptor for the Fc portion of IgG (FcγR) has been widely documented in fibroblasts (Keller et al. 1976; Furukawa et al. 1975), only one report concerning the induction of an FcγR in endothelial cells was available to date (Ryan et al. 1981). Moreover, in this latter study, endothelial cells of bovine origin were inoculated with laboratory strains of CMV (Ryan et al. 1981). In the studies presented here, it was shown that infection of human endothelial cells with both clinical isolates or the laboratory strain of CMV, AD169, resulted in the induction of a FcγR in both the cytoplasm, and at the surface of the inoculated cells. The use of flow cytometry to detect the surface form of the FcγR allowed negative and positive Fc binding populations to be distinguished on the basis of the histogram profiles. This was important because the percentage of endothelial cells actively infected with either C1FE or AD169 never reached 100%. This was particularly noticeable when CMV strain AD169 was used to inoculate the endothelial cells, because <5% of the endothelial cells exposed to the virus manifested CMV specific antigen. Although this block could be partially overcome by treatment of the cells with sodium butyrate (see Chapter 3), a large proportion of the cells remained uninfected, and so it was necessary to examine whether Fc-binding subpopulations of cells existed within the CMV-inoculated endothelial cell cultures.

Using the technique described above, it was found that endothelial cells inoculated with CMV strain AD169 only expressed FcγR activity when viral replication had been initiated by sodium butyrate treatment of the cells. Furthermore, the FcγR induced on the surface of the endothelial cells appeared to be similar to that induced on the surface of fibroblasts, in that treatment of both cell types with trypsin resulted in loss of FcγR activity. The similarity between the percentage of endothelial cells actively infected with either AD169 or C1FE, and the number of cells expressing surface FcγR, suggested that the proportion of endothelial cells which did not express the FcγR under these conditions were the uninfected cells. This was supported by the finding that the percentage of fibroblasts expressing surface FcγR activity 4 days after inoculation, a time when maximum FcγR activity occurred both in the cytoplasm and at the cell surface, was dependent on the initial virus-to-cell ratio, although the overall level of FcγR expression

on the positive cells showed little change with increasing virus dose. Moreover, the ability to bind IgG via the Fc portion of the molecule in both cell types was strictly confined to the infected cell, and was not manifested by the bystander uninfected cells, as demonstrated by double immunofluorescence labelling of permeabilised cells. The fact that ganciclovir abrogated the activity of the CMV-induced FcγR at the cell surface suggested that CMV late gene expression was responsible for the FcγR activity seen in these studies.

It has been suggested that the CMV induced FcγR is located predominantly in the cytoplasm of the host cell, rather than at the cell surface (Keller et al. 1976; Furukawa et al. 1975). A report describing the FcγR induced by murine CMV supported this view, as expression of the FcγR was detected more strongly and more consistently in the cytoplasm than on the plasma membrane of murine CMV-infected cells (Thale et al. 1994). The present study showed that this was not the case for the human virus, as strong binding of human Fc fragments to both the cytoplasmic and surface forms of the FcγR was detected following infection of human umbilical vein endothelial cells and fibroblasts with CMV. However, as mentioned above, the addition of trypsin to the CMV infected cells severely reduced the surface FcγR activity. The FcγRs induced by HSV-1 and VZV have previously been shown to be affected by trypsin in a similar manner (Litwin et al. 1990; McTaggart et al. 1978). Thus, the differences in the expression of the FcγRs induced by the murine and human virus at the different cell locations could perhaps reflect the extreme sensitivity of the surface form of the FcγR to proteolytic cleavage. Indeed, in a recent study concerning the murine CMV induced FcγR, Fc binding activity could not be detected at the surface of fibroblasts following trypsin digestion (Crnkovic-Mertens et al. 1998), thus supporting the above assumption.

An extensive panel of murine monoclonal antibodies, which were capable of recognising the three different forms of human cellular FcγR distributed on different cell types, did not react with the CMV-induced FcγR at the surface of either fibroblasts or endothelial cells. These murine antibodies also failed to react with the cytoplasm of CMV-infected fibroblasts, when the ligand binding region of the CMV-induced FcγR had been blocked prior to the addition of each antibody. Thus, the FcγR present in the cytoplasm or at the surface of CMV infected cells was not one of the previously

identified cellular FcγR. Hence, it could be inferred that the FcγR induced by CMV was probably the product of a virally encoded gene, and was sufficiently different from the three known cellular FcγRs to avoid detection by monoclonal antibodies raised against them. A similar lack of reactivity with the cellular FcγR's has been reported for the FcγR induced by varicella zoster virus (Litwin et al. 1990).

Some of the aforementioned murine monoclonal antibodies, however, reacted with the cytoplasmic form of the CMV-induced FcγR when the receptor had not been blocked by prior incubation with human Fc fragments. This suggested that these antibodies were binding via their Fc region, rather than through their antigen-specific binding regions. This prompted a review on the ability of the CMV-induced FcγR to bind to immunoglobulin of homogeneous and xenogeneic origin. It has previously been reported that the FcγRs induced by HSV-1 reacted with IgG from rabbit, swine, sheep, and goat sera, but did not react with mouse, guinea pig, or hamster sera (Johansson et al. 1985). The FcγR induced by CMV has been reported to react with IgG from hamster and guinea pig sera, in addition to IgG from human and rabbit sera (Furukawa et al. 1975). The data shown here indicated that the reactivity of the cytoplasmic form of the CMV induced FcγR with human and rabbit Fc fragments was greater than that of the cytoplasmic form of the HSV-1 gE/gI FcγR, even when the fibroblasts had been inoculated with CMV at a lower multiplicity of infection than HSV-1.

Little is known about the ability of the CMV induced FcγR to react with IgG from mouse sera. The results presented here showed that there was a difference in the ability of murine Fc fragments, and murine IgG1 and IgG3 molecules, to bind to CMV- and HSV-1-infected fibroblasts. The binding of these molecules to the cytoplasmic form of the CMV induced FcγR was concentration dependent, and was only observed when murine IgG1 molecules or murine Fc fragments were used at concentrations >20μg/ml. Murine IgG3 molecules, however, were able to bind at lower concentrations. The binding of a proportion of the antibodies specific for cellular FcγRs to the CMV-infected fibroblasts could thus be explained; All were of the IgG1 murine subclass, and only bound at concentrations >20μg/ml. No detectable binding was observed when the same murine IgG molecules and Fc fragments were incubated with the HSV-1-infected cells, supporting previous literature reports (Johansson et al. 1985). Murine IgG1 and IgG3

molecules also reacted with the cytoplasm of the CMV infected endothelial cells. The results presented here show that, in addition to the blocking steps recommended when probing CMV and HSV-1 infected samples with rabbit or human sera (Gallo, 1986; Furukawa et al. 1975), similar steps should be employed when probing fixed CMV-infected samples with murine IgG antibodies.

The binding of murine Fc fragments or murine IgG at the surface of CMV infected fibroblasts and endothelial cells could not be detected, even when concentrations as high as 40µg/ml were used. In contrast, binding of both human and rabbit Fc fragments to the surface of CMV-infected fibroblasts was consistently detectable during similar experiments. The reason for the difference between the cytoplasmic and surface pattern of murine IgG reactivity obtained in this study is unclear, but could reflect the different procedures used to monitor the CMV induced FcγR at the different locations in the cell. Since acetone fixation results in the dehydration of lipid membranes, it is possible that artificial aggregation of molecules which were previously spacially separated may have occurred and, thereby, created receptors with higher affinity for Fc fragments in the cytoplasm of the infected cell. The different reactivity patterns could also be due to post-translational modifications of the receptor. Excessive sialylation of both the HSV-1 and varicella zoster virus induced FcγRs is known to abrogate Fc receptor activity (Litwin et al. 1992; McTaggart et al. 1978). Thus immature CMV FcγR molecules present in the cytoplasm, and which have not been fully processed, could have better affinity for the murine IgG1 and IgG3 subclasses than the mature surface form of the CMV induced FcγR. Indeed, the FcγR induced by murine CMV has been shown to be heavily glycosylated (Thale et al. 1994).

The biological function of the CMV-induced FcγR is at present unclear. These FcγR's may protect the infected cells from antibody-mediated destruction by binding the Fc portion of non-virus specific antibody, thereby impeding the attachment of specific antiviral IgG to the cell surface (Van Vliet et al. 1992; Frank, Friedman, 1989; Adler et al. 1978). Moreover, the simultaneous binding of virus specific antibody to the viral antigen and to the virus-induced FcγR could prevent the functional engagement of the Fc portion of the antibody, thus preventing its participation in antibody-dependent cell mediated

cytotoxicity or antibody plus complement-mediated lysis of the infected cells (Murayama et al. 1987; Dubin et al. 1991).

Support for an important biological role of the CMV-induced FcγR comes from studies in mice. Although the FcγR region of murine CMV was found to be dispensable for growth *in vitro*, after infection of experimental animals, FcγR deletion mutants exhibited reduced growth characteristics in various organs (Crnkovic-Mertens et al. 1998). This, however, was not due to the antibody binding properties of the FcγR, as a similar effect occurred when mutant mice which lacked antibodies were infected with the same FcγR-deleted viral strain (Crnkovic-Mertens et al. 1998). Thus, the FcγR induced by CMV may subserve functions other than binding IgG. One such mechanism might be in the promotion of cell to cell spread of the virus, thus enabling the virion to avoid recognition by extrinsic virus-specific antibodies. Indeed, such fusion properties have been shown for the HSV-1 encoded FcγR, gE and gI (Dingwell et al. 1995; Dingwell et al. 1994) and appear to be the main function of the pseudorabies gE/gI homologue, gp63/gI (Zsak et al. 1992; Zuckermann et al. 1988). Although the proteins responsible for the CMV-induced FcγR have not been identified, a recent study indicated that CMV pUS9 and HSV-1 gE had analogous functions in promoting virus spread across lateral membranes of polarized retinal epithelial cells (Maidji et al. 1996). Similar properties may be conferred on infected cells following induction of the CMV-FcγR, and thus these proteins might aid in viral dissemination through selected cell types without provoking an immune response.

In summary, a trypsin sensitive FcγR was induced on the surface of endothelial cells and fibroblasts following infection with CMV. Double-labelling experiments showed strong expression of the CMV FcγR in the perinuclear region of infected cells but not in bystander uninfected cells. The CMV FcγR did not react with a panel of monoclonal antibodies directed against the known human cellular FcγRs, FcγRI, FcγRII and FcγRIII. The cytoplasmic form, but not the cell surface form the CMV-induced FcγR bound murine IgG moderately, and murine IgG1 more weakly, while both forms bound rabbit IgG almost as strongly as human IgG.

CHAPTER 6.

THE EFFECT OF CMV INFECTION ON THE EXPRESSION OF ADHESION MOLECULES PRESENT AT THE SURFACE OF ENDOTHELIAL CELLS AND FIBROBLASTS.

6.1. Introduction.

Cell adhesion molecules play an important role in directing the immune response to an invading pathogen. The first step in all leukocyte recruitment is the induction of molecules which interact loosely with flowing leukocytes to promote reduced movement through the bloodstream. This in turn enables firmer binding of the leukocytes to the endothelium, which facilitates transendothelial cell migration (Butcher, 1991; Hogg, 1992). However, aberrant adhesion molecule expression has been detected in tissues undergoing allograft rejection (Heemann et al. 1994).

In vivo, CMV has been implicated in both provoking allograft rejection, and in promoting altered adhesion molecule expression (Koskinen, 1993; Yilmaz et al. 1996). Altered adhesion molecule expression following infection of fibroblasts with CMV has also been demonstrated *in vitro* (Grundy, Downes, 1993; Craigen, Grundy, 1996). However, only a few studies have investigated the ability of CMV to alter adhesion molecule expression at the surface of endothelial cells, and what little is known is largely controversial (Shahgasempour et al. 1997a; Sedmak et al. 1994a; Scholz et al. 1992a). Furthermore, the kinetics of adhesion molecule expression has not been properly investigated in these studies, because the virus strains used were either inefficient at infecting endothelial cells, or were cell associated. Therefore, the effect of CMV infection on the expression of the endothelial cell adhesion molecules ICAM-1, LFA-3, ICAM-2, PECAM-1 and E-selectin was investigated. Endothelial cells were infected with different viral strains (AD169, C1FE or Toledo.E) and the cell surface expression of the various adhesion molecules was determined at different times post inoculation, using flow cytometric techniques. Where indicated in the text, fibroblasts were inoculated with similar viral strains (AD169, C1F and Toledo) for purposes of comparison. In addition, the effect of cell-free supernatants taken from C1FE-inoculated endothelial cells on the ability to alter adhesion molecule expression was also investigated. This was to determine whether any changes observed were a direct viral effect, or mediated by soluble factors released from infected cells.

6.2 The effect of CMV infection on the expression of ICAM-1 at the surface of endothelial cells at different times post inoculation.

To determine the effect of CMV infection on ICAM-1 expression at the surface of endothelial cells, with respect to time, the cells were inoculated with C1FE at an MOI of 4, and the expression of ICAM-1 was then analysed at various days post inoculation by flow cytometry. Using these methods, it was determined that uninfected endothelial cells displayed a broad, low basal level of ICAM-1 expression for all time points studied (Figure 6.2.1). In contrast, C1FE-inoculated endothelial cells progressively displayed a narrower, increased level of ICAM-1 expression when compared to the uninfected counterparts (Figure 6.2.1). When the logarithmically acquired data from three experiments performed in triplicate were converted to a linear form for statistical analysis, a significant increase in the expression of ICAM-1 was evident at day 1 post inoculation and continued throughout the study ($p=0.01$, $n=9$). The greatest difference between the C1FE-inoculated and uninfected endothelial cells occurred at day 3-5 post inoculation (Figure 6.2.1). Detection of CMV IEA using cytospin preparations of the endothelial cells revealed that 70% of the infected cells were positive for CMV IEA at day 3 post infection (data taken from the experiment shown in Figure 6.2.1). The uninfected endothelial cells, as expected, were negative for this viral antigen. However, despite there being a mixed population of CMV antigen positive or negative cells within the virally inoculated cell population, two distinctive populations of ICAM-1 expression were not observed. When the Toledo.E strain was used to inoculate the endothelial cells, a similar increase in the expression of ICAM-1 occurred from day 1 to day 5 (data not shown). This data therefore showed that CMV infection of endothelial cells led to an increase in the cell surface expression of ICAM-1.

6.3. The expression of LFA-3 on the surface of endothelial cells following infection with CMV.

To determine the effect of CMV infection on LFA-3 expression at the surface of endothelial cells, with respect to time, the cells were inoculated with CMV strain C1FE, as described above, and the expression of LFA-3 was then analysed at various days post inoculation by flow cytometry. The uninfected endothelial cells displayed a

narrow, low basal level of LFA-3 expression for all time points tested (Figure 6.3.1), and no change in LFA-3 expression was observed following inoculation with C1FE at any time point studied (Figure 6.3.1). Although a difference in the level of LFA-3 expression could be observed at the surface of C1FE-inoculated endothelial cells at 4 days post inoculation when compared with the uninfected counterparts (Figure 6.3.1), conversion of the logarithmically acquired data, taken from four experiments performed in triplicate, to a linear form (for statistical analysis) indicated that these were not significantly different (n=12). The fact that LFA-3 was unaltered during these experiments was not due to a poor infection rate, because 70% of the infected cells were positive for CMV IEA at day 3 post infection (data taken from the experiment shown in Figure 6.3.1). When the Toledo.E strain of CMV was used to inoculate the endothelial cells, the results were similar to those seen for C1FE (data not shown). Thus, in contrast to the results seen for ICAM-1, CMV infection of endothelial cells with two low passage clinical isolates did not alter the cell surface expression of LFA-3.

6.4. The expression of LFA-3 and ICAM-1 on the surface of fibroblasts following infection with different strains of CMV.

The above results suggested that LFA-3 expression was not altered following infection of endothelial cells with the endothelial adapted strains of CMV, C1FE and Toledo.E. However, a previous report which used AD169-inoculated fibroblasts indicated that LFA-3 expression was in fact up-regulated during the experimental time period, as was the expression of ICAM-1 (Grundy, Downes, 1993). This suggested that either endothelial cells do not alter LFA-3 expression, or that the strain of virus may influence the results. To test this latter possibility, fibroblasts were inoculated at a MOI of 2 with either the laboratory strain of CMV, AD169, or the fibroblast-passaged clinical isolates C1F and Toledo. The expression of LFA-3 was then analysed at the surface of the fibroblasts at various time points post-inoculation, as previously described. In addition, the expression of ICAM-1, which was up-regulated following CMV-infection of endothelial cells, was similarly monitored on the fibroblasts, and served as a control.

The results indicated that, as was seen for the endothelial cells, uninfected fibroblasts displayed a narrow, low basal level of LFA-3 expression (data not shown). However, following CMV-inoculation of fibroblasts, a differential LFA-3 staining pattern was observed, depending upon which viral strain had been used to inoculate the cells (Figure 6.4.1). Thus, only those cells which had been infected with the laboratory strain AD169, or the clinical isolate C1F, consistently displayed an increased level of LFA-3 expression at the cell surface, when compared to the uninfected counterparts (Figure 6.4.1). When the logarithmically acquired data from two experiments performed in triplicate were converted to a linear form for statistical analysis, a significant increase in LFA-3 expression at the surface of either the AD169- or C1F-inoculated fibroblasts was detected at 3-4 days post inoculation ($p=0.05$, $n=6$). In contrast, no significant effect on LFA-3 expression ($n=6$) was observed following infection with the Toledo strain of CMV. Although no data could be obtained for the Toledo-inoculated fibroblasts at 5 days post inoculation, due to marked lysis of these infected cells, it was unlikely that this influenced the results at the earlier time points.

When the fibroblasts were analysed on the basis of ICAM-1 expression, however, a different result was observed. In this case, all CMV-inoculated fibroblasts progressively displayed increased levels of ICAM-1 expression when compared to the uninfected counterparts, regardless of the strain used or the time point studied (Figure 6.4.1). Furthermore, when the logarithmically acquired data from two experiments performed in triplicate were converted to a linear form for statistical analysis, ICAM-1 expression was significantly increased at all time points studied ($p=0.05$, $n=6$). This is in agreement with the results obtained for the endothelial cells (c.f. Section 6.2). Taken together, these results suggested that the expression of ICAM-1 at the surface of fibroblasts was increased following infection with either laboratory or clinical isolates of CMV, whereas a strain dependant effect on LFA-3 expression at the surface of CMV- infected fibroblasts was observed.

6.5. The expression of LFA-3 and ICAM-1 at the surface of fibroblasts following infection with either an endothelial cell passaged or a fibroblast passaged clinical isolate.

The above results suggested that the viral strain used to inoculate fibroblasts was important in determining the subsequent effect on LFA-3 expression, but only partially explained why CMV-infection of endothelial cells did not alter the expression of this molecule. In particular, the fact that the Toledo strain of virus did not increase LFA-3 expression at the surface of fibroblasts explained why infection of endothelial cells with Toledo.E (which was derived from Toledo) also failed to increase LFA-3. In contrast, C1F infection of fibroblasts resulted in an increase in LFA-3 expression, whereas C1FE infection of endothelial cells did not, even though the latter strain was derived from C1F. This again suggested that either endothelial cells were incapable of altering LFA-3 expression, or that C1F and C1FE exerted different effects on LFA-3. To test the latter possibility, fibroblasts were inoculated (MOI of 2) with either C1F or C1FE, and the expression of LFA-3 was determined at the cell surface 3 days later, the time when maximal expression of this molecule was consistently observed. In addition, the expression of ICAM-1 was also monitored by similar methods, for reasons of comparison.

As expected, LFA-3 expression was once again increased at the cell surface three days post inoculation following inoculation with C1F, when compared to the uninfected fibroblasts, as was the expression of ICAM-1 (Figure 6.5.1). In contrast, the inoculation of fibroblasts with CMV strain C1FE did not result in increased LFA-3 expression at this time, even though an increased expression of ICAM-1 was observed (Figure 6.5.1). When the logarithmically acquired data from two experiments performed in triplicate were converted to a linear form for statistical analysis, a significant increase in LFA-3 expression was only observed at the surface of the C1F-inoculated fibroblasts ($p=0.05$, $n=6$). Thus C1F, but not C1FE, could increase expression of LFA-3 at the surface of fibroblasts, whereas both viral strains were capable of altering ICAM-1 expression on the same cell type. Unfortunately, a similar set of experiments could not be performed using endothelial cells, as C1F does not efficiently infect this cell type (see Chapter 3). The above data, however,

suggested that the virus strains, rather than cell type, was important in determining the effect of CMV on the expression of LFA-3.

6.6. The expression of ICAM-2 at the surface of endothelial cells following infection with CMV.

To determine the effect of CMV infection on the constitutive expression of ICAM-2 at the surface of endothelial cells, with respect to time, the cells were inoculated at an MOI of 4 with C1FE, and the expression of ICAM-2 was then analysed at various days post inoculation by flow cytometry. In addition, cytospin preparations of the C1FE-infected endothelial cells showed that 60% of these cells were positive for CMV IEA at 4 days post inoculation.

The results indicated that uninfected endothelial cells displayed a broad, high constitutive expression of ICAM-2 at all time points tested (Figure 6.6.1). In contrast, endothelial cells which had been inoculated with C1FE displayed a decrease in the expression of this molecule when compared to the uninfected counterparts (Figure 6.6.1). When the logarithmically acquired data from three experiments performed in triplicate were converted to a linear form for statistical analysis, the expression of ICAM-2 at the surface of C1FE-inoculated endothelial cells was found to be significantly decreased from day 1 through to day 5 ($p=0.01$, $n=9$). No evidence of dual levels of expression of this molecule was observed in any of the studies, despite the fact that a mixed population of uninfected and infected cells existed following inoculation of the endothelial cells (see above). A similar down-regulation of ICAM-2 expression occurred at the surface of endothelial cells which had been inoculated with Toledo.E (data not shown). Thus, the constitutive expression of ICAM-2 was decreased at the endothelial cell surface following inoculation with endothelial cell adapted CMV strains.

6.7. The expression of PECAM-1 at the surface of endothelial cells following infection with CMV.

To determine the effect of CMV infection on the constitutive expression of PECAM-1 at the surface of endothelial cells, with respect to time, the cells were inoculated at an MOI of 4 with C1FE, and the expression of PECAM-1 was then

analysed at various days post inoculation by flow cytometry. In addition, cytopsin preparations of the C1FE-infected endothelial cells showed that 60% of these cells were positive for CMV IEA at 4 days post inoculation.

The effect of CMV infection on the cell surface expression of PECAM-1 was essentially the same as that described above for ICAM-2. Uninfected endothelial cells consistently showed a broad, high constitutive expression of PECAM-1, whereas the C1FE-inoculated counterparts progressively decreased the expression of this molecule from day 1 through day 5 (Figure 6.7.1). When the logarithmically acquired data from three experiments performed in triplicate were converted to a linear form for statistical analysis, the expression of ICAM-2 at the surface of C1FE-inoculated endothelial cells was significantly decreased from day 1 through to day 5 ($p=0.01$, $n=9$). A similar down-regulation of PECAM-1 expression occurred at the surface of endothelial cells which had been inoculated with Toledo.E (data not shown). Thus, these results indicated that the constitutive expression of PECAM-1 was down-regulated at the endothelial cell surface following inoculation with endothelial cell adapted CMV strains.

6.8. The effect of ganciclovir on the ability of CMV to alter the expression of ICAM-1, PECAM-1 and ICAM-2 at the endothelial cell surface.

In order to determine whether CMV late antigen expression had any effect on the changes observed in ICAM-1, ICAM-2 and PECAM-1 expression following infection of endothelial cells with CMV, the cells were inoculated at a MOI of 5 with Toledo.E and either treated with the antiviral drug ganciclovir, or left untreated. This viral strain was used because it was consistently susceptible to ganciclovir treatment, as indicated in Chapter 4. The expression of either ICAM-1, ICAM-2 or PECAM-1 was then determined at 5 days post-inoculation, as previously described. The expression of LFA-3 was not investigated here because inoculation with Toledo.E did not produce any changes in the surface expression of this molecule (Section 6.3). Analysis of cytopsin preparations of these inoculated cells at day 5 post inoculation showed that only the untreated, Toledo.E-inoculated cells expressed CMV late (gB) antigen in 60% of the cells. The similarly inoculated, ganciclovir treated cells only expressed CMV IEA (70%), but no late antigen.

The addition of ganciclovir to the culture medium did not prevent the increased expression of ICAM-1 at the surface of the Toledo.E-inoculated endothelial cells, when compared to similarly treated but uninfected cells (Figure 6.8.1). In fact, when the ganciclovir treated, Toledo.E-inoculated cells were compared to the similarly inoculated cells which had not been treated with the drug, the expression of ICAM-1 was further increased in the former case with respect to the latter (Figure 6.8.1). Although ICAM-1 expression was apparently increased on drug treated uninfected endothelial cells when compared to the untreated counterpart, conversion of the logarithmic data to a linear form revealed no difference between the two (Figure 6.8.1). Furthermore, when the logarithmically acquired data from three experiments performed in triplicate were converted to a linear form for statistical analysis, there was a significant difference between the levels of ICAM-1 expression for the Toledo.E-inoculated cells which had either been treated with ganciclovir or not ($p=0.01$, $n=9$), whereas no difference in ICAM-1 expression was observed for either the uninfected, untreated or the uninfected, drug treated cells ($n=9$). Thus, the treatment of CMV-inoculated endothelial cells with ganciclovir did not prevent the up-regulation of ICAM-1 expression, but rather resulted in a further increase in the expression of this molecule at the surface of the CMV-inoculated endothelial cells. Thus, CMV late gene expression was not responsible for the up-regulation of ICAM-1 seen in these studies.

The addition of ganciclovir to the culture medium also failed to prevent the decrease in either ICAM-2 or PECAM-1 expression observed at the surface of the Toledo.E-inoculated endothelial cells, when compared to the similarly treated but uninfected cells (Figure 6.8.1). Once again, an apparent difference in the level of expression of both ICAM-2 or PECAM-1 was observed when the uninfected ganciclovir treated cells were compared to the untreated counterparts (Figure 6.8.1), although conversion of the logarithmic data to a linear form revealed no difference between the two (Figure 6.8.1). Furthermore, when the logarithmically acquired data from three experiments performed in triplicate was converted to a linear form for statistical analysis, no significant difference between the expression of these molecules was observed when the inoculated, ganciclovir treated cells were compared with the inoculated, untreated cells ($n=9$). There was a significant difference,

however, when the inoculated cells (either ganciclovir treated or untreated) were compared to the uninfected counterparts ($p=0.01$, $n=9$). Thus, ganciclovir treatment did not prevent the down regulation of either ICAM-2 or PECAM-1 at the surface of CMV-infected endothelial, suggesting that this effect was influenced by either the IE or the early stage of CMV infection, as was the up-regulation of ICAM-1.

6.9. The effect of cell-free, CMV-infected cell supernatants on the expression of ICAM-1, ICAM-2 and PECAM-1 at the endothelial cell surface.

To determine if the CMV-induced changes in the expression of the adhesion molecules ICAM-1, ICAM-2 and PECAM-1 were mediated by soluble factors which were released into the medium during the course of CMV infection of endothelial cells, supernatants from C1FE-infected endothelial cells were tested for their ability to alter the expression of these adhesion molecules. The medium was removed from either C1FE-inoculated or uninfected endothelial cells at two days post inoculation, centrifuged to remove any cell debris and then passed through a 0.2μ filter. The supernatants were then added onto fresh, uninfected endothelial cells for a further three days, after which adhesion molecule expression was determined using flow cytometric techniques.

The results indicated that neither the levels of ICAM-1, ICAM-2 nor PECAM-1 were changed following incubation with supernatants from C1FE-inoculated or uninfected endothelial cells (Figure 6.9.1). Although it could be argued that a soluble factor may have been produced after 2 days, the fact that adhesion molecule expression was altered as early as day 1 post inoculation suggested that any soluble factor involved in this process would already be present at 2 days. Furthermore, during the same experiments, the expression of MHC Class I was increased after supernatant transfer (Section 4.6), indicating that CMV-induced soluble factors were present. Thus, the above data suggested that a soluble factor was not responsible for the changes to the adhesion molecules which were consistently observed following infection of endothelial cells with CMV, but rather that a direct viral effect was involved.

6.10. The expression of E-selectin on the surface of endothelial cells following infection with CMV.

The ability of endothelial cells to alter the surface expression of E-selectin following infection with CMV was also investigated. This molecule was studied separately because, unlike the other molecules, E-selectin was not constitutively expressed at the surface of endothelial cells, but could be transiently induced by the cytokine IL-1 β . Initial experiments which were undertaken to determine the optimum stimulatory effect of IL-1 β , with respect to time and concentration, consistently showed that the maximum stimulation occurred after 4-6 hours of treatment with 10ng/ml of IL-1 β (data not shown). However, because not all of the cells showed induction of this molecule at one particular time (Figure 6.10.1), the percentage of cells which were either positive or negative for E-selectin expression after 4 hours of IL-1 β stimulation was determined in reference to a marker which was set using the isotype matched negative control antibody. For the experiments described below, endothelial cells were inoculated at an MOI of 4 with the clinical isolate C1FE, and at various time points post inoculation, the cells were treated with IL-1 β . The cell surface expression of E-selectin was then determined 4 hours later by flow cytometry.

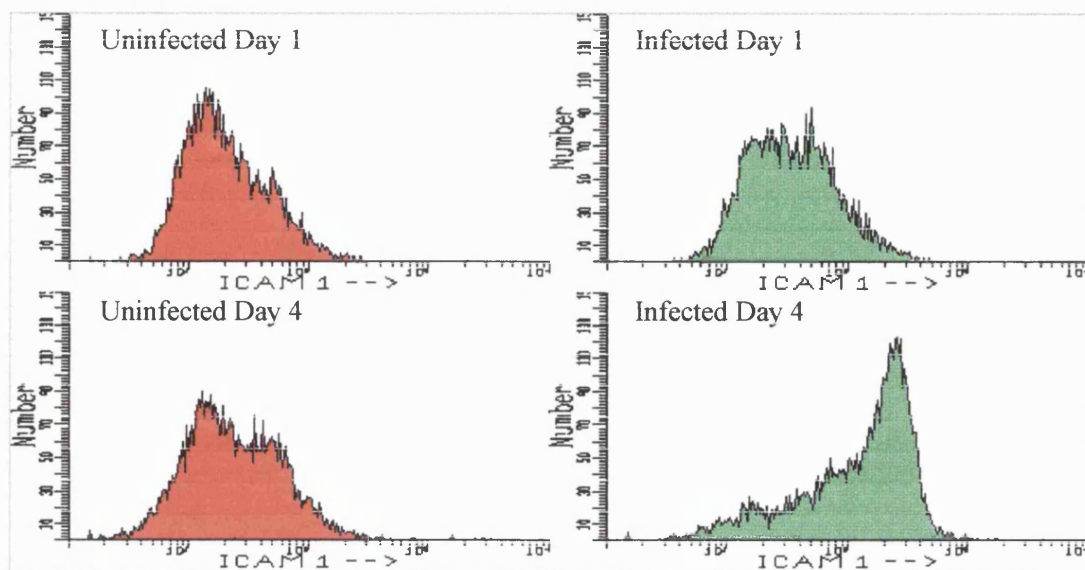
The results indicated that E-selectin expression only occurred on those cells which had been treated with IL-1 β , for both uninfected and CMV-inoculated endothelial cells (Figure 6.10.1). Less than 2% of uninfected endothelial cells expressed this molecule in the absence of IL-1 β stimulation, as did similarly untreated C1FE-inoculated cells, at all time points tested (day 1-4). In contrast, the expression of this molecule was markedly reduced on the endothelial cells which had been inoculated with C1FE and subsequently stimulated with IL-1 β , when compared to the cytokine-stimulated uninfected cells (Figure 6.10.1). When the logarithmically acquired data from three experiments performed in triplicate were converted to a linear form for statistical analysis, a significant reduction in E-selectin expression occurred at all time points ($p=0.01$, $n=9$). Furthermore, the percentage of uninfected endothelial cells which expressed E-selectin was found to be 76%, 68%, 61% and 54% for days 1-4, respectively, whereas the percentage on the C1FE-inoculated endothelial cells was only 34%, 32%, 34% and 23%, days 1-4, respectively (data

taken from the experiment shown in Figure 6.10.1). Cytospin preparations of C1FE-inoculated endothelial cells taken at day 4 post inoculation revealed that 65-70% were positive for CMV IEA, whereas the uninfected cells were negative for this antigen. No direct correlation could be established between the percentage of cells expressing CMV IEA and the percentage of cells which could no longer be induced to express E-selectin, and may partly be due to the fact that not all the uninfected cells originally expressed E-selectin following IL-1 β stimulation. Taken together, however, these results did show that CMV infection of endothelial cells did not induce E-selectin, but rather prevented the induction of this molecule in a proportion of the cells following IL-1 β stimulation.

6.11. The effect of ganciclovir on the ability of CMV to alter the expression of E-selectin at the surface of cytokine stimulated endothelial cells.

Finally, the effect of the antiviral agent ganciclovir on the ability of CMV to prevent the induction of E-selectin on cytokine stimulated endothelial cells was investigated. Endothelial cells were inoculated at an MOI of 5 with Toledo.E and either treated with ganciclovir for 4 days, or left untreated. All cells were then stimulated with IL-1 β , and the expression of E-selectin was determined as described previously. The results indicated that Toledo.E, like C1FE, reduced the expression of E-selectin at the endothelial cell surface, when compared to the uninfected cells, and that the presence of ganciclovir had no effect on the ability of Toledo.E to prevent the induction of E-selectin expression on the surface of endothelial cells (Figure 6.11.1). Furthermore, the percentage of cells which were positive for E-selectin expression was not altered following ganciclovir treatment, when either the uninfected cells (41% and 43%, untreated and treated, respectively) or the Toledo.E-inoculated cells (12% and 14%, untreated and treated, respectively) were compared. These results indicated that infection with CMV prevented the induction of E-selectin on a proportion of the endothelial cells, and late viral antigen was not required to produce this effect. Thus, CMV IE or early gene expression influenced the ability of endothelial cells to induce E-selectin in response to IL-1 β stimulation.

A.



B.

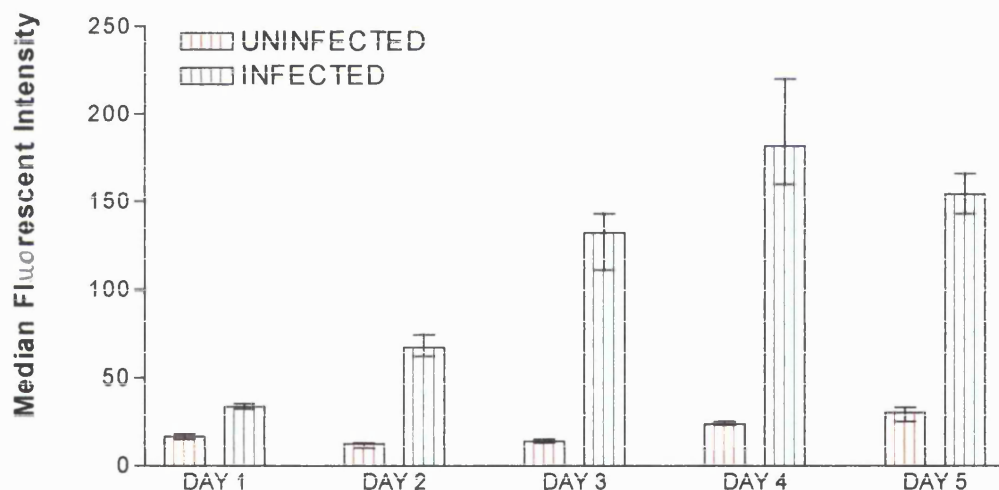
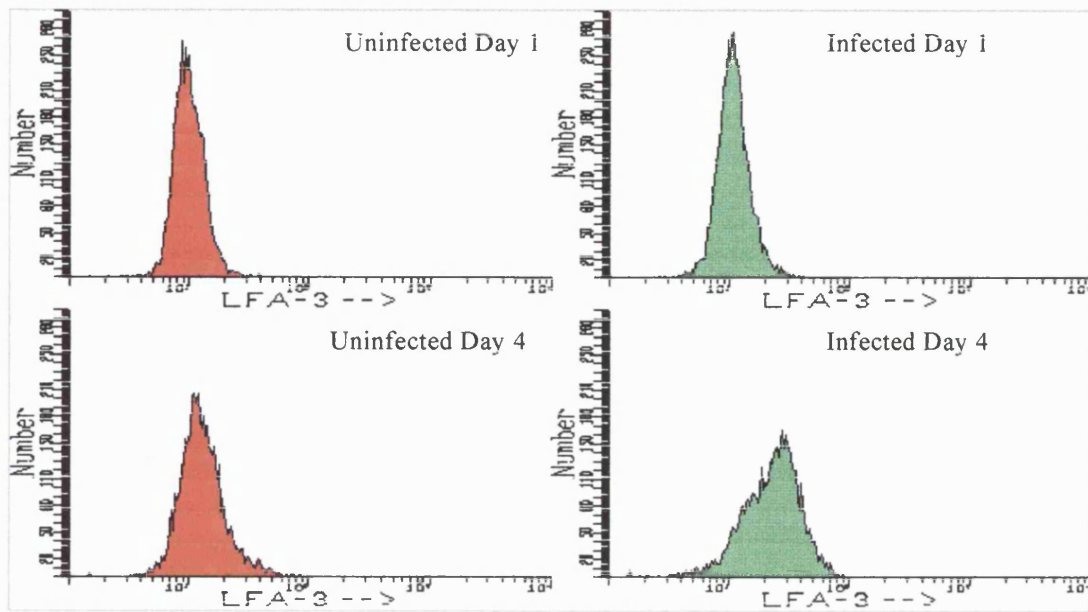


Figure 6.2.1. The expression of ICAM-1 at the surface of endothelial cells following infection with CMV for various time periods.

Endothelial cells were inoculated at an MOI of 4 with CMV strain C1FE, and the expression of ICAM-1 was determined at the cell surface using flow cytometric techniques. In part A, representative histogram profiles of the data are shown at day 1 and day 4 post inoculation, where the ordinate represents the number of fluorescence events (scale 0-150), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). In part B, the data represent the median fluorescent intensity of triplicate samples for a representative experiment, at days 1-5 post inoculation. The error bars show the range of values obtained.

A.



B.

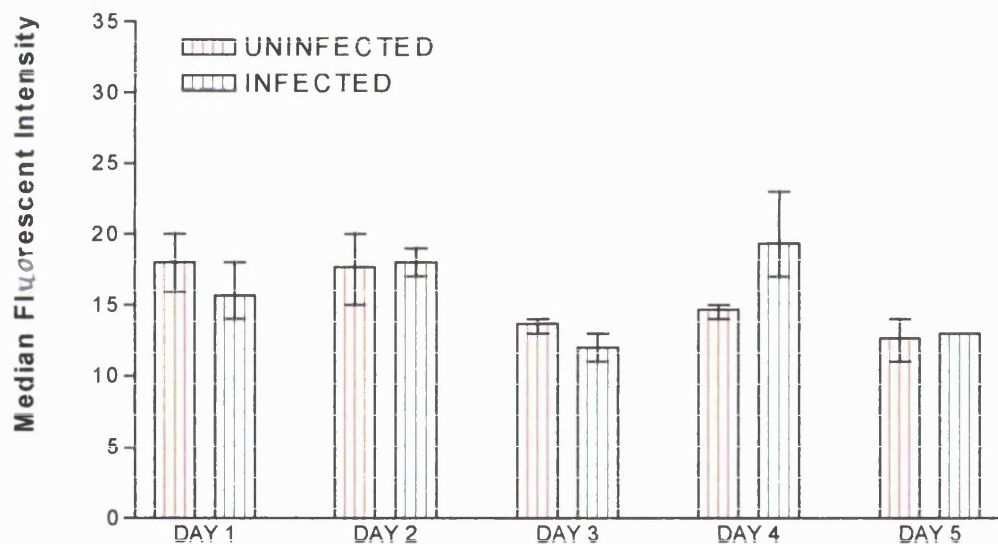
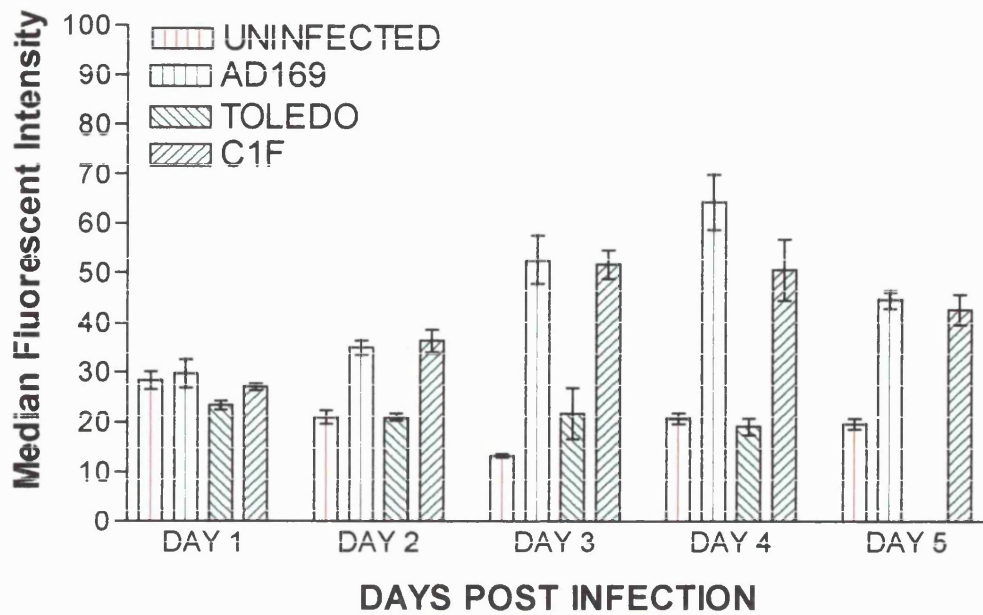


Figure 6.3.1. The expression of LFA-3 at the surface of endothelial cells following infection with CMV for various time periods.

Endothelial cells were inoculated at an MOI of 4 with CMV strain C1FE, and the expression of LFA-3 was determined at the cell surface using flow cytometric techniques. In part A, representative histogram profiles of the data are shown at day 1 and day 4 post inoculation, where the ordinate represents the number of fluorescence events (scale 0-300), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). In part B, the data represent the median fluorescent intensity of triplicate samples for a representative experiment, at days 1-5 post inoculation. The error bars show the range of values obtained.

A. LFA-3



B. ICAM-1

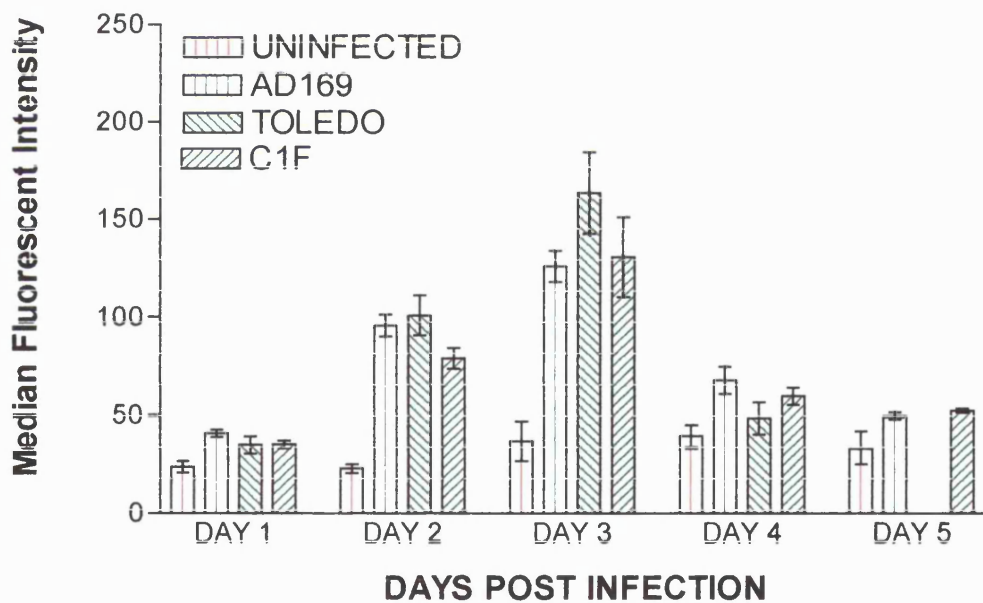
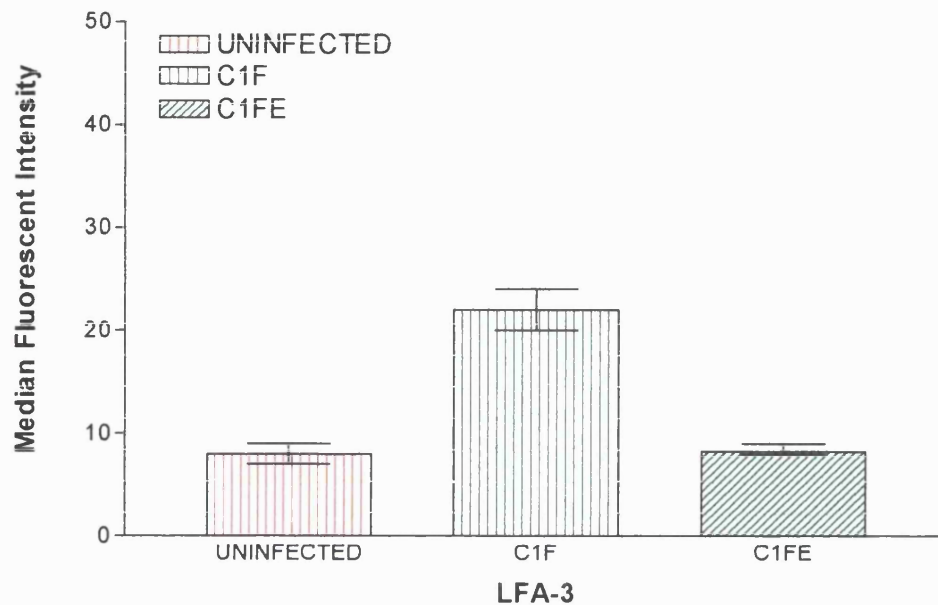


Figure 6.4.1. The effect of infection with different strains of CMV on the expression of LFA-3 or ICAM-1 at the surface of fibroblasts.

Fibroblasts were inoculated at an MOI of 2 with CMV strains AD169, Toledo or C1F, and the expression of LFA-3 (Part A) or ICAM-1 (Part B) was determined at the cell surface using flow cytometric techniques. The results are expressed as the median fluorescent intensity of triplicate samples for a representative experiment, at days 1-5 post inoculation. The error bars show the range of values obtained.

A.



B.

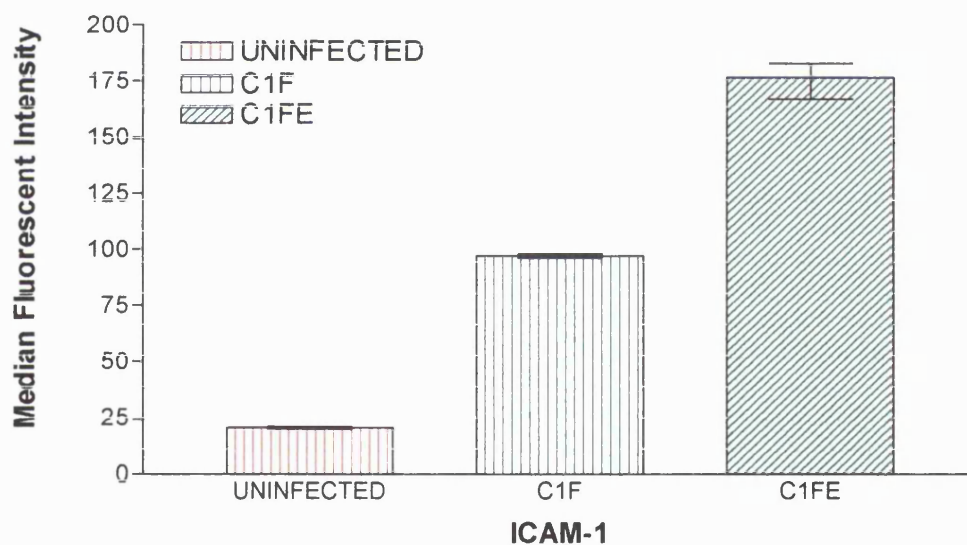


Figure 6.5.1. The expression of LFA-3 or ICAM-1 on the surface of fibroblasts following infection with either an endothelial-adapted or a fibroblast-adapted strain of CMV.

Fibroblasts were inoculated at an MOI of 2 with the fibroblast adapted strain C1F or the endothelial adapted strain C1FE, and the expression of LFA-3 (Part A) or ICAM-1 (Part B) was determined at the cell surface 3 days post inoculation, using flow cytometric techniques. The data is expressed as the median fluorescent intensity of triplicate samples for a representative experiment. The error bars show the range of values obtained.

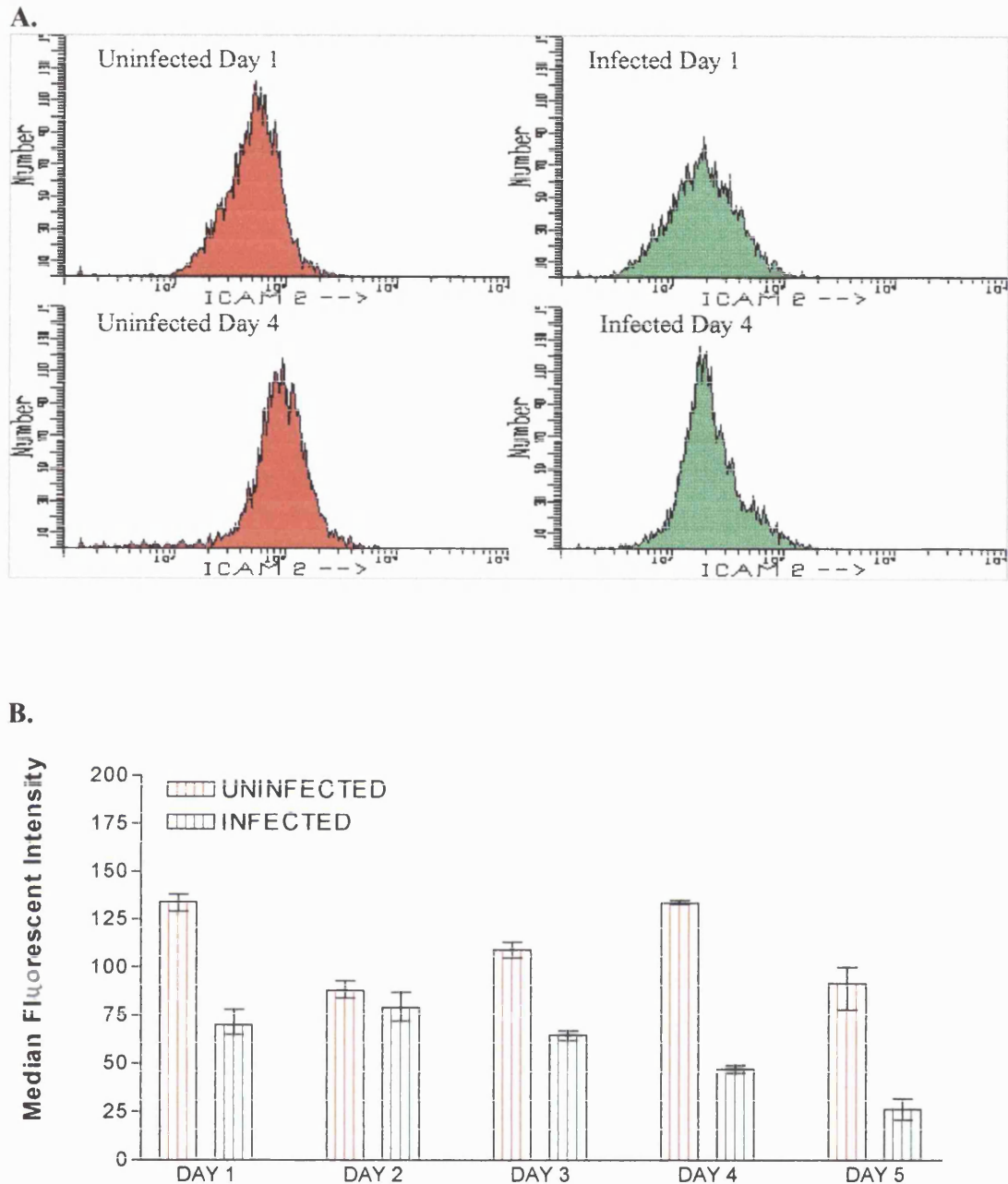
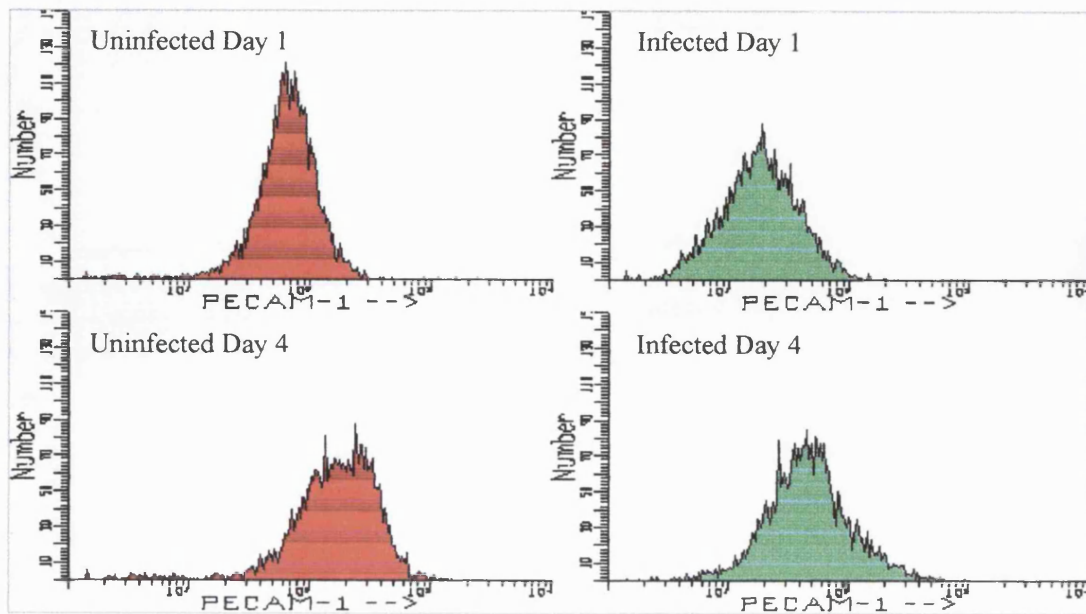


Figure 6.6.1. The expression of ICAM-2 at the surface of endothelial cells following infection with CMV for various time periods.

Endothelial cells were inoculated at an MOI of 4 with CMV strain C1FE, and the expression of ICAM-2 was determined at the cell surface using flow cytometric techniques. In part A, representative histogram profiles of the data are shown at day 1 and day 4 post inoculation, where the ordinate represents the number of fluorescence events (scale 0-150), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). In part B, the data are expressed as the median fluorescent intensity of triplicate samples for a representative experiment, at days 1-5 post inoculation. The error bars show the range of values obtained.

A.



B.

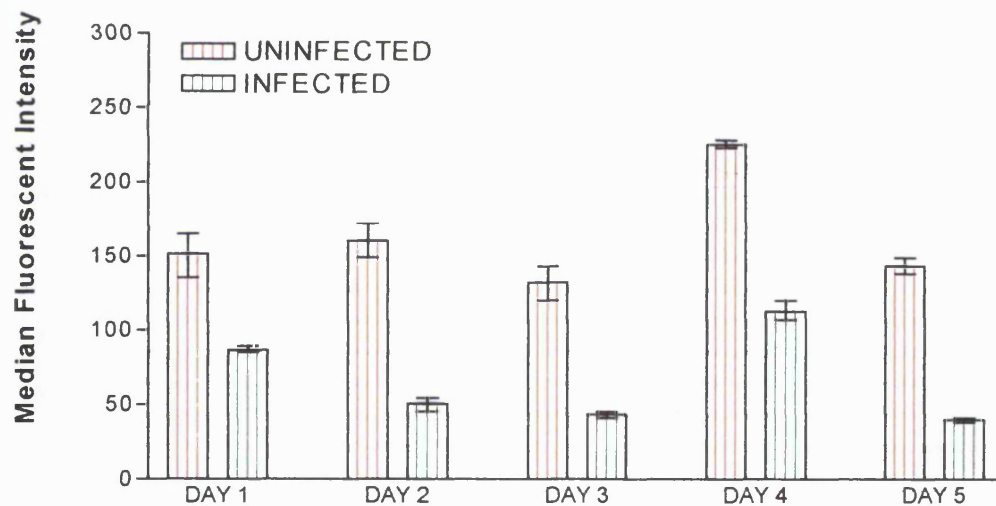


Figure 6.7.1. The expression of PECAM-1 at the surface of endothelial cells following infection with CMV at various times post infection.

Endothelial cells were inoculated at an MOI of 4 with CMV strain C1FE, and the expression of PECAM-1 was determined at the cell surface using flow cytometric techniques. In part A, representative histogram profiles of the data are shown at day 1 and day 4 post inoculation, where the ordinate represents the number of fluorescence events (scale 0-150), and the abscissa represents $\log_{10}$ fluorescence intensity in arbitrary units (scale 10^0 - 10^4). In part B, the data are expressed as the median fluorescent intensity of triplicate samples for a single experiment, at days 1-5 post inoculation. The error bars show the range of values obtained.

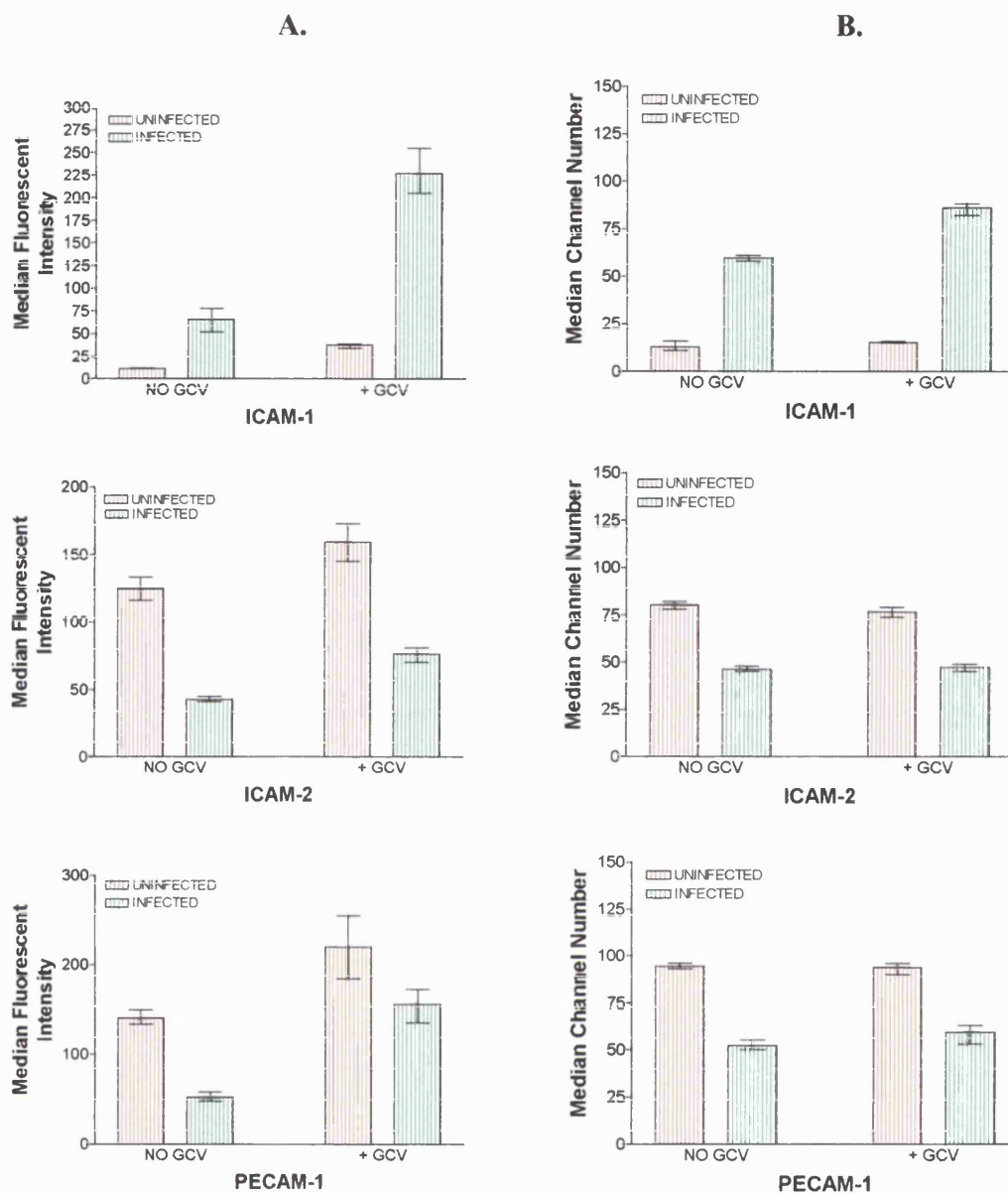


Figure 6.8.1. The effect of ganciclovir treatment on the expression of ICAM-1, ICAM-2 or PECAM-1 following infection of endothelial cells with CMV.

Endothelial cells were inoculated at an MOI of 5 with CMV strain Toledo.E and either treated with ganciclovir (+GCV) or left untreated (no GCV). The expression of ICAM-1, ICAM-2 or PECAM-1 at the Toledo.E-inoculated or uninfected endothelial cell surface was determined 5 days later, using flow cytometric techniques. In panel A, the data are expressed as the median fluorescent intensity of triplicate samples for a representative experiment. The data were obtained using a $\log_{10}$ scale. In panel B, the same data were converted to linear median channel values, minus the values obtained using the irrelevant control antibody. The error bars show the range of values obtained.

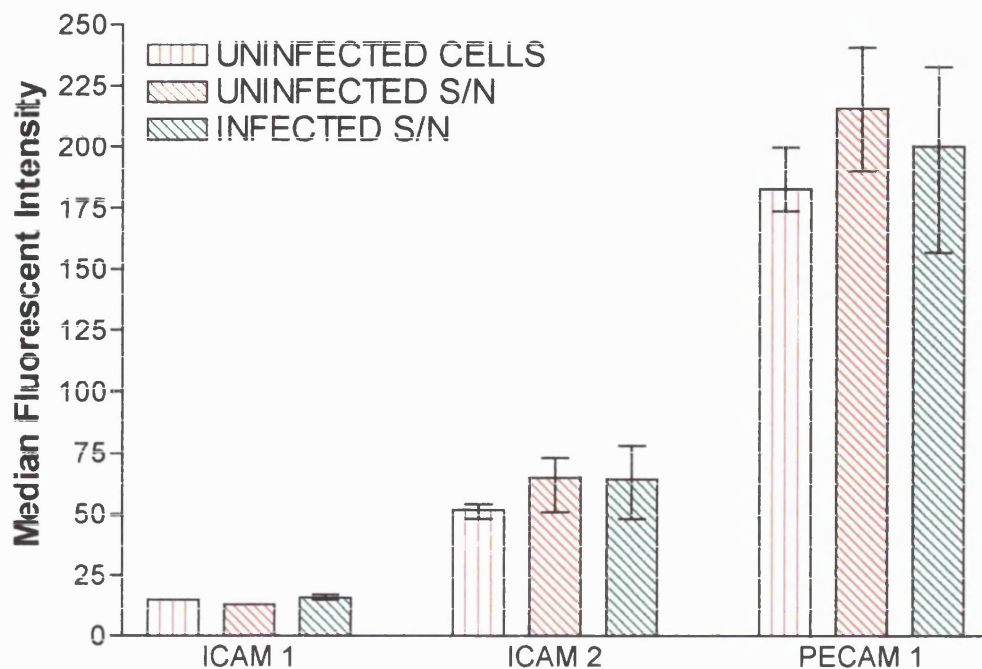


Figure 6.9.1. The effect of soluble factors produced following infection of endothelial cells with CMV on the expression of adhesion molecules.

Endothelial cells were inoculated at an MOI of 5 with C1FE and the culture supernatant was removed at day 2 post inoculation, any cell debris removed by centrifugation, and the supernatants passed through a 0.2 μ filter. Supernatants from uninfected endothelial cells were treated in a similar manner. The supernatants from uninfected or C1FE-infected cells (uninfected S/N and infected S/N, respectively) were then transferred to fresh uninfected endothelial cells, and the expression of ICAM-1, ICAM-2 or PECAM-1 was determined 3 days later, using flow cytometric techniques. In addition, the expression of these molecules was determined for uninfected endothelial cells which had been growing for 5 days in growth medium (uninfected cells). The data are expressed as the median fluorescent intensity of triplicate samples for a representative experiment. The error bars show the range of values obtained.

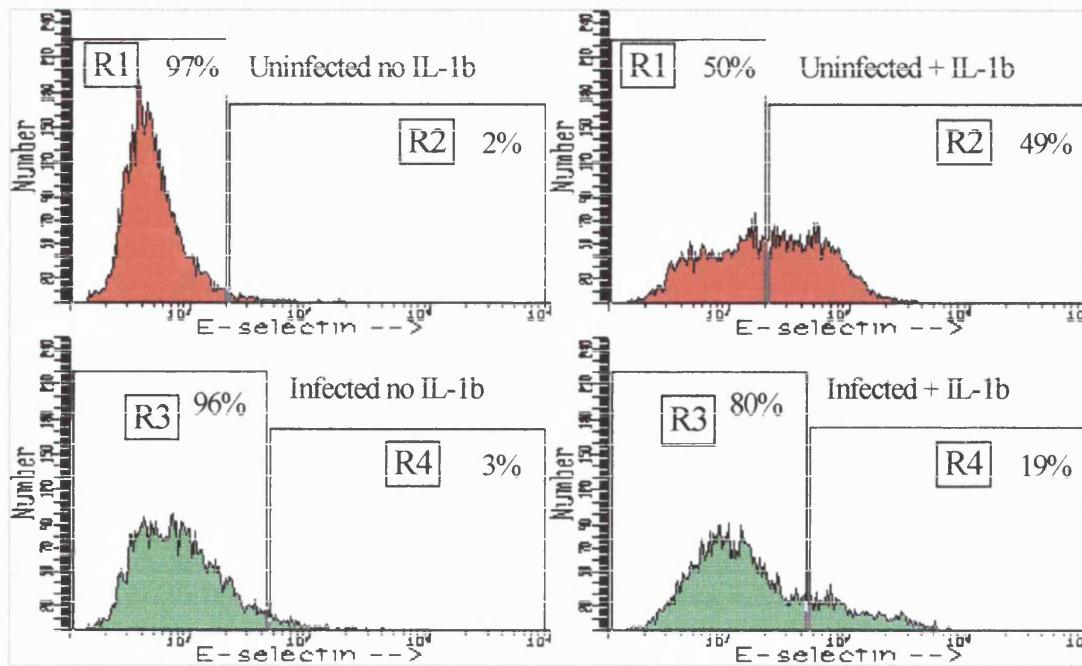


Figure 6.10.1. The effect of CMV infection on the expression of E-selectin at the surface of endothelial cells.

Endothelial cells were inoculated at an MOI of 4 with CMV strain C1FE, and at 4 days post inoculation, the cells were either treated for 4 hours with IL-1 β (+ IL-1b), or left untreated (no IL-1b). The expression of E-selectin was then determined at the cell surface, using flow cytometric techniques. Representative histogram profiles from a single experiment are shown for uninfected or C1FE-inoculated cells. Both the negative population (R1 & R3, uninfected and infected, respectively) or the positive population (R2 & R4, uninfected and infected, respectively) was determined by using the appropriate IgG1 isotype control antibody as a negative marker (data not shown). The percentage of cells contained within each population is shown. The ordinate represents the number of fluorescence events (scale 0-250), and the abscissa represents log₁₀ fluorescence intensity in arbitrary units (scale 10⁰-10⁴).

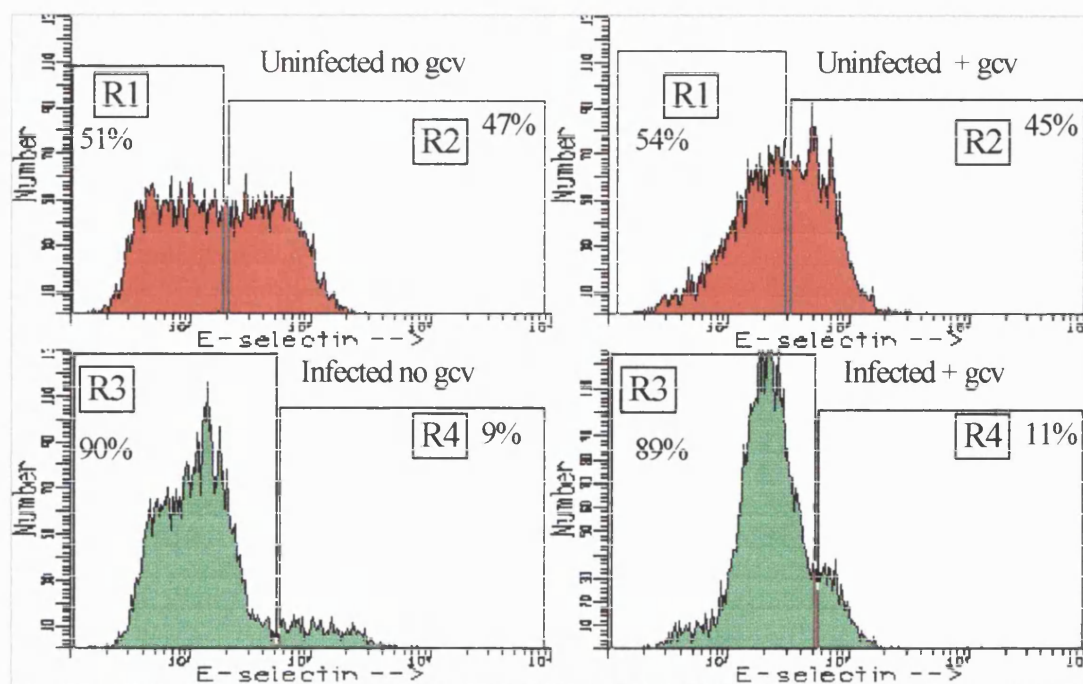


Figure 6.11.1. The effect of ganciclovir on the ability of CMV to alter the expression of E-selectin at the surface of IL-1 β stimulated endothelial cells.

Endothelial cells were inoculated at an MOI of 5 with CMV strain Toledo.E, and either treated with ganciclovir (+gcv) or left untreated (no gcv). At 4 days post inoculation, the cells were treated for 4 hours with IL-1 β , and the expression of E-selectin was then determined at the cell surface using flow cytometric techniques. Representative histogram profiles from a single experiment are shown for uninfected or C1FE-inoculated cells. Both the negative population (R1 & R3, uninfected and infected, respectively) or the positive population (R2 & R4, uninfected and infected, respectively) was determined by using the appropriate IgG1 isotype control antibody as a negative marker (data not shown). The percentage of cells contained within each population is shown. The ordinate represents the number of fluorescence events (scale 0-130), and the abscissa represents log₁₀ fluorescence intensity in arbitrary units (scale 10⁰-10⁴).

6.12. Discussion.

In this chapter, it was shown that the constitutive expression of ICAM-1 was significantly increased at the surface of endothelial cells following infection with two different strains of CMV, beginning at day 1 post infection and continuing through the duration of the study (day 5). Similar results were obtained following infection of fibroblasts with three other CMV strains. Conversely, the expression of LFA-3, which had previously been shown to increase at the surface of fibroblasts following infection with the laboratory strain AD169 (Grundy, Downes, 1993), was not altered on the surface of endothelial cells following infection with two 'endothelial tropic' strains of CMV, C1FE and Toledo.E. This was not a cell-type effect, nor was it due exclusively to the use of the endothelial tropic strains, because both the fibroblast-propagated Toledo strain, and the endothelial cell-propagated C1FE strain, failed to increase the expression of LFA-3 at the surface of fibroblasts. These results suggested that the up-regulation of LFA-3 was CMV strain dependent, and also demonstrated that the CMV-induced increase in ICAM-1 and LFA-3 expression involved different mechanisms, as the former effect was observed at the surface of both endothelial cells and fibroblasts, regardless of the strain used.

Increased expression of ICAM-1 has been detected at the surface of proximal tubular epithelial cells (van Dorp et al. 1993), lung fibroblasts (Ito et al. 1995; Grundy, Downes, 1993), and endothelial cells (Scholz et al. 1992) following infection with the AD169 strain of CMV. Furthermore, using a different clinical isolate to those employed here, it was shown that increased ICAM-1 expression in a mixed culture containing both uninfected and infected endothelial cells was exclusively located to those cells expressing CMV antigens (Sedmak et al. 1994b). A similar conclusion was reached when fibroblasts were inoculated with CMV strain AD169, because it was shown that neither virus-free supernatants nor UV-inactivated virus were capable of up-regulating the expression of the adhesion molecules ICAM-1 or LFA-3 (Craig, Grundy, 1996; Ito et al. 1995). In agreement with these findings, cell-free supernatants taken from C1FE-inoculated endothelial cells did not alter the expression of ICAM-1 during this study, although they did increase the expression of Class I MHC during the same experiments. Thus, cytokines which may have an effect

on ICAM-1 expression, such as IL-1, TNF- α or IFN- γ (Dustin et al. 1986) were not responsible for the observed increase in the expression of this molecule following infection of endothelial cells with CMV.

Cytokine-induced up-regulation of LFA-3 has not been reported for either fibroblasts or endothelial cells, and reports of cytokine-related effects in other cell types are rare; one report suggested that IL-4 can alter LFA-3 expression in Burkitt's lymphoma cell lines (Rousset et al. 1989), whilst a separate report showed that a combination of IFN- γ and TNF- α increased LFA-3 on epithelial cells (Kirby et al. 1993). However, it was shown that none of these cytokines were secreted following infection of fibroblasts with CMV strain AD169 (Craigden, Grundy, 1996; Duncombe et al. 1990), and thus it is extremely unlikely that any of these cytokines would be responsible for the up-regulation of LFA-3 following infection of fibroblasts seen here. Furthermore, the fact that two strains of CMV (Toledo and C1FE) failed to up-regulate LFA-3 expression on fibroblasts, and the fact that no up-regulation of this molecule was observed following inoculation of endothelial cells with CMV, would suggest that when LFA-3 expression was up-regulated, it was due to a direct virus-mediated effect, rather than being cytokine induced.

A number of other viruses can modulate the expression of ICAM-1, although only in a few cases has the mechanism responsible been attributed to a specific viral protein. Infection with Hepatitis B virus increased ICAM-1 expression on hepatocytes due to the transactivation of the ICAM-1 gene by the hepatitis B virus X protein (Hu et al. 1992; Volpes et al. 1992). However, this was not specific for ICAM-1 because the X protein was also capable of transactivating a number of other cellular genes, including the IL-8 gene (Mahe et al. 1991). ICAM-1 and LFA-3 expression on the T-lymphoblastoid line Jurkat was also up-regulated, in this case by the Tax1 protein of human T cell leukaemia virus type I (Tanaka et al. 1995). The latent membrane protein of Epstein Barr virus has also been shown to increase the expression of LFA-1 and ICAM-1 on B-lymphoblastoid lines (Wang et al. 1988). In contrast, increased adhesion molecule expression by the secretion of cytokines during a viral infection appears to be more commonly reported. ICAM-1 expression was enhanced on pulmonary epithelial cells infected with respiratory syncytial virus via IL-1 α

induction (Patel et al. 1995), whilst measles virus infection of astrocytes appeared to induce ICAM-1 by both a direct mechanism, and by the secretion of Type I interferon (Kraus et al. 1992). Interestingly, no increase in ICAM-1 expression was observed following measles virus infection of cerebral endothelium (Brankin et al. 1995). The induction of ICAM-1 expression on fibroblasts following infection with West Nile virus was shown to be due to a direct viral effect at the early stages of infection, and to a soluble factor at later time points (Shen et al. 1995), whereas the same virus was only able to induce the expression of this molecule by the former mechanism following infection of endothelial cells (Shen et al. 1997). These data suggested that the effect of some viruses on the expression of ICAM-1 changed according to the type of cell infected. In the studies presented here, increased ICAM-1 expression was not related to cell type, as CMV-infection of both fibroblasts and endothelial cells produced similar reproducible effects.

With regards to the constitutive expression of the other adhesion molecules studied here, both PECAM-1 and ICAM-2 were down-regulated at the surface of the inoculated endothelial cells, beginning at day 1 post infection and continuing through to day 5. Decreased expression of cellular genes following CMV infection has been reported previously, although in these cases, integrins rather than members of the immunoglobulin superfamily were studied. Thus, endothelial cell expression of $\alpha 5\beta 1$ and $\alpha 2\beta 1$, and fibroblast expression of $\alpha 1\beta 1$ was also decreased at the cell surface following infection with CMV (Warren et al. 1994b; Shahgasempour et al. 1997b). The expression of ICAM-2 at the surface of endothelial cells has been reported as being refractory to cytokine regulation (Nortamo et al. 1991), whereas PECAM-1 expression can be redistributed and down-regulated at the surface of endothelial cells by the combination of the cytokines IFN- γ and TNF- α (Rival et al. 1996; Romer et al. 1995). However, it was extremely unlikely that IFN- γ and TNF- α were responsible for the decrease observed in the present study, because the cellular sources of these cytokines are leukocytes, not endothelial cells (Sen, 1997; Tracey, 1997), and leukocytes were absent from the endothelial cell cultures. Furthermore, supernatants obtained from CIFE-infected cells failed to have any effect on the expression of ICAM-2 or PECAM-1, again suggesting that the secretion of cytokines from the

CMV-infected cells was not responsible for these effects. The ability of other viruses to alter the expression of these two adhesion molecules has not been extensively investigated. In the one reported case where PECAM-1 expression was modulated during a viral infection, an increase in the expression of PECAM-1 on murine corneal cells was observed following infection with HSV-1, and this was thought to be due to secretion by IFN- γ from infiltrating leukocytes (Tang, Hendricks, 1996). As stated above, this cytokine was unlikely to have been produced in the endothelial cultures used here, therefore the present study appears to be the first report indicating that direct viral modulation of these molecules can occur.

During the studies described here, it was shown that the addition of the antiviral drug ganciclovir did not prevent the decreased expression of either ICAM-2 or PECAM-1 molecules at the surface of inoculated endothelial cells, suggesting that they were selectively modulated by CMV immediate early or early genes. A similar conclusion was drawn for the effect on ICAM-1 expression, as ganciclovir also failed to prevent the up-regulation of this molecule at the surface of CMV-inoculated endothelial cells. However, in this latter case, increased expression of ICAM-1 at the surface of infected cells was both prolonged and enhanced in the presence of the drug, whereas no difference in the extent of down-regulation of ICAM-2 or PECAM-1 was observed when the inoculated cells were treated with the drug, or left untreated. A similar effect of ganciclovir on the expression of ICAM-1 at the surface of CMV-infected fibroblasts has previously been reported (Craig, Grundy, 1996). Although the CMV gene responsible for these effects was not identified in the present or the latter studies, the results described here suggest that ICAM-1, ICAM-2 and PECAM-1 expression was modulated by different mechanisms.

With regards to the expression of the endothelial cell molecule E-selectin, no induction of this molecule was observed at the surface of CMV inoculated endothelial cells in the absence of cytokine stimulation, a finding which is in agreement with some reports (Sedmak et al. 1994b; Scholz et al. 1992b) but in contrast to others (Shahgasempour et al. 1997a; Span et al. 1991a). In one of these studies, increased expression of E-selectin was attributed to the secretion of IL-1 by the virally infected cells (Span et al. 1991a). Although it has been shown that endothelial cells can

produce cytokines such as IL-1 α and IL-1 β after activation with various stimuli (Miossec et al. 1986), the majority of reports suggest that CMV does not induce the expression of IL-1. Thus, although the induction of IL-1 β mRNA by CMV IE1 genes has been reported in monocytes (Iwamoto et al. 1990), the actual protein was not secreted, a finding consistent with other studies involving monocytes or lymphocytes (Kapasi, Rice, 1988; Kline et al. 1994). Furthermore, IL-1 β has not been detected in the supernatants from AD169-infected fibroblasts or endothelial cells (Span et al. 1991a; Craigen, Grundy, 1996a), or from endothelial cells infected with a clinical isolate (Woodroffe et al. 1993); in fact, in this latter report, IL-1 β expression was suppressed until 24 hours post inoculation. Thus, the lack of E-selectin expression at the surface of CMV-inoculated endothelial cells observed during these studies is consistent with the finding that IL-1 β is not produced by CMV infected endothelial cells.

The addition of exogenous IL-1 β to uninfected endothelial cells transiently induced the expression of E-selectin, in agreement with previous findings (Bevilacqua et al. 1987; Bevilacqua et al. 1989). Following CMV-inoculation of endothelial cells, however, E-selectin was refractory to induction by IL-1 β . A similar effect on E-selectin, and VCAM-1 expression was observed following stimulation of CMV-infected endothelial cells with the cytokine TNF- α (Sedmak et al. 1994b). In contrast, it has been shown that both E-selectin and VCAM-1 up-regulation at the surface of endothelial cells following infecting with the flavivirus, West Nile Virus, can be further increased following the addition of these cytokines (Shen et al. 1997). Cytokines could also mediate increased VCAM-1 expression on simian immunodeficiency virus-infected cerebral cells (Sasseville et al. 1994), and a small increase in both ICAM-1 and E-selectin expression was observed following infection of endothelial cells with Influenza virus Type A (Colden-Stanfield et al. 1993). Thus, CMV appears to selectively prevent the expression of E-selectin (and VCAM-1) in response to the inflammatory cytokines, IL-1 β and TNF- α , a phenomenon which is not a general effect of virus infection.

The inability of CMV-inoculated endothelial cells to express E-selectin following stimulation with IL-1 β was unlikely to have occurred due to CMV-induced

secretion of the IL-1 receptor antagonist (Kline et al. 1994), as endothelial cells are the only cell types to date which do not appear to produce this factor (Tocci, Schmidt, 1997). Furthermore, the fact that the expression of this molecule was refractory to stimulation by another cytokine, TNF- α (Sedmak et al. 1994b) suggests that the manner by which CMV inhibits E-selectin (and VCAM-1) induction was not at the cytokine receptor level, because TNF- α and IL-1 β are structurally unrelated and bind to separate receptors (Tocci, Schmidt, 1997). Thus, the pathway of E-selectin regulation which is affected by CMV infection of endothelial cells has to be common to both TNF- α and IL-1 β . Interestingly, it has been shown that the elevation of cAMP within endothelial cells inhibited the TNF- α mediated induction of E-selectin and VCAM-1, although ICAM-1 expression was concomitantly increased (Pober et al. 1993; Ghersa et al. 1994). This pattern of altered adhesion molecule expression, as stated previously, was observed in the present study following infection of endothelial cells with CMV, and CMV infection of fibroblasts is associated with increased cAMP levels (Albrecht et al. 1990). A second possible block, however, is at the level of transcription. The E-selectin promoter has been shown to possess 3 NF- κ B sites, whilst the VCAM-1 promoter possesses 2 such sites (Neish et al. 1995; Collins et al. 1995). In contrast, one NF- κ B site has been shown to be required for ICAM-1 expression, and this latter site is 'atypical', as are sites on the IL-8 and GM-CSF genes (Ledebur, Parks, 1995). Thus, these latter three genes preferentially bind c-REL/p65 heterodimers, as opposed to p65 homodimer or p65/p50 heterodimer binding in the former cases (Ledebur, Parks, 1995). Hence, different genes may be activated by different transcription dimers, and CMV may preferentially increase the levels of one transcription factor over another. The elucidation of the mechanisms involved in the alterations to endothelial cell adhesion molecule expression following infection with CMV was, however, outside the scope of this study, and thus warrants further investigation.

In summary, infection of endothelial cells and fibroblasts with CMV alters the expression of a variety of adhesion molecules at the cell surface, some being selectively up-regulated by the virus, whilst others are down-regulated. Although these alterations might be mediated by different mechanisms, the CMV IE or early

gene products are strongly implicated in promoting these events, as the addition of the antiviral drug ganciclovir failed to prevent the alterations in all cases. The functional consequences of the changes to adhesion molecule expression are studied in the subsequent chapter, and the possible *in vivo* relevance discussed in Chapter 8.

CHAPTER 7.

THE EFFECT OF CMV INFECTION OF ENDOTHELIAL CELLS ON LEUKOCYTE ADHERENCE AND TRANSENDOTHELIAL CELL MIGRATION.

7.1. Introduction.

The recruitment of appropriate subsets of leukocytes to the site of infection contributes to the elimination of an invading pathogen. However, inappropriate leukocyte recruitment may contribute to chronic inflammatory disorders and immunopathology (Luscinskas, Gimbrone, Jr. 1996). One such disorder, which appears to be a result of such recruitment, is the mononuclear cell endothelialitis associated with both chronic rejection (Hruban et al. 1990; Billingham, 1994) and CMV infection (Koskinen et al. 1994; Paavonen et al. 1992).

In the previous chapter, it was shown that infection with CMV led to alterations in both the constitutive and inducible expression of adhesion molecules at the surface of endothelial cells. This raised the prospect that the altered endothelium could ultimately affect leukocyte adherence and/or leukocyte recruitment to the vicinity of the CMV-infected endothelial cell. In order to address these possibilities, the ability of ^{51}Cr -labelled leukocytes (neutrophils, monocyte-depleted peripheral blood mononuclear cells (PBL), or T-lymphocytes) to adhere to, or migrate through endothelial cells which had been infected with CMV (strain C1FE) was determined. No other virus strain was tested because it had already been established that Toledo.E did not differ from C1FE in the ability to alter the endothelial cell adhesion molecule expression, and CMV strain AD169, as explained previously, does not replicate well in this cell type. In addition, the ability of C1FE-inoculated endothelial cells to transmit the virus to neutrophils was investigated, as both these cell types have been implicated in the dissemination of CMV.

7.2. The ability of neutrophils to adhere to endothelial cells following infection with CMV.

In order to determine whether CMV infection of endothelial cells resulted in altered neutrophil adherence, neutrophils were first purified from healthy donors using a double density gradient, and then labelled with ^{51}Cr . Endothelial cells were inoculated (MOI of 2 or 4) with CMV strain C1FE, and the subsequent binding of ^{51}Cr -labelled neutrophils to the unstimulated endothelium was determined, using a gamma counter. All the assays were carried out at 4 days post inoculation, as the maximum alterations in the expression of the adhesion molecules were consistently

observed at this time point (Chapter 6). In addition, uninfected or C1FE-inoculated endothelial cells were also treated with the cytokine IL-1 β for 4 hours prior to the neutrophil adhesion assay, to determine the effect of CMV infection on the ability of neutrophils to bind to activated endothelium. The data from representative experiments are shown in Figure 7.2.1.

In two separate experiments performed in triplicate (n=6), the results which were observed following the addition of neutrophils to the uninfected endothelial cells revealed that only a small proportion of these leukocytes (median 1.3%, range 0.6%-3.2%) were able to form an adhesive interaction with the unstimulated endothelial cells. The binding of neutrophils to the unstimulated C1FE-inoculated endothelial cells, however, was significantly increased (median 2.7%, range 2.3%-5.6%) with respect to the number of neutrophils which bound to the uninfected cells (p=0.05, n=6), when the former cells were showing 25-30% CMV late cytopathic effect. In order to determine whether an higher infection rate led to even greater neutrophil binding, the proportion of infected endothelial cells was increased to 60-65% (using an higher viral dose), whilst still maintaining the integrity of the endothelial cell monolayer. Once again, there was a significant increase (p=0.01, n=9, based on the analysis of three experiments performed in triplicate) in neutrophil binding to the unstimulated C1FE-inoculated endothelial cells when compared to the untreated uninfected counterparts (median 1.3%, range 0.75%-2.3% and median 2.9%, range 2%-9%, uninfected and infected, respectively). However, there was no significant difference (n=6, based on two experiments performed in triplicate) between neutrophil adherence to the C1FE-inoculated endothelial cells at the two viral doses. This low level of adhesion was not due to a limitation in the number of leukocytes which were added, because stimulation of the endothelial cell monolayers with IL-1 β greatly increased leukocyte binding to these uninfected endothelial cells in both cases, as indicated below. Indeed, the optimal cell concentrations employed during these studies was first determined using IL-1 β -stimulated endothelial cells (data not shown).

The addition of IL-1 β to both uninfected and C1FE-inoculated monolayers (at both viral doses) prior to the addition of the neutrophils greatly increased the adhesive

interaction between the two cell types, when compared to the unstimulated counterparts (Figure 7.2.1). However, when the percentage of neutrophils which bound to the cytokine-stimulated uninfected endothelial cells were compared to the percentage of neutrophils which bound to the C1FE-inoculated equivalent, an interesting pattern was revealed (Figure 7.2.1). In two separate experiments performed in triplicate, significantly less ($p=0.05$, $n=6$) neutrophil binding to the C1FE-inoculated (MOI of 2) endothelial cells was observed, when compared to the number of neutrophils which bound to similarly treated, but uninfected endothelial cells (median 13.3%, range 11%-32% and median 11.9%, range 8.8%-17%, uninfected and infected, respectively). At the higher viral dose (MOI of 4), a similar result ($p=0.01$, $n=9$, based on three experiments performed in triplicate) was obtained following cytokine stimulation of the endothelial cells (median 11%, range 10.2%-12.7% and median 5.4%, range 1.3%-9.1%, uninfected and infected, respectively).

Finally, when the binding of the neutrophils to the uninfected or to the infected endothelial cells was observed using a light microscope, it was found that very few cells adhered to the uninfected cells in the absence of cytokine stimulation (Figure 7.2.2). In contrast, the neutrophils appeared to form adhesive clusters around enlarged endothelial cells which were present following inoculation with CMV (Figure 7.2.2). After the endothelial cells had been treated with IL-1 β for 4-6 hours, the neutrophils bound to both the uninfected and to the infected endothelial cells in an apparently indiscriminate fashion (Figure 7.2.2). These visual observations are consistent with results of the adhesion assays. Thus it appeared that infection with CMV enhanced the adherence of neutrophils to unstimulated endothelial cells, but decreased the ability of the same leukocyte type to adhere to cytokine stimulated endothelial cells, when compared to the uninfected counterparts.

7.3 The ability of peripheral blood lymphocytes (PBL) to adhere to endothelial cells following infection with CMV.

In order to test whether CMV infection of endothelial cells resulted in altered PBL adherence, mononuclear cells were isolated from healthy donors using a single density gradient, the monocytes removed through adherence to plastic, and the non-adherent fraction (PBL) labelled with ^{51}Cr .

Endothelial cells were inoculated (MOI = 2 or 4) with C1FE, and the ability of ^{51}Cr -labelled, monocyte-depleted PBL to adhere to endothelial cell monolayers was determined, using a gamma counter. In addition, both C1FE-inoculated and uninfected endothelial cells were stimulated with IL-1 β for 1 day, to determine whether PBL showed altered binding to activated endothelium. The data from representative experiments are shown in Figure 7.3.1.

In two separate experiments performed in triplicate, the results which were observed following the addition of PBL onto confluent monolayers of uninfected endothelial cells indicated that there was limited binding to the unstimulated endothelial cells (median 11.1%, range 6%-13.2%). However, there was a small but insignificant increase in the amount of PBL binding to the unstimulated C1FE-inoculated endothelial cells (median 12.4%, range 7%-14.2%) when compared to the similarly untreated uninfected cells (n=6). These results were obtained using a confluent endothelial cell monolayer which was showing 25-30% CMV late cytopathic effect (MOI of 2). When the proportion of infected endothelial cells displaying this late cytopathic effect was increased to 60-65% (MOI of 4), there was a small but significant increase (p=0.05, n=9, based on the analysis of three experiments performed in triplicate) in the number of PBL which bound to the untreated C1FE-inoculated endothelial cells, when compared to the similarly untreated but uninfected endothelial cells (median 14%, range 9.2%-16.9% and 15.7%, range 11.5%-19.2%, uninfected and infected, respectively). Thus, at the higher viral dose, a slightly greater effect on PBL binding was observed.

The addition of IL-1 β to the uninfected endothelial cell monolayer one day prior to the addition of the PBL greatly ^{increased} the adhesive interaction between the two cell types (Figure 7.3.1). However, the addition of this cytokine to the C1FE-inoculated endothelial cells only significantly (p=0.05, n=6, based on the analysis of two experiments performed in triplicate) increased PBL binding at the lower (MOI of 2), but not the higher (MOI of 4, n=9, based on the analysis of three experiments performed in triplicate) viral dose. However, when the percentage of PBL which bound to the IL-1 β treated uninfected cells were compared to the percentage which

bound the C1FE-inoculated equivalent, a significantly lower level of PBL binding to the latter was observed, when either a MOI of 2 ($p=0.05$, $n=0.6$, based on the analysis of two experiments performed in triplicate), or a MOI of 4 ($p=0.01$, $n=9$, based on the analysis of three experiments performed in triplicate) was used. At the higher viral dose, this effect was more marked (median 25.4%, range 19.1%-28.2% and median 11.7%, range 9.7%-23.8%, uninfected and infected, respectively) than when the lower viral dose was used (median 23.5%, range 19.8%-27.8% and median 18.3%, range 13.8%-22.7%, uninfected and infected, respectively). Thus it appeared that infection with CMV slightly enhanced the adherence of PBL to unstimulated endothelial cells, and more strongly reduced the ability of the same leukocyte type to adhere to cytokine stimulated endothelial cells.

Finally, when the binding of the PBL to the uninfected or to the infected endothelial cells was observed using a light microscope, it was found that a moderate number of PBL adhered to both the uninfected and the infected endothelial cells in the absence of cytokine stimulation in an apparently indiscriminate fashion (Figure 7.3.2). After the endothelial cells had been treated with IL-1 β for one day, the number of PBL which bound to both the uninfected and to the infected endothelial cells appeared to increase, although there was no obvious change to the indiscriminate pattern of binding which had been observed previously (Figure 7.3.2). These observations are consistent with the results from the adhesion assays. Thus it would appear that infection with CMV slightly increased the adherence of PBL binding to unstimulated endothelial cells, but decreased the ability of the same leukocyte type to adhere to cytokine activated endothelial cells, when compared to the uninfected counterpart. These results are therefore similar to those obtained for the neutrophil studies described above.

7.4. The ability of neutrophils to migrate through CMV infected endothelial cells.

The ability of neutrophils to transmigrate across endothelial cell monolayers following infection with CMV was investigated next. Endothelial cells were inoculated (MOI of 4) with CMV strain C1FE, and seeded onto Transwell filters at 3 days post inoculation. The ability of ^{51}Cr -labelled neutrophils to migrate through

these confluent endothelial cell monolayers was determined the following day (4 days post inoculation), using a gamma counter. In some cases, uninfected endothelial cells were treated with IL-1 β prior to the migration assay, and served as a control. In addition, the barrier function of the endothelial cell monolayers was tested, and in each case was shown to permit <5% iodinated bovine serum albumin to pass through, indicating that any results obtained were not due to altered permeability of the endothelial cell monolayers following infection with CMV. Analysis of cytospin preparations of the C1FE-inoculated endothelial cells revealed that 40-60% of the cells were expressing CMV IEA and late (gB) antigen at four days post inoculation. Infection rates above this level resulted in a loss of endothelial cell monolayer integrity. Due to the limited number of endothelial cells which were available for use in these assays, the effect of IL-1 β on the ability of neutrophils to migrate through the C1FE-inoculated endothelial cells was not studied during these experiments. A representative experiment of the data obtained is shown in Figure 7.4.1.

In three separate experiments performed in triplicate, the results which were obtained when neutrophils had been added to uninfected endothelial cell monolayers showed that within a two hour incubation period, few neutrophils (median 2.3%, range 0.1%-3.9%) had transmigrated through the endothelial cell monolayer. However, the number of neutrophils which were found to transmigrate during the same period of time was significantly increased ($p=0.01$, $n=9$) following C1FE-inoculation of the endothelial cells (median 6.3%, range 4.8%-12.8%). Thus, inoculation of endothelial cells with CMV (strain C1FE) enhanced the transendothelial cell migration of neutrophils, when compared to the uninfected cells.

7.5. The ability of T-lymphocytes to transmigrate through CMV infected endothelial cells.

The PBL used in the above studies contained a mixed population of B-lymphocytes, T-lymphocytes, and natural killer cells. An attempt was made to monitor the migration of these populations separately, as preferential migration of one population over the others might occur. The ability of T-lymphocytes to transmigrate across endothelial cell monolayers following infection with CMV was therefore investigated, using similar methods to those described above. Unfortunately, the other

populations were not studied here, as these assays had not yet been developed. Endothelial cells were inoculated (MOI of 4) with CMV strain C1FE, seeded onto Transwell filters, and ^{51}Cr -labelled T-lymphocytes were added the following day (4 days post inoculation). The percentage of T-lymphocytes which migrated through the endothelial cells was determined 18-24 hours later, as the migration of lymphocytes takes place more slowly than migration of neutrophils. In these experiments, all monolayers maintained an intact barrier function (<5% transmission of iodinated bovine serum albumin), and 40-60% of the C1FE-inoculated endothelial cells expressed CMV IEA and late (gB) antigen at four days post inoculation. Due to the limited number of endothelial cells which were available for use in these assays, the effect of IL-1 β on the ability of T-lymphocytes to migrate through the C1FE-endothelial cells was not consistently studied during these experiments. A representative experiment of the data obtained is shown in Figure 7.5.1.

In two separate experiments performed in triplicate, the results which were obtained when the T-lymphocytes were added to the uninfected endothelial cells showed that, within an incubation period of 18-24 hours, a moderate number of T-lymphocytes (median 11.3%, range 0.6-15.4%) transmigrated through the endothelial cell monolayer. During these same assays, a significant increase ($p=0.05$, $n=6$) in the percentage of T-lymphocytes which migrated through the infected endothelial cells (median 26.45%, range 1.8%-28.1%) was observed. Thus, inoculation of endothelial cells with CMV (strain C1FE) enhanced the transendothelial cell migration of T-lymphocytes, when compared to the uninfected cells.

7.6. The ability of culture supernatants to promote leukocyte transmigration through endothelial cells.

A possible explanation for the increase in transendothelial cell migration observed in the studies presented above (sections 7.4 and 7.5) was that CMV infection of endothelial cells led to the secretion of soluble chemoattractant factors. Therefore, the ability of culture supernatants from C1FE-inoculated or uninfected endothelial cells to promote either neutrophil or T-lymphocyte transmigration was studied. Supernatants were obtained from endothelial cells which had been infected with CMV for 6 hours, a time which had previously been shown to produce

chemoattractant soluble factors in fibroblasts (Craigien et al. 1997). Furthermore, at this time, no virion replication would have occurred, nor would the nutrients present in the medium have to be replaced in order to maintain endothelial cell growth.

The results from these experiments indicated that the supernatants from the C1FE-inoculated endothelial cells were able to increase the percentage of both neutrophils or T-lymphocytes which migrated through uninfected, unstimulated endothelial cells (Figure 7.6.1). Moreover, when these supernatants were incubated with fresh, uninfected endothelial cells for the same period of time as used in the migration assay, the expression of the adhesion molecules ICAM-1, ICAM-2, PECAM-1 and E-selectin remained the same (Figure 7.6.2). Therefore, these results suggested that changes to adhesion molecule expression were not responsible, but that a chemoattractant soluble factor was responsible, at least in part, for the increased transmigration of both neutrophils and T-lymphocytes through CMV-infected endothelial cells.

7.7. Transmission of CMV to neutrophils.

Neutrophils have been implicated in the dissemination CMV within immunocompromised individuals, although the source of the virus has not been elucidated. Therefore, as the above results suggested that CMV-infected endothelial cells both secrete factors which are chemotactic for neutrophils, and also promote increased adhesion to unstimulated endothelial cells, the ability of endothelial cells to transmit the virus to the neutrophils was investigated. Endothelial cells were inoculated (MOI of 4) with CMV strain C1FE and cultured for 5-6 days until extensive cytopathic effect was observed. The neutrophils were then co-cultured with the C1FE-infected cells for approximately 16 hours, after which they were again separated from the endothelial cells. The neutrophils were then analysed for the presence of either CMV IEA, which represents initial viral replication, or the structural protein pp65, which in the absence of CMV IEA expression, represents virion uptake.

The CMV pp65 protein could be routinely detected in neutrophils which were co-cultured with CMV-inoculated endothelial cells at neutrophil:endothelial cell ratios of 50-100:1. This CMV-specific antigen was localised within the characteristic

multi-lobed nucleus of the neutrophils, although a minority of the cells exhibited weaker staining in the cytoplasm (Figure 7.7.1). In some cases, a few endothelial cells were also present in the separated preparations, but these could clearly be distinguished from the neutrophils due to their larger size, and by the fact that pp65 was predominantly expressed in the cytoplasm, presumably reflecting the synthesis of new pp65 protein (Figure 7.7.1). In contrast, in the same experiments, CMV IEA expression could not be demonstrated in the neutrophils (data not shown). Parallel co-culture experiments with neutrophils and infected fibroblasts failed to transmit pp65 to the neutrophils under the same conditions (data not shown). Therefore, it was concluded that infected endothelial cells were able to transmit pp65 to neutrophils, probably through the uptake of virion structural proteins, but that viral replication was not initiated in the neutrophil.

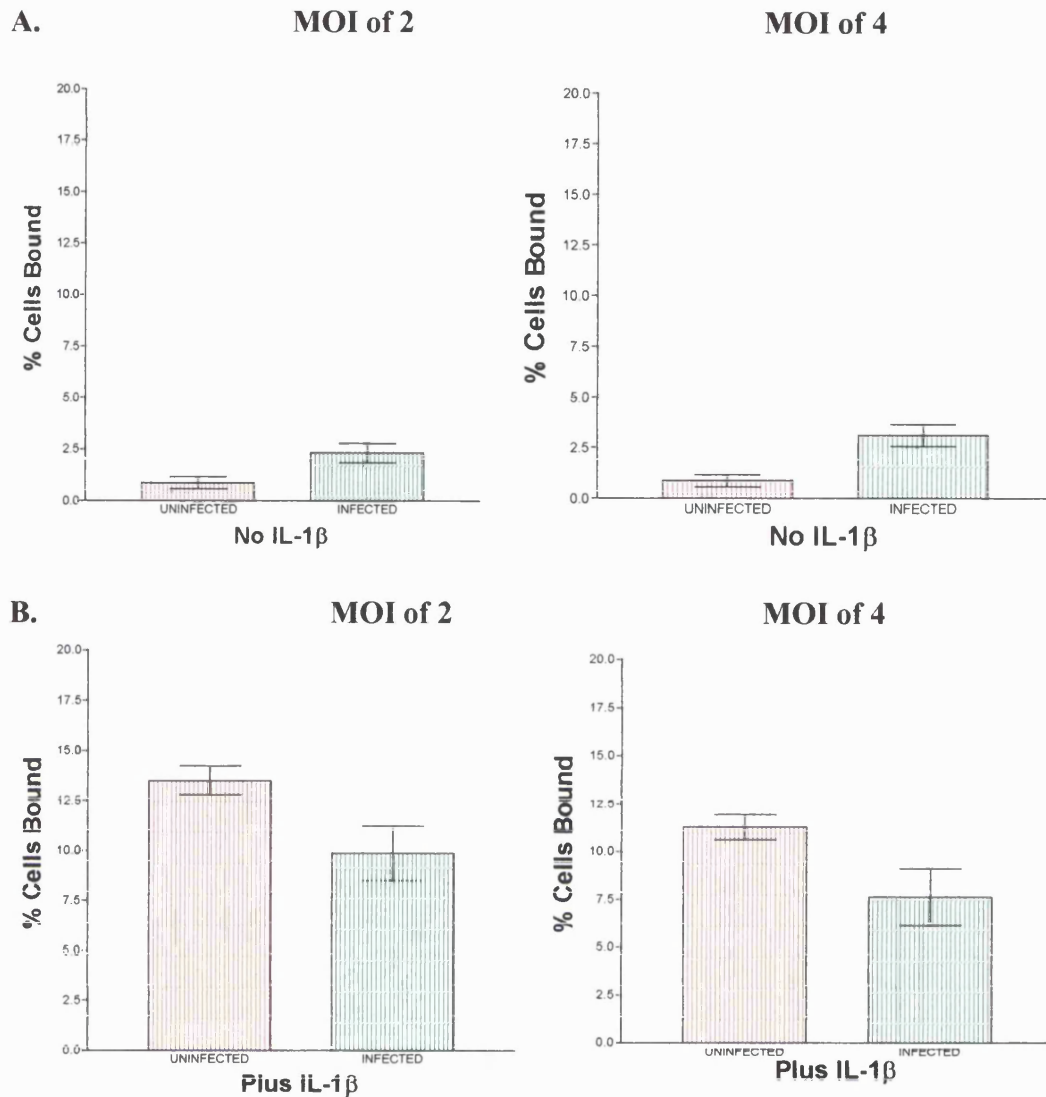


Figure 7.2.1. The adhesion of neutrophils to endothelial cells following infection with CMV.

Endothelial cells were inoculated with CMV strain C1FE to give 30% (MOI of 2) or 65% (MOI of 4) infection at 4 days post inoculation. 1×10^5 ^{51}Cr -labelled neutrophils were added per 4×10^4 endothelial cells, and the percentage of neutrophils bound was determined using a gamma counter. The data represent the median percentage of cells bound for a representative experiment performed in triplicate. In part A, the data were obtained for unstimulated endothelial cells (no IL-1 β). In part B, the data were obtained after the endothelial cells had been stimulated for 4-6 hours with 10ng/ml of IL-1 β prior to the addition of the neutrophils (plus IL-1 β). The error bars show the range of values obtained.

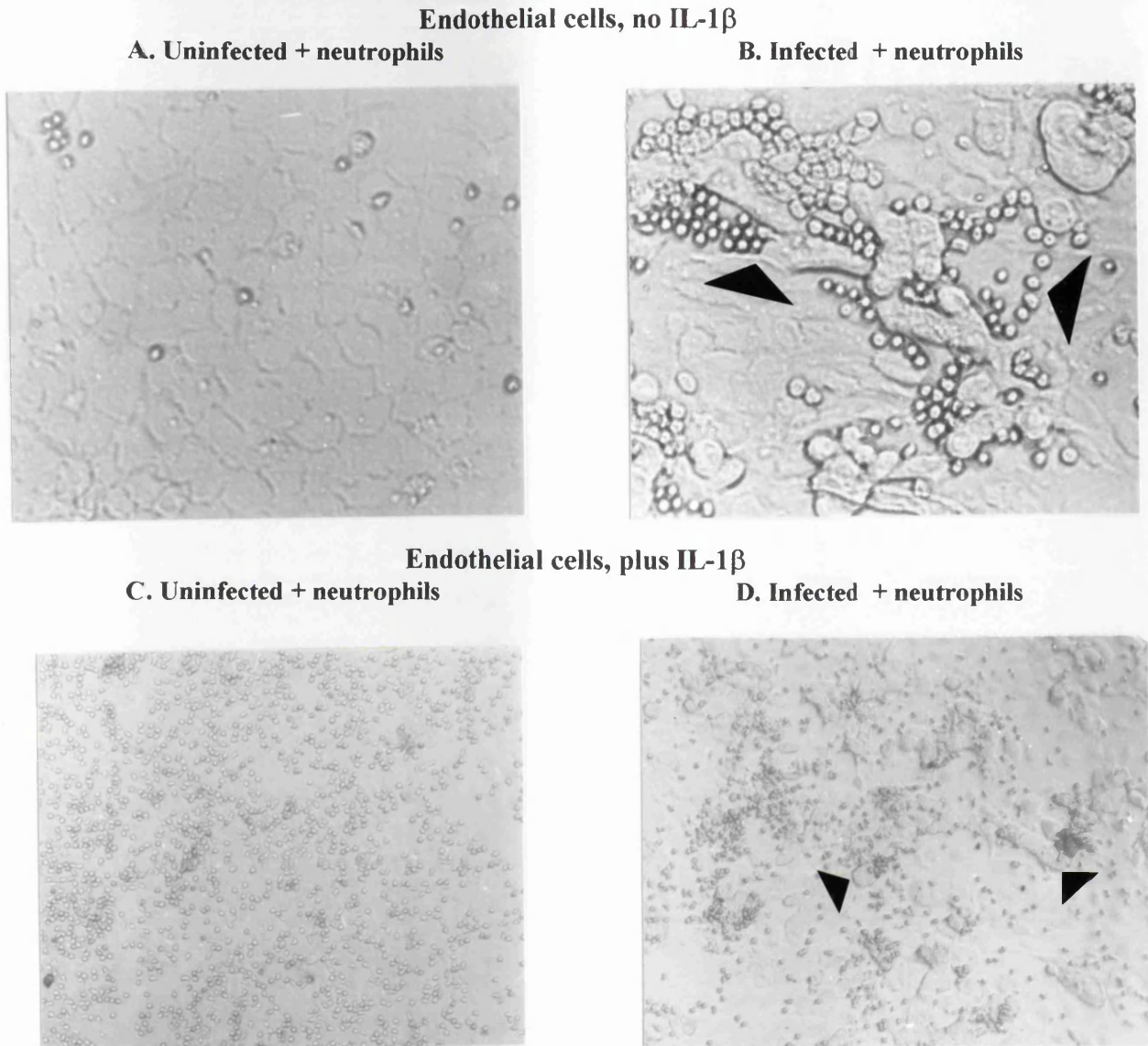


Figure 7.2.2. Photomicrographs showing neutrophil adhesion to CMV infected or uninfected endothelial cells.

Endothelial cells were inoculated with CMV strain C1FE to give 30% infection at 4 days post inoculation, and either treated with IL-1 β for 4 hours, or left untreated. Neutrophils were then added for 2 hours, and any unbound neutrophils were removed by aspiration. In parts A & B, the photomicrographs depict neutrophils bound to either uninfected (A) or C1FE-infected endothelial cells (B) in the absence of IL-1 β stimulation (magnification x100). In parts C & D, the photomicrographs depict neutrophils bound to either uninfected (C) or C1FE-infected endothelial (D) cells following 4 hours of IL-1 β stimulation (magnification x 40). The arrows indicate the accumulation of neutrophils around enlarged cells (B & D).

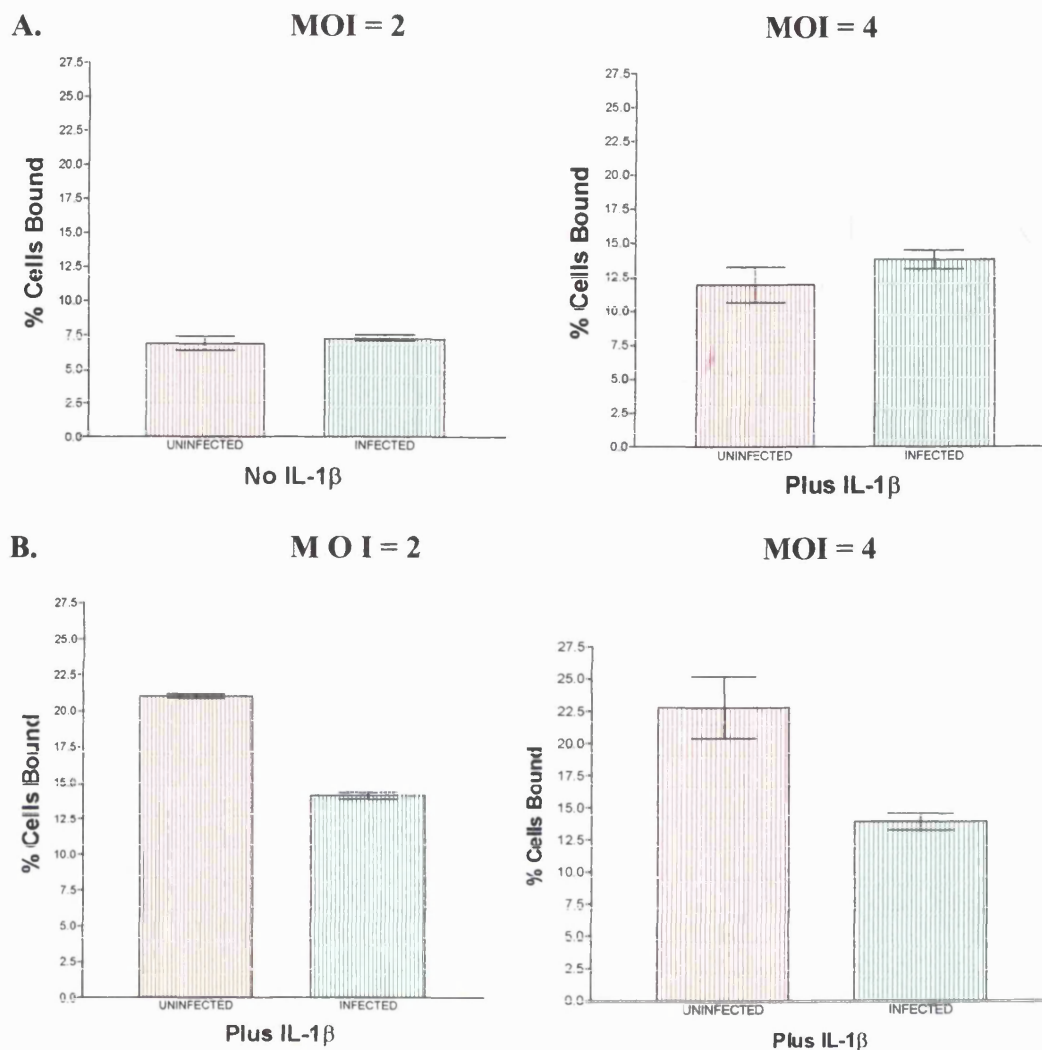


Figure 7.3.1. The adhesion of PBL to endothelial cells following infection with CMV.

Endothelial cells were inoculated with CMV strain C1FE to give 30% (MOI of 2) or 65% (MOI of 4) infection at 4 days post inoculation. 1×10^6 ^{51}Cr -labelled PBL were added per 4×10^4 endothelial cells, and the percentage of PBL bound was determined using a gamma counter. The data represent the median percentage of cells bound for a representative experiment performed in triplicate. In part A, the data were obtained for unstimulated endothelial cells (no IL-1 β). In part B, the data were obtained after the endothelial cells had been stimulated for 1 day with 10ng/ml of IL-1 β prior to the addition of the PBL (plus IL-1 β). The error bars show the range of values obtained.

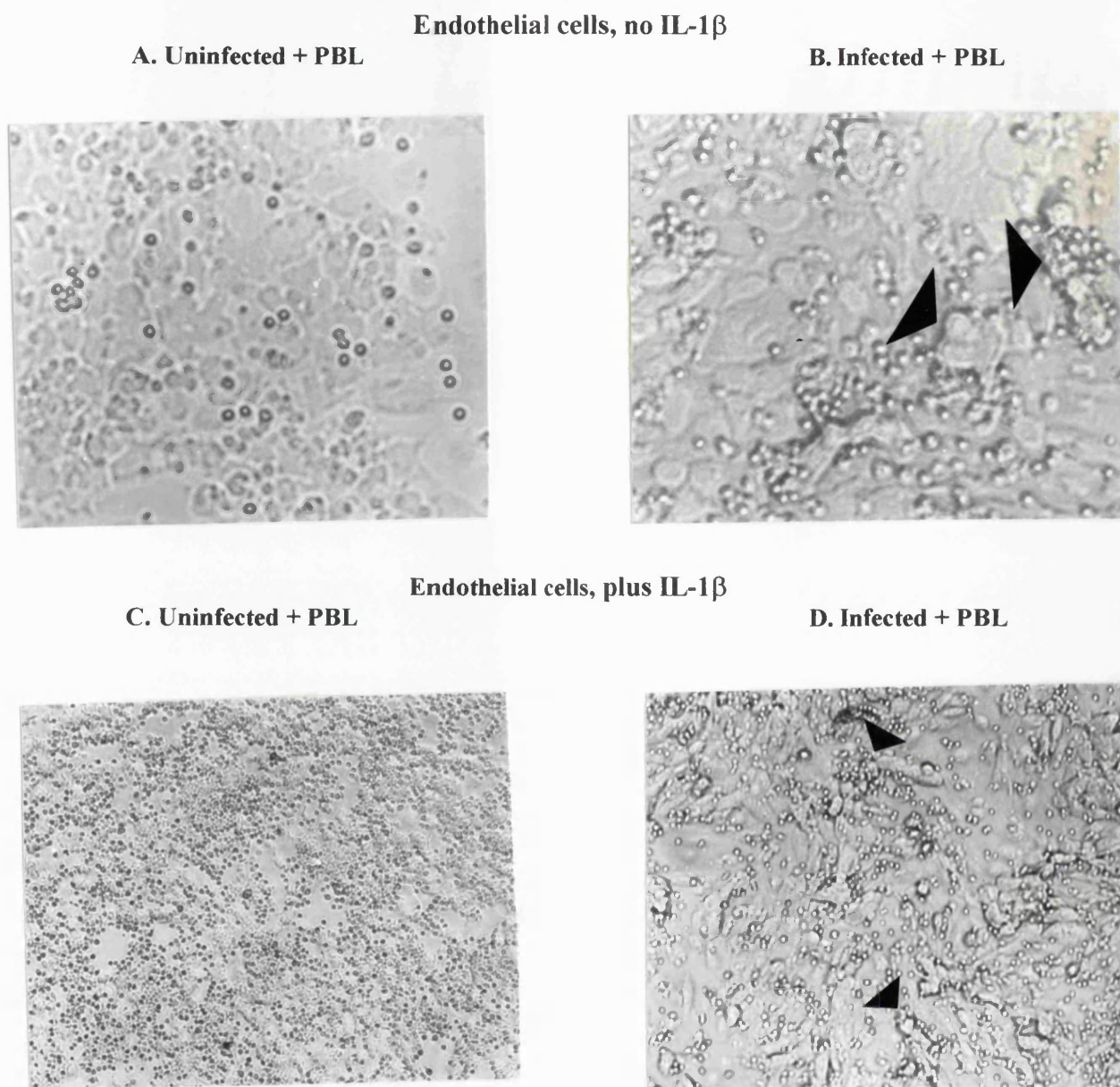


Figure 7.3.2. Photomicrograph of PBL adhesion to CMV infected or uninfected endothelial cells.

Endothelial cells were inoculated with CMV strain C1FE to give 30% infection at 4 days post inoculation. The cells had either been treated with IL-1 β for 1 day, or left untreated. PBL were added for 2 hours, and any unbound PBMC were removed by aspiration. In parts A & B, the photomicrographs depict PBL bound to either uninfected (A) or C1FE-infected endothelial cells (B) in the absence of IL-1 β stimulation (magnification x100). In parts C & D, the photomicrographs depict PBL bound to either uninfected (C) or C1FE-infected endothelial cells (D) following 1 day of IL-1 β stimulation (magnification x 40). The arrows indicate the accumulation of PBL around the enlarged cells (B & D).

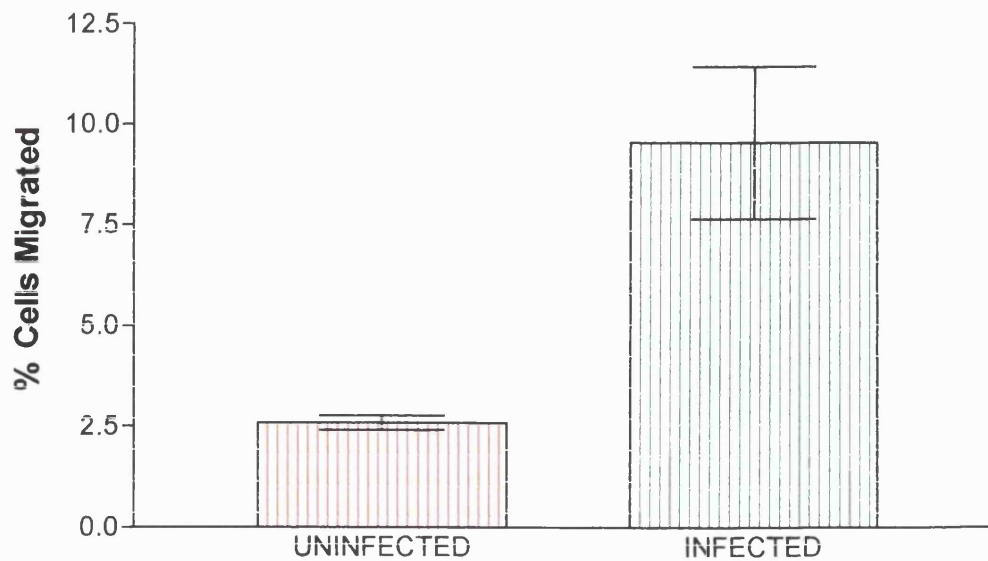


Figure 7.4.1. Neutrophil transmigration through endothelial cells infected with CMV.

Transwell filters coated with C1FE-inoculated (MOI of 4) or uninfected endothelial cells were added to wells containing RPMI medium + 10% FCS. 1×10^5 ^{51}Cr -labelled neutrophils were added to the upper chamber, and the percentage of neutrophils which migrated through the endothelial cell monolayer into the lower chamber was determined after 2 hours, using a gamma counter. The graph shows the median percentage migration for a representative experiment performed in triplicate. The error bars show the range of values obtained.

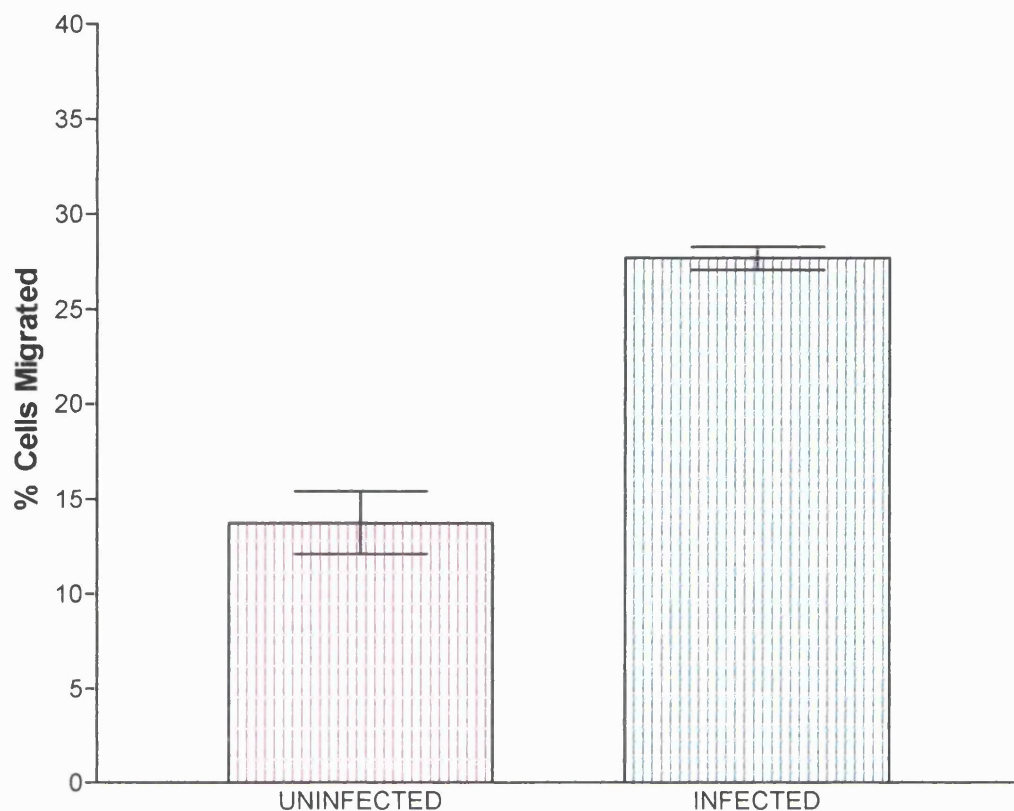
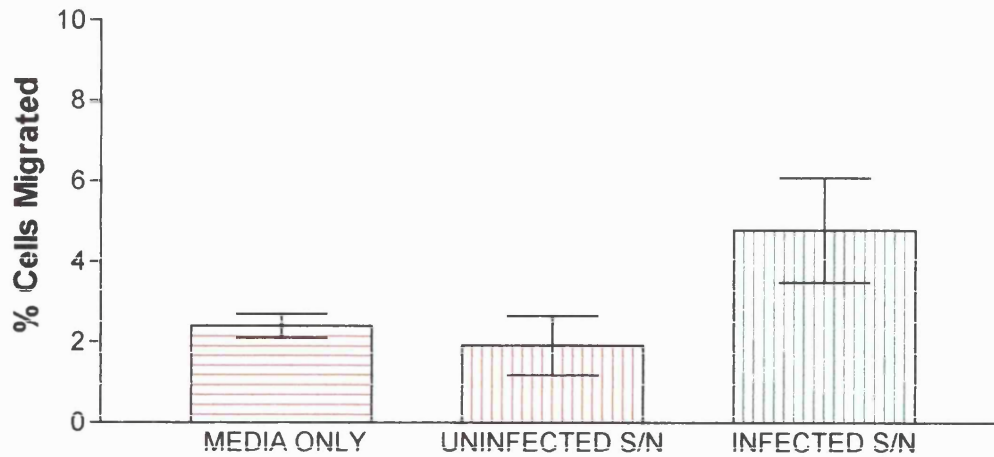


Figure 7.5.1. T-lymphocyte migration through endothelial cells infected with CMV.

Transwell filters coated with C1FE-inoculated (MOI of 4) or uninfected endothelial cells were added to wells containing RPMI medium + 10% FCS. 1×10^6 ^{51}Cr -labelled T-lymphocytes were added to the upper chamber, and the percentage of T-lymphocytes which migrated through the endothelial cell monolayer into the lower chamber was determined after 18 - 24 hours, using a gamma counter. The graph shows the median percentage migration for a representative experiment performed in triplicate. The error shows the range of values obtained.

A. Neutrophils.



B. T-lymphocytes.

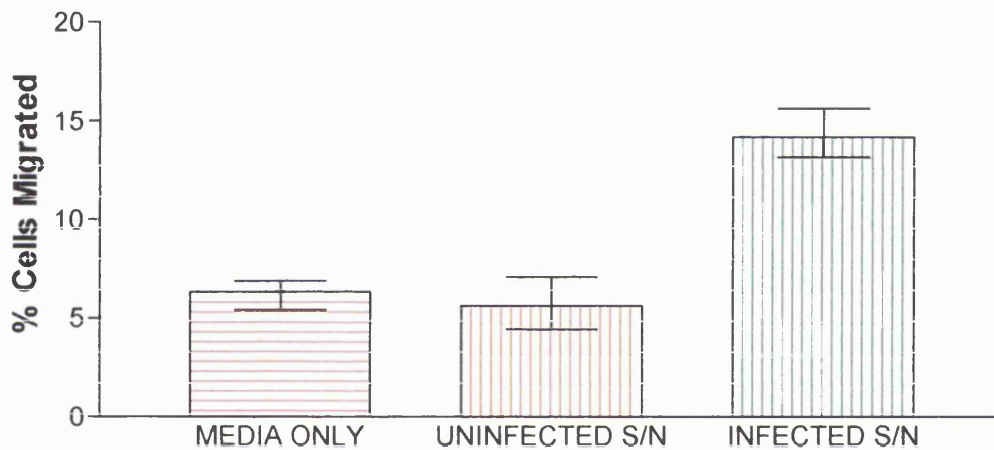


Figure 7.6.1. The effect of soluble factors on transendothelial migration following infection of endothelial cells with CMV.

Supernatants were harvested from cultures of C1FE-infected or uninfected endothelial cells at 6 hours post inoculation. Uninfected endothelial cells were grown on TransWell filters, and placed over the supernatants (S/N) or fresh growth media (media only). Migration of Cr -labelled neutrophils (part A) or T-lymphocytes (part B) across uninfected endothelial monolayers towards each supernatant was measured after 2 hours or 18 hours, respectively. The data are expressed as the median percentage of cells migrated for a representative experiment performed in triplicate. The error bars show the range of values obtained.

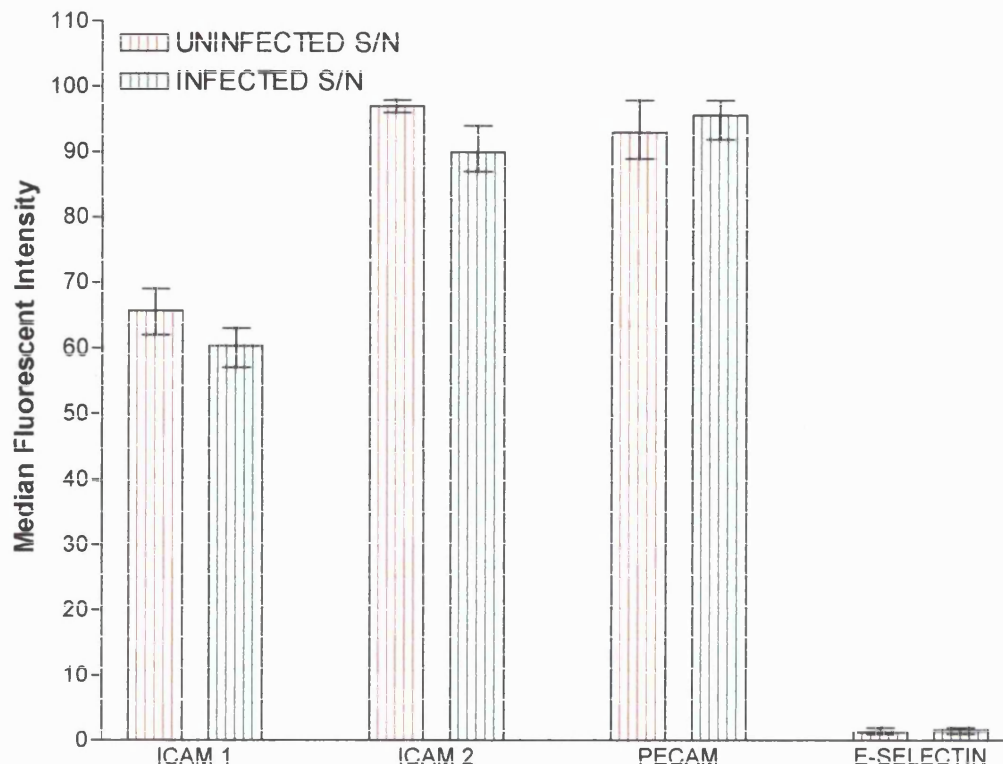


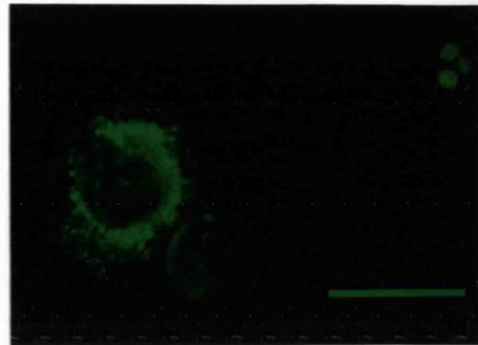
Figure 7.6.2. The effect of soluble factors on the expression of adhesion molecules following infection of endothelial cells with CMV.

Endothelial cells were inoculated at an MOI of 5 with C1FE and the culture supernatant was removed at 6 hours post infection. Supernatants from uninfected cells were also removed at the same time. The supernatants from uninfected or C1FE-infected cells (uninfected S/N and infected S/N, respectively) were then transferred onto fresh uninfected cells, and the expression of ICAM-1, ICAM-2, PECAM-1 or E-selectin was determined 2 hours later, using flow cytometric techniques. The data represent the median fluorescent intensity of triplicate samples for a representative experiment. The error bars show the range of values obtained.

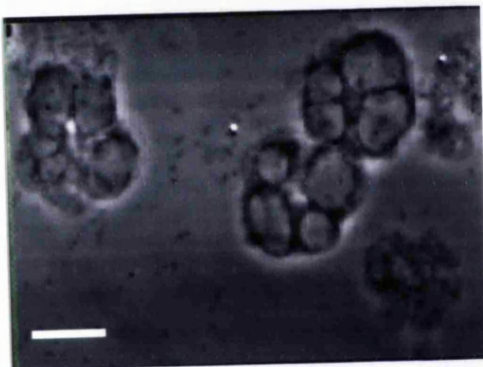
A. Phase contrast



B. pp65 fluorescence



C. Phase contrast



D. pp65 fluorescence

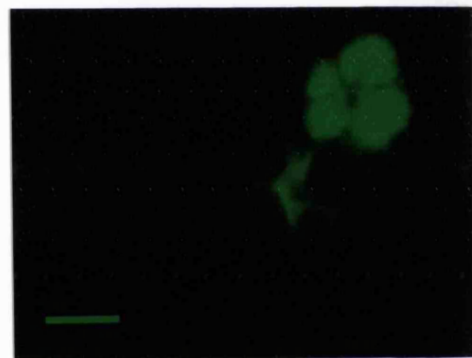


Figure 7.7.1. Photomicrograph of pp65 staining of neutrophils following co-culture with CMV infected endothelial cells.

Endothelial cells were inoculated with CMV strain C1FE until 70-80% of cells showed cytopathic effect. The infected endothelial cells were then co-cultured with neutrophils for 16 hours, and the cells separated prior to analysis of pp65 expression by immunofluorescence. The figure shows confocal scanning laser photomicrographs with simultaneously acquired phase contrast (parts A & C) and immunofluorescent images (parts B & D) of neutrophils after the co-culture period. A pp65 positive neutrophil can be seen in the upper right hand corner of part B, along with an enlarged residual infected endothelial cell expressing cytoplasmic pp65 (lower left hand corner). In part D, the nuclear localisation of pp65 in the neutrophil can be seen. Scale bar = 50 microns, parts A & B, Scale bar = 25 microns, parts C & D.

7.8. Discussion.

The main aim of this chapter was to determine whether any changes to leukocyte adhesion or transendothelial migration occurred following infection with the endothelial tropic strain of CMV, C1FE. The results indicated that both adhesion and transmigration of neutrophils was increased, following infection of endothelial cells with this viral strain in the absence of prior cytokine stimulation. Furthermore, the adhesion of PBL to unstimulated endothelial cells was marginally increased after virus infection, whereas the transmigration of T-lymphocytes was markedly increased through the CMV-infected endothelial cells. Cell-free supernatants obtained from these CMV-infected endothelial cells at early (6 hour) times post inoculation also promoted the transmigration of neutrophils and T-lymphocytes through fresh, uninfected endothelial cells, suggesting that a soluble factor was partially involved in this latter process. However, after cytokine activation (either 4 or 24 hours) of the CMV-infected endothelial cells, the adhesion of both neutrophils or PBL was decreased with respect to the uninfected counterpart, which is in contrast to the results obtained for the 'unactivated' endothelial cells.

Taken separately, the adhesion studies indicated that the binding of purified neutrophils and PBL to uninfected endothelial cells was low in the absence of cytokine stimulation, although a slightly higher basal adhesion to the uninfected endothelial cells was evident in the latter case. This trend in spontaneous leukocyte adherence is in agreement with current literature reports, where it has been shown that natural killer cells bound preferentially to unactivated endothelial cells, lymphocytes less well, and polymorphonuclear cells minimally (Allavena et al. 1991; Bender et al. 1987). Furthermore, it has been shown that under conditions of stasis the predominant receptor/ligand interactions responsible for basal leukocyte adhesion consist of the β 2-integrins on the leukocyte and ICAM-1 on the unactivated endothelial cell (Oppenheimer Marks et al. 1990; Hakkert et al. 1991; Smith et al. 1988). However, other molecules may also contribute to these adhesive events, because both neutrophils and lymphocytes obtained from patients suffering from a congenital CD18 (β 2-integrin) deficiency were also capable of binding to unstimulated endothelium (Kavanaugh et al. 1991; Smith et al. 1988). Thus, it was

unclear as to the exact molecules responsible for the neutrophil and PBL binding seen here with unstimulated, uninfected endothelial cells.

Following CMV infection, increased neutrophil and PBL binding to the unstimulated endothelial cells was observed. This effect on granulocyte binding has been reported previously after endothelial cells were infected with a laboratory strain of CMV, and was attributed to a direct viral effect because CMV-free medium obtained from the infected cells failed reproduce this binding (Span et al. 1991b; Span et al. 1989b). The observation presented here, that neutrophils and PBL accumulated around the enlarged, and presumably infected endothelial cells, rather than across the whole monolayer, would appear to support this assumption. It was considered unlikely that E-selectin played a role in this adherence, as suggested in one study (Span et al. 1991a) because, as indicated in Chapter 6, no increased expression of E-selectin was observed following infection with CMV. Thus, a more likely mechanism for this effect was the increased expression of ICAM-1 observed at the surface of endothelial cells following infection with CMV (Chapter 6). Increased binding of neutrophils to parainfluenza virus-infected endothelial cells has previously been reported to occur partly via an ICAM-1-dependant mechanism (Tosi et al. 1992), as has the adherence of lymphocytes following HSV-1 infection of cerebral endothelial cells (Brankin et al. 1995), measles virus infection of glial cells (Kraus et al. 1992), and Epstein-Barr virus infection of B-lymphocytes (Wang et al. 1988).

Heterotypic lymphocyte binding to various cell types, including endothelial cells, also occurs through other receptor-ligand interactions. One such interaction involves the CD2/LFA-3 pathway (Parra et al. 1993; Springer et al. 1987), and appears to be involved in the immune surveillance of several viruses. Infection of B-lymphoblastoid cells with either Influenza virus (van Kemenade et al. 1993) or Epstein-Barr virus (Wang et al. 1988) resulted in increased conjugate formation with T-lymphocytes via the CD2/LFA-3 pathway, whereas Burkitt's lymphoma cells with normal levels of ICAM-1 but low levels of LFA-3 were poor targets for Epstein Barr virus-specific CTL's (Gregory et al. 1988). Furthermore, increased binding of peripheral blood mononuclear cells to CMV-infected fibroblasts was found to be largely ICAM-1 independent, but was dependant upon CD2+ leukocytes interacting

with the increased levels of LFA-3 displayed at the fibroblast surface (Grundy, Downes, 1993). In the present study, however, these molecules were unlikely to have contributed to the increased adherence of PBL to the CMV-infected endothelial cells because, as demonstrated in Chapter 6, LFA-3 expression was not up-regulated by the endothelial tropic C1FE strain of CMV used in the adherence experiments.

In all of the above viral studies, the ability of leukocytes to adhere to endothelial cells was carried out in the absence of cytokine stimulation. Therefore, in the present study, the ability of leukocytes to adhere to uninfected or CMV-infected endothelial cells following activation with IL-1 β was also investigated. Stimulation with agents such as TNF- α , IL-1 β and LPS are known to induce the expression of molecules which mediate leukocyte adhesion and transmigration. Cytokine-induced E-selectin expression was reported to be responsible for increased neutrophil binding to endothelial cells after 4-6 hours of stimulation (Bevilacqua et al. 1989; Bevilacqua et al. 1987), whilst at later times, ICAM-1-mediated binding predominated (Jones et al. 1996; Luscinskas et al. 1991; Smith et al. 1988). Similarly, the increased binding of lymphocytes (Kavanaugh et al. 1991; Oppenheimer Marks et al. 1990) and natural killer cells (Leeuwenberg et al. 1990b; Pinola et al. 1994b; Lucin et al. 1992b) to 24 hour-stimulated endothelium was preferentially mediated by VCAM-1/VLA-4 and ICAM-1/LFA-1 interactions. In agreement with these previous findings, increased binding of neutrophils and PBL over baseline levels was observed here following IL-1 β stimulation of uninfected endothelial cells for either 4 or 24 hours, respectively, and this presumably occurred by similar mechanisms to those reported above.

Interestingly, however, there was a decrease in the adherence of both neutrophils and PBL to the CMV-inoculated cells following stimulation with IL-1 β , when compared to the similarly treated but uninfected counterparts. Furthermore, it was observed that those leukocytes which did bind to the CMV-inoculated endothelial cells again appeared to accumulate around the enlarged and presumably infected cells, as opposed to the apparently uninfected areas. One explanation for this effect was that reduced levels of E-selectin (Sedmak et al. 1994b; Tosi et al. 1992b) or VCAM-1 (Sedmak et al. 1994b) expression at the surface of the cytokine treated, CMV-inoculated endothelial cells contributed to the decreased neutrophil or PBL

binding, respectively. Indeed, it was shown in the latter study that E-selectin and VCAM-1 expression was decreased not only on the CMV-infected cells, but on some surrounding uninfected cells as well (Sedmak et al. 1994b). The slightly increased binding to the cytokine-treated inoculated cells described here, in comparison to the similarly inoculated but untreated cells, could be due to a proportion of the uninfected cells still capable of expressing E-selectin or VCAM-1, whereas preferential leukocyte binding to the infected cells could be mediated by ICAM-1 (Chapter 6).

An alternative explanation for the decreased leukocyte adhesion to the cytokine-treated, CMV inoculated cells seen here, however, was that infection of endothelial cells with CMV altered the binding properties of the leukocytes so that they no longer interacted with the IL-1 β activated endothelial cells. It has previously been shown that, whereas the chemokine IL-8 increased the adherence of normal polymorphonuclear cells to unactivated endothelial cells (Carveth et al. 1989), the same chemokine inhibited the binding of polymorphonuclear cells to IL-1 β -activated endothelial cells (Gimbrone, Jr. et al. 1989). Although it was originally reported that a direct effect on E-selectin expression was responsible for these effects (Gimbrone, Jr. et al. 1989), later studies suggested that IL-8 affected the expression of a neutrophil binding protein (Luscinskas et al. 1992; Hebert et al. 1990). Thus, it was suggested that a reverse chemotactic gradient was produced, leading to leukocyte deactivation at the sites where large accumulations of the gradient occurred (Smith et al. 1991). This phenomenon has also been described *in vivo*, where polymorphonuclear cell extravasation was promoted when IL-8 was given locally, but inhibited if administered systemically by the intravenous route (Ley et al. 1993; Hechtman et al. 1991). Thus, high levels of systemic IL-8 may produce a reverse chemotactic gradient and leukocyte deactivation, representing a feedback mechanism to protect against tissue damage (Mantovani et al. 1997). Interestingly, it has been shown that increased levels of C-X-C chemokines are produced by fibroblasts and endothelial cells following infection with CMV (Grundy et al. 1998; Craigen et al. 1997), although the contribution of these molecules to the decreased leukocyte adhesion seen here remains to be determined. Thus, further studies are required in order to understand the

mechanism of decreased leukocyte binding to cytokine-activated, CMV-inoculated endothelial cells.

Leukocyte migration has been less frequently investigated than leukocyte adhesion, especially concerning viral infections. In the study presented here, it was shown that, in the absence of cytokine stimulation, the transmigration of neutrophils and T-lymphocytes was increased through CMV-inoculated endothelial cell monolayers in comparison to the uninfected cells. As both neutrophil (Hakkert et al. 1991; Luscinskas et al. 1991) and lymphocyte (Kavanaugh et al. 1991; Oppenheimer Marks et al. 1991) transmigration has been shown to be partially dependant upon ICAM-1, the increased expression of this molecule on the CMV-inoculated cells might have been responsible for the increased level of transmigration observed here.

However, it has also been shown that the adhesion molecule PECAM-1 is important in transendothelial cell leukocyte migration. A decrease in PECAM-1 expression at the endothelial cell surface led to a decrease in the migration of polymorphonuclear cells (Rival et al. 1996), and the addition of anti-PECAM-1 antibodies prevented the migration but not the adhesion of leukocytes to endothelial cells (Berman et al. 1996; Muller et al. 1993). Furthermore, the migration of neutrophils through HSV-1 infected cerebral cortex cells was prevented by the addition of anti-PECAM-1 antibodies, whereas the adhesion of neutrophils, via ICAM-1, to the same cells was not (Tang, Hendricks, 1996). Thus, given the fact that the expression of PECAM-1 was reduced following infection with CMV (Chapter 6), it was possible that the leukocytes were using the adhesion molecules expressed on the uninfected cells, rather than the CMV-infected cells, for migration. In support of this latter supposition, supernatants obtained from the CMV-infected cells promoted increased neutrophil and T-lymphocyte migration through uninfected endothelial cells in the absence of altered adhesion molecule expression. Thus activation of neutrophils and T-lymphocytes by a soluble factor (Tanaka et al. 1993a; Detmers et al. 1991a; Huber et al. 1991a) may have promoted an intrinsic locomotor capacity to migrate randomly through uninfected cells. Recent studies from this laboratory have confirmed that C-X-C chemokines, produced following infection of endothelial cells with CMV, are capable of promoting neutrophil transendothelial migration (Grundy

et al. 1998). However, it has also been proposed that the redistribution of PECAM-1 away from cell to cell junctions may reduce the adhesive interactions between adjacent endothelial cells, thus enhancing the diapedesis of neutrophils by facilitating easier passage (Romer et al. 1995). Thus further studies are necessary in order to determine whether migration occurs through the infected or the uninfected cells.

Finally, it was shown here that the co-culture of neutrophils with infected endothelial cells for 1 day resulted in the acquisition of the CMV pp65 structural protein by the neutrophil. Although not presented here, these studies have subsequently been extended to show that neutrophils which migrated through CMV-infected endothelial cells also acquired this protein (Grundy et al. 1998). However, the fact that the neutrophils did not express the CMV IEA protein, p72, suggested that viral replication did not occur in this cell type, which is in agreement with previous *in vivo* findings (Turtinen et al. 1987). The transmission of endothelial cell derived virus to the neutrophils required cell to cell contact (Grundy et al. 1998), suggesting that the increased adhesion of neutrophils seen in this study functioned to promote viral transmission.

The transmission of virus from endothelial cells to monocytes also been reported previously, and it was proposed that the monocytes which had acquired CMV were able to subsequently disseminate the virus throughout the body (Waldman et al. 1995b). Although it was unclear whether a similar bi-directional transmission of virus occurred following neutrophil acquisition of CMV in this study, subsequent data from this laboratory indicated that the pp65-containing neutrophils could transmit infectious virus to fibroblasts (Grundy et al. 1998). Taken together, this data suggests a basis for the pp65 antigenemia assay which has been used *in vivo* to diagnose CMV infection in transplant recipients (Koskinen et al. 1993c; Grefte et al. 1992c; Grefte et al. 1992c), in that neutrophil contact with infected endothelial cells is the likely viral source. What has not yet been determined, and warrants further investigation, is the effect that pp65 acquisition has on the neutrophil. This viral protein has been attributed kinase activity (Furukawa et al. 1994), and may thus promote gene expression in the neutrophil. As neutrophils are capable of secreting numerous

cytokines which are capable of influencing subsequent immune reactions (Fantone, 1997), the 'passive' uptake of pp65 may promote secretion of cytokines which recruit mononuclear cells to the site of infection, and may thus indirectly contribute to the endothelialitis associated with allograft rejection.

CHAPTER 8.
GENERAL DISCUSSION.

Human cytomegalovirus (CMV) causes widespread infection in the general population, although such infection is not usually associated with overt symptoms of disease in the immunocompetent host (Weller, 1971). In the immunocompromised host, however, CMV infection is often associated with significant morbidity and mortality (Sonnabend et al. 1983; Jacobson, Mills, 1988; Ho, 1977), suggesting that the immune system plays an important role in limiting viral spread in the healthy individual. At the cellular level, viral replication is dependent upon a complex, and in part obscure, series of regulatory factors associated both with the virus itself, and with the host cell in which the virus resides (Lafemina, Hayward, 1988; Albrecht et al. 1990). Thus, the type and physiology of the cell that the virus encounters may also influence the course of infection and disease. Evidence that CMV interacts with the host at both the immunological level, and at the cellular level, is presented in this thesis.

In Chapter 3, it was shown that CMV was able to infect endothelial cells *in vitro*, but that the outcome of such an infection, in relation to subsequent productive or abortive replication, was dependant both upon the viral strain used, and upon the activation state of the endothelial cells. Highly fibroblast-passaged laboratory strains of CMV were inefficient at infecting endothelial cells, although replication could be initiated in a proportion of these cells following treatment with the short chain fatty acid, sodium butyrate. Thus the metabolic state of the host cell clearly affects CMV replication. On the other hand, low fibroblast-passaged clinical isolates retained their capacity to infect endothelial cells, suggesting that CMV strain variation might influence the outcome of infection. That this was not an *in vitro* phenomenon was indicated by the ability of an *ex vivo* source of CMV to infect both fibroblasts and endothelial cells, although to a greater extent in the former case than in the latter. These results have important implications for designing studies to investigate the interactions of CMV with endothelial cells *in vitro*, as some viral strains will not infect this cell type, and this could influence the results produced. Indeed, in early studies, highly fibroblast-passaged strains of CMV were used as the source of the viral inoculum, and so it was concluded that CMV could not replicate in endothelial cells (Smiley et al. 1988; Friedman et al. 1981).

Other studies have shown that low passaged strains of CMV replicated more efficiently in various cell types than did higher passaged laboratory strains (Waldman et al. 1989; Einhorn, Ost, 1984; Mocarski et al. 1993), indicating that extensive fibroblast passaging may adversely affect the tropism for other cell types. Thus it is desirable to both limit the number of CMV passages *in vitro*, and where possible, to propagate the virus through the cell type under investigation. Furthermore, because the endothelial cell propagated CMV strains also infected fibroblasts with an efficiency equal to that of the laboratory strains, endothelial cells may be the best choice for the propagation of clinical isolates. However, it is not yet known whether extensive *in vitro* passaging through endothelial cells would lead to the large scale deletions observed in fibroblasts (Cha et al. 1996), and this may have clinical relevance. The laboratory strains of CMV, AD169 and Towne, were highly passaged *in vitro* with the strategy of attenuating wild type virulence, and indeed, inoculation of the Towne strain into healthy volunteers led to an asymptomatic seroconversion, although relatively high viral doses were necessary in order to elicit a protective response in healthy adults and children (Adler et al. 1998a; Quinnan, Jr. et al. 1984a). Similar inoculation of the low passage Toledo strain of CMV, however, was associated with clinical disease in previously seropositive males (Quinnan, Jr. et al. 1984), although at lower viral doses, illness was confined to seronegative individuals (Plotkin et al. 1989; Plotkin et al. 1985). This might partly have reflected different levels of replication *in vivo* by the separate viral strains (Brown et al. 1995), which, in turn, may have been influenced by the ability to infect different cell types. Thus an understanding of the factors controlling CMV tropism *in vitro* might help us to understand the complex interaction which takes place between the virus and the host *in vivo*, and which ultimately determines whether the infection is resolved, or leads to disease.

The anatomical localisation of endothelial cells at the junction of the blood vessels and underlying tissues makes them a pivotal cell type during the generation of an inflammatory reaction. Leukocytes are first required to interact with molecules present at the surface of the endothelial cells in order to infiltrate into the underlying tissues (McEver, 1992; Shimizu et al. 1992; Springer, 1990), and endothelial cells are capable of secreting a number of soluble factors which potentiate this process

(Mantovani et al. 1997). In addition, the cell surface of vascular endothelial cells is a point of first contact between the transplanted tissue and its host immune system, and this cell type has also been implicated as a primary target of graft destruction during transplant rejection (Adams et al. 1989b; Forbes et al. 1983b; Hayry et al. 1993b). Thus changes to cell surface molecules following infection of endothelial cells with CMV can have important consequences for all these processes.

Using the *in vitro* culture system described above, it was shown in the present study that CMV infection of endothelial cells led to the modulation of a number of molecules involved in immune regulation. Although the expression of LFA-3 was not altered following infection of endothelial cells or fibroblasts with the clinical strains C1FE, Toledo.E., and Toledo, increased expression of this molecule was observed following inoculation of fibroblasts with the clinical strain C1F (from which C1FE was derived) and the laboratory strain AD169. This suggested that the ability of CMV to alter LFA-3 expression was strain dependent, and further supports the existence of quasi-strains of CMV in recent clinical isolates. The ability of some strains of CMV to prevent the induction of LFA-3 might represent an immune evasion strategy, as the expression of this molecule is associated with natural killer cell activation and migration (Somersalo et al. 1995; Li et al. 1993; Lanier et al. 1997). Indeed, it has been reported by members of this laboratory that natural killer cell adhesion to and lysis of CMV-infected cells correlated with the increased expression of LFA-3 (Fletcher et al. 1998; Grundy et al. 1993). Furthermore, a decrease in the ability of natural killer cells to lyse cells infected with the strains of CMV which did not induce LFA-3 expression (C1FE and Toledo) was also demonstrated (Fletcher et al. 1998). As the ability of transplant recipients to mount a strong natural killer response against a CMV infection has been correlated with favourable clinical outcome (van den Berg et al. 1993; Quinnan, Jr. et al. 1981; Quinnan, Jr. et al. 1982), the existence of CMV strains which are not susceptible to lysis by natural killer cells warrants further investigation.

In the present study, it was also shown that infection with the low passage clinical strains of CMV altered both the constitutive and inducible expression of the endothelial cell adhesion molecules, ICAM-2, PECAM-1 and E-selectin. These three molecules were either down-regulated at the CMV-infected endothelial cell surface,

or else were refractory to cytokine stimulation. Although the biological significance of the CMV-mediated inhibition of the expression of these adhesion molecules is unclear at present, the inability of the infected cell to express E-selectin might reduce the early 'rolling' events associated with leukocyte recruitment (Patel et al. 1995). Furthermore, because ICAM-2 and PECAM-1 have been implicated in the constitutive trafficking of resting T-lymphocytes, and the migration of both granulocytes and mononuclear cells through the endothelium (Berman et al. 1996; Somersalo et al. 1995; Zocchi et al. 1996; Muller et al. 1993; Christofidou Solomidou et al. 1997), it is likely that the CMV mediated downregulation of these two molecules reduces the number of leukocytes which migrate into the infected tissues.

In addition, the inability of endothelial cells to express E-selectin in response to either IL-1 β stimulation (the present study), or E-selectin and VCAM-1 in response to TNF- α stimulation (Sedmak et al. 1994b), might reflect a general non-responsiveness to these cytokines following infection with CMV. This warrants further investigation, as these cytokines are the classical pro-inflammatory mediators of the immune response, and indeed, both neutrophil and PBL adhesion to infected endothelial cells was reduced with respect to the uninfected cells following IL-1 β stimulation, as shown in the present study. Furthermore, the fact that ganciclovir had no effect on the ability of CMV to inhibit E-selectin expression suggested that an IE/early gene was responsible for this lack of induction. As the expression of IE/early genes have been identified in a number of cell types, such as monocytes and neutrophils (Rice et al. 1984; Dankner et al. 1990; Gerna et al. 1992), without necessarily leading to productive infection, cells which display this limited gene expression might still be non-responsive to IL-1 β and TNF- α . This, in turn, might partly explain the generalised immunosuppression associated with CMV infection (Rinaldo, Jr. et al. 1980; Carney, Hirsch, 1981), as these cytokines are required for efficient macrophage activation and function.

During the present study, however, it was also found that ICAM-1 expression was increased at the infected endothelial cell surface following infection with CMV. Thus, although the decreased expression of some adhesion molecules could dampen the immune response to the virally infected cells, on the other hand, the increased

expression of ICAM-1 would be expected to promote leukocyte adherence, and to prolong leukocyte residency in the proximity of the infected tissues. Indeed, as indicated in Chapter 7, increased adherence of neutrophils and PBL to endothelial cells occurred following infection with CMV in the absence of prior cytokine stimulation. However, the relative contribution of ICAM-1 to the increased adhesion of these cell types has yet to be determined.

Whilst not presenting a potential problem in the immunocompetent individual, increased expression of ICAM-1 during allograft rejection could be a contributing factor to the ongoing inflammatory process. Increased levels of this molecule have been observed on epithelium, hepatocytes, myocytes and endothelium in rejecting hepatic (Steinhoff et al. 1993; Adams et al. 1989), renal (Brockmeyer et al. 1993), or heart allografts (Taylor et al. 1992; Tanio et al. 1994). All these cell types have also been implicated as primary targets of the rejection process. Support for the proposal that ICAM-1 might indeed play a role in allograft rejection comes from *in vivo* animal studies using monoclonal antibodies that block the function of this molecule. Administration of ICAM-1- and LFA-1-specific antibodies to mice with heart allografts promoted cardiac engraftment with respect to mice which did not receive such treatment (Isobe et al. 1992), whilst an ICAM-1 antibody alone delayed lymphocyte infiltration and inhibited rejection of xenogeneic islet cells in diabetic mice (Zeng et al. 1994).

Increased expression of ICAM-1 during allograft rejection is generally believed to be a result of cytokine production by infiltrating leukocytes. However, as the present studies indicate, ICAM-1 up-regulation at the surface of CMV-infected endothelial cells occurred despite the absence of contaminating leukocytes, and was not modulated by soluble factors secreted from the CMV-infected endothelial cells. A similar finding has been reported elsewhere for endothelial cells (Sedmak et al. 1994b), whilst the ability of CMV to induce ICAM-1 on *in vitro* cultured epithelial cells (van Dorp et al. 1993) and fibroblasts (Grundy, Downes, 1993) was shown to be independent of cytokine secretion in the latter cell type (Craig, Grundy, 1996). Thus, CMV may influence the initial infiltration of inflammatory cells by directly promoting ICAM-1/LFA-1 interactions. Once mononuclear cells have been recruited, however, the activated leukocytes can secrete soluble factors which enhance adhesion

molecule expression on surrounding 'uninfected' cells. Indeed, it has been shown that increased ICAM-1 expression on endothelial cells which lacked CMV antigen was a result of cytokine-dependent mechanisms, as indicated by co-culture of CMV-infected endothelial cells with CD4⁺ leukocytes from seropositive individuals (Waldman, Knight, 1996; Waldman et al. 1995). Hence, CMV infection of various cell types would not only promote the expression of this molecule on the infected cells, but would subsequently be responsible for up-regulated ICAM-1 expression on surrounding uninfected cells as well.

The recruitment of cells during an inflammatory response is also influenced by the induction of chemoattractant molecules (Kunkel et al. 1997). Indeed, it was shown in the present study that the infected endothelial cells produced soluble factors which promoted the migration of neutrophils and T-lymphocytes in the absence of altered adhesion molecule expression. Subsequent studies by members of this laboratory showed that the neutrophil promoting factor was in part due to the secretion of the C-X-C chemokines, IL-8 and Gro-1 α (Grundy et al. 1998). Increased production of IL-8 has also been reported following infection of fibroblasts (Craigien et al. 1997) or monocytes with CMV (Murayama et al. 1997). Furthermore, IL-8 enhanced the replication of CMV in human embryonic lung fibroblasts, and selectively induced an IL-8 receptor transcript in the same cells (Murayama et al. 1994). It would thus be interesting to see whether IL-8 secretion had a similar effect on the production of infectious virions from endothelial cells.

The production of IL-8 from CMV-infected cells, however, may also have important consequences in the immunosuppressed individual, as neutrophils have been documented as constituents of the inflammatory infiltrate in liver transplant tissues prior to acute rejection episodes (Adams et al. 1990). Furthermore, IL-8 is also a potent chemoattractant molecule for smooth muscle cells (Yue et al. 1994), endothelial cells (Koch et al. 1992) and subsets of T-lymphocytes (Qin et al. 1996; Xu et al. 1995). All these cell types have been implicated in the endothelialitis associated with the development of chronic allograft rejection (Colvin, 1990; Nemlander et al. 1982; Hruban et al. 1990; Billingham, 1994). Acute CMV infection has also been associated with a T-lymphocyte and monocyte endothelialitis of allograft vascular structures following heart transplantation in man (Koskinen et al. 1994; Paavonen et

al. 1992), with a concomitant increase in arterial endothelial cell proliferation (Koskinen et al. 1993; Koskinen et al. 1993). Similar inflammatory infiltrates in rat allografts, together with vascular intimal proliferation, have been documented following infection with rat CMV (Li et al. 1996; Lemstrom et al. 1994; Koskinen et al. 1996). As shown in the present study, transendothelial migration of T-lymphocytes was promoted following infection with CMV, and was influenced by the secretion of soluble factors from the infected cells, although the exact factors responsible for this have yet to be identified. Thus, an understanding of the soluble factors produced during CMV infection of endothelial cells, together with their effect on leukocyte migration, warrants further investigations.

The recruitment of neutrophils to the site of endothelial cell infection, via IL-8 secretion, might also facilitate the spread of virus. Indeed, it was shown in this thesis that CMV infected endothelial cells were capable of transmitting the viral protein pp65 to co-cultured neutrophils. Subsequent studies by members of this laboratory demonstrated that cell to cell contact was required for this process and that, although CMV IEA could not be detected in this study, the pp65 antigen positive neutrophils were able to transmit the virus to fibroblasts (Grundy et al. 1998). Isolation of CMV from blood of viraemic patients also indicates that infectious virus is associated with white blood cells, especially polymorphonuclear cells, *in vivo* (Gerna et al. 1992; Saltzman et al. 1988). Although the detection of CMV pp65 antigen in peripheral blood neutrophils, via the antigenaemia test (Grefte et al. 1992a; Grefte et al. 1992a), has been used as a predictor of CMV-associated disease *in vivo* (Niubo et al. 1996; Podzamczar et al. 1997; Koskinen et al. 1993), the reason behind this association has not been clear. The data presented here suggests that neutrophil acquisition of pp65 reflects virus infection of endothelial cells, and that the endothelial cell transmits CMV to leukocytes, which can then disseminate the virus to other sites in the body (Waldman et al. 1995b; Grundy et al. 1998b).

However, it is clear from the above studies, and from those of others, that the cells of the innate immune system, such as neutrophils and monocyte/macrophages are not capable of destroying free infectious virions, but are rather a source^{of} CMV infection (Sinzger et al. 1996; Gerna et al. 1992; Saltzman et al. 1988; Grundy et al. 1998). Therefore, this part of the immune system is not effective in eliminating the

virus, and hence the adaptive immune response to CMV is also important in resolving CMV infection (Koszinowski et al. 1991). However, this thesis also provides evidence that CMV interferes with molecules involved in the adaptive immune response. In Chapter 5, it was found that infection of endothelial cells with CMV led to the induction of receptors for the Fc portion of IgG. Although the function of the FcγR induced by CMV is unclear, it has been suggested that the presence of a FcγR offers a survival advantage to the virus inducing such a receptor, by preventing antibody-mediated cellular cytotoxicity, and antibody plus complement mediated lysis (Dubin et al. 1991; Adler et al. 1978). However, other reports have suggested that virally induced FcγR actually contributes towards infected cell lysis (Rouse et al. 1977; Visser et al. 1989; Cines et al. 1982; Rager Zisman et al. 1976), and thus the insertion of FcγR molecules into the outer membrane of CMV infected cells could also contribute to the tissue injury (Ross, 1993; Leeuwenberg et al. 1990; Harlan, 1987). Although it is still not known whether the presence of a virally induced FcγR contributes to immune evasion, or to the pathogenesis of disease, the fact that CMV is able to produce such a novel FcγR in a strategically important location like the endothelium suggests that its biological role needs to be further investigated.

The studies presented here also indicated that CMV can interfere with the adaptive immune response by altering the expression of MHC Class I and Class II molecules. In particular, a cytokine-independent down-regulation of MHC Class I, and a reduced induction of MHC Class II in response to IFN-γ, was evident at the surface of CMV-inoculated endothelial cells. Previous reports on the effect of CMV infection on the expression of Class I at the surface of various cell types, however, have been conflicting. The data presented in this thesis suggests a reason for these apparent discrepancies. Class I down-regulation was found to be due to the expression of CMV IE/early genes, as the addition of the antiviral drug ganciclovir failed to prevent Class I down-regulation following infection of endothelial cells with the clinical CMV strain, Toledo.E. Furthermore, when the laboratory strain of CMV was used to inoculate endothelial cells, obvious Class I down-regulation only occurred after viral replication was induced by sodium butyrate treatment of the inoculated cells. In addition, the type of analysis used to monitor Class I expression was also

found to be important. When only a fraction of cells were infected, measuring the average intensity of Class I expression from the total cell population, which contained a mixture of both low (infected) and high (uninfected) Class I expressing cells, resulted in 'skewed' readings that gave an overall impression of Class I up-regulation. Hence studies which analysed an overall level of Class I MHC expression following inoculation of cells with CMV (Tuder et al. 1994; Tuder et al. 1991; van Dorp et al. 1993; van Dorp et al. 1989), would show an increase in Class I expression if the cultures in question contained a large number of uninfected bystander cells. Thus it is perspicacious to realise that the use of viral strains which do not readily express immediate early/early genes in certain cell types would appear to have different effects on Class I expression (Ustinov et al. 1991; Hosenpud et al. 1991), depending on the type of analysis used.

Other investigators have previously shown that CMV encodes a number ^{cf} genes which result in inefficient antigen presentation via Class I molecules. The tegument protein pp65 specifically blocks presentation of peptides derived from the IE genes (Gilbert et al. 1996), gpUS3 retains MHC Class I molecules in the endoplasmic reticulum (Ahn et al. 1996; Jones et al. 1996), pUS2 and pUS11 dislocate the heavy chains and divert them back to the cytosol for proteosomal degradation (Jones et al. 1995; Wiertz et al. 1996), and US6 prevents TAP transportation through the endoplasmic reticulum (Wiertz et al. 1997). Thus, the sequential expression of these genes during viral replication prevent expression of MHC Class I molecules at the surface of the infected cell, and one or all of these viral proteins are likely to be responsible for the reduction in endothelial cells Class I expression observed in this study. *In vitro*, this reduced expression of Class I MHC at the surface of fibroblasts correlates with increased resistance to lysis of the infected target cells by virus specific CTL (Warren et al. 1994a; Del Val et al. 1989a), and CMV-infected endothelial cells also specifically escaped immune destruction following co-culture with allogeneic CTL (Waldman et al. 1997). The reason for so many different viral proteins achieving the same function is unclear, but may reflect the fact that CMV infects different cell types to differing degrees, thus necessitating different mechanisms to override Class I expression in all these cell types.

In addition, CMV purportedly interferes with IFN- γ signal transduction pathways by unknown viral mechanisms (Miller et al. 1998), and this was presumably the reason why CMV infected endothelial cells were refractory to Class II induction by this specific cytokine, as determined in both the present and other studies (Sedmak et al. 1994a; Scholz et al. 1992a). Reduced expression of endothelial cell Class II antigens should, in turn, render the infected endothelial cells resistant to the effects of CD4⁺ lymphocytes, and also reduce the cytokine milieu generated during the course of an infection. Furthermore, IFN- γ up-regulates a number of other molecules, including ICAM-1 (Dustin et al. 1986), and enhances the immune potential of T-lymphocytes, natural killer cells and macrophages. Thus, CMV interference of the IFN- γ signalling pathway in different cell types might affect other immunomodulatory functions produced by this cytokine, and thus warrants further investigation.

In vivo, however, the generation of CMV-specific CTL's, and to a lesser extent CD4⁺ effector cells, is associated with a better resolution of CMV infection and disease (Koszinowski et al. 1991). This fact is in apparent contradiction to the effects predicted from the above results. One possible explanation for this discrepancy comes from the *in vitro* finding that the addition of Type I and Type II interferons, as well as TNF- α , prior to infection partially restores antigen presentation via the Class I pathway in CMV infected fibroblasts (Hengel et al. 1994). Furthermore, secretion of Type I interferons from infected cells following infection with CMV (Craig, Grundy, 1996; Barnes, Grundy, 1992) would increase the expression of Class I on the surrounding uninfected cells, potentially allowing recently infected cells to present CMV antigen via Class I. Indeed, increased Class I expression was observed in a proportion of the CMV inoculated (presumably uninfected) cells during the study presented here, via the secretion of a soluble factor from the infected endothelial cells. Furthermore, it has been shown that co-cultivation of peripheral blood mononuclear cells or CD4⁺ T-lymphocytes with CMV infected endothelial cells resulted in the secretion of IFN- γ from the immune cells, and promoted both Class I and Class II expression on cells not infected with the virus (Tuder et al. 1991; Waldman, Knight, 1996). Thus, cytokine secretion by host cells

in response to CMV infection is likely to be a countermeasure by the host for these virally controlled immune evasion mechanisms.

In immunosuppressed individuals, however, cytokine induced MHC up-regulation on donor derived tissues has been associated with allograft rejection (Shoskes, Wood, 1994; Gouw et al. 1988; Labarrere et al. 1995), resulting in the proliferation of allogeneic T-lymphocytes (Salomon et al. 1991). An increase in MHC antigen expression during CMV infection has also been suggested to trigger rejection episodes *in vivo* (Manez et al. 1993; O'Grady et al. 1988; von Willebrand et al. 1986). Furthermore, studies in mice and man have shown that CMV infection can lead to polyclonal T-lymphocyte activation and the generation of allospecific CTL (Waldman et al. 1992; Ouwehand et al. 1994; Yang et al. 1989). Thus, although CMV prevents the expression of Class I and Class II MHC molecules at the surface of productively infected cells, cytokines produced by the infected cells (as shown in the present study), and also by CMV-activated T-lymphocytes (Waldman, Knight, 1996) are capable of enhancing MHC antigen expression on other, uninfected cells. In the context of the transplant recipient, this would increase the alloantigenicity of the donor derived tissue, thus promoting T-lymphocyte activation.

Finally, the results from the present study indicated that the alterations to both adhesion molecule and MHC antigen expression, observed following infection of endothelial cells with CMV, were not prevented by the addition of the antiviral drug, ganciclovir. Indeed, in the case of ICAM-1, the expression of this molecule was both enhanced and prolonged, with respect to those CMV-inoculated cells which had not been treated with the drug. Furthermore, the secretion of soluble factors, which were capable of promoting transendothelial cell migration of both neutrophils and T-lymphocytes, occurred at early times (6 hours) post inoculation, suggesting that the prevention of late gene expression by ganciclovir would not impede this process. Thus, whilst ganciclovir treatment of CMV-infected cells suppresses virus DNA replication (Crumpacker, 1996), the antiviral drug does not prevent a previously infected cell from displaying substantially different properties, with regards to cell surface molecule and cytokine expression, to their uninfected counterparts.

In a situation where CMV-induced pathology may be mediated by cells of the immune system, as has been suggested for pneumonitis in bone marrow transplant

recipients and for accelerated atherosclerosis in solid organ transplant recipients (Hayry et al. 1984; Grundy et al. 1987), the results from the present study suggest that ganciclovir treatment might not necessarily be effective. Indeed, 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 clinical outcome (Slavin et al. 1993; Churchill et al. 1987; Shepp et al. 1985). Furthermore, ganciclovir treatment of established CMV disease was shown to have no effect on chronic rejection of lung allografts (Duncan et al. 1994), nor on the development of accelerated atherosclerosis in heart transplant recipients (Koskinen et al. 1993b). Although these facts might suggest that CMV infections are not associated with chronic rejection episodes, the data in this thesis also supports the notion that, if given during episodes of established CMV disease, inhibitors of viral DNA synthesis would fail to prevent many of the pro-inflammatory effects of the virus (Grundy, 1998). In particular, although ganciclovir treatment would lead to a reduction in the amount of infectious virus produced by the host, the infected cell would still be relatively resistant to CMV-specific T-lymphocytes, by virtue of decreased MHC Class I and Class II expression, and so the virus would not be cleared from the body. However, the infected cell would be altered in such a way as to promote leukocyte adhesion and migration towards the site of infection, and thus could contribute to an ongoing inflammatory process.

In support of this assumption, it has been shown that ganciclovir prophylactic therapy completely abolished CMV-enhanced aortic allograft atherosclerosis in rats, whereas ganciclovir treatment initiated during the infection only partially inhibited the development of intimal lesions (Lemstrom et al. 1997; Lemstrom et al. 1994). Furthermore, the use of CMV intravenous immunoglobulin in combination with ganciclovir has also dramatically improved the success of treatment of CMV pneumonitis in bone marrow transplant recipients (Emanuel et al. 1988; Reed et al. 1988), possibly by neutralising a viral antigen on lung cells recognised by CTL (Grundy, 1998), or by affecting cytokine production (Mouthon et al. 1996; Andersson et al. 1996). Thus, in situations where CMV might provoke an immunopathological process, both early antiviral administration (to prevent virus dissemination) combined with immunotherapeutic treatment (to reduce the pro-inflammatory effects of the

virus) might be necessary to resolve CMV-induced disease. Hence, a fuller understanding of the mechanisms behind these CMV-induced disease processes are required in order to develop effective treatment strategies.

In summary, although an immunocompetent host mounts a rapid immune response following infection with CMV, the virus has evolved mechanisms which limit this response, such as the down-regulation of MHC and adhesion molecule expression, and the *de novo* generation of a FcγR. This enables a few infected cells to escape immune destruction, but does not result in the host being destroyed. Once the host immune system has been compromised, however, this equilibrium is perturbed. In the setting of allograft transplantation, the combination of increased MHC molecules and ICAM-1 expression on the graft tissue following infection with CMV, together with increased cytokine production and leukocyte recruitment, may exacerbate an ongoing chronic inflammatory reaction. These effects should be taken into account if the CMV-induced disease is to be counteracted.

CHAPTER 9.
REFERENCES.

- Adam E, Melnick JL, Probstfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH, DeBaakey ME (1987) High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. *Lancet* 2:291-293.
- Adams DH, Hubscher SG, Shaw J, Rothlein R, Neuberger JM (1989a) Intercellular adhesion molecule 1 on liver allografts during rejection. *Lancet* 2:1122-1125.
- Adams DH, Wang L, Hubscher SG, Neuberger JM (1989b) Hepatic endothelial cells. Targets in liver allograft rejection? *Transplantation* 47:479-482.
- Adams DH, Wang LF, Burnett D, Stockley RA, Neuberger JM (1990) Neutrophil activation--an important cause of tissue damage during liver allograft rejection? *Transplantation* 50:86-91.
- Adler R, Giorioso JC, Cossman J, Levine M (1978) Possible role of Fc receptors on cells infected and transformed by herpesvirus: escape from immune cytolysis. *Infect Immun* 21:442-447.
- Adler SP, Hempfling SH, Starr SE, Plotkin SA, Riddell S (1998a) Safety and immunogenicity of the Towne strain cytomegalovirus vaccine. *Pediatr Infect Dis J* 17:200-206.
- Adler SP, Hur JK, Wang JB, Vetrovec GW (1998b) Prior infection with cytomegalovirus is not a major risk factor for angiographically demonstrated coronary artery atherosclerosis. *J Infect Dis* 177:209-12.
- Adlish JD, Lahijani RS, St.Jeor SC (1990) Identification of a putative cell receptor for human cytomegalovirus. *Virology* 176:337-345.
- Ahn K, Angulo A, Ghazal P, Peterson PA, Yang Y, Fruh K (1996) Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc Natl Acad Sci U S A* 93:10990-10995.
- Albrecht T, Boldogh I, Fons M, AbuBakar S, Deng CZ (1990) Cell activation signals and the pathogenesis of human cytomegalovirus. *Intervirology* 31:68-75.
- Allavena P, Paganin C, Martin-Padura I, Peri G, Gaboli M, Dejana E, Marchisio PC, Mantovani A (1991) Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J Exp Med* 173:439-448.
- Andersson J, Skansen-Saphir U, Sparrelid E, Andersson U (1996) Intravenous immune globulin affects cytokine production in T lymphocytes and monocytes/macrophages. *Clin Exp Immunol* 104 Suppl 1:10-20.
- Angulo A, Suto C, Boehm MF, Heyman RA, Ghazal P (1995) Retinoid activation of retinoic acid receptors but not of retinoid X receptors promotes cellular differentiation and replication of human cytomegalovirus in embryonal cells. *J Virol* 69:3831-3837.
- Aschan J, Ringden O, Ljungman P, Lonnqvist B, Ohlman S (1992) Foscarnet for treatment of cytomegalovirus infections in bone marrow transplant recipients. *Scand J Infect Dis* 24:143-50.
- Bach MC, Bagwell SP, Knapp NP, Davis KM, Hedstrom PS (1985) 9-(1,3-Dihydroxy-2-propoxymethyl)guanine for cytomegalovirus infections in patients with the acquired immunodeficiency syndrome. *Ann Intern Med* 103:381-2.
- Baggiolini M (1998) Chemokines and leukocyte traffic. *Nature* 392:565-568.

- Balfour HH, Jr. (1992) The clinical significance of infections with cytomegalovirus strains resistant to antiviral drugs. *Res Virol* 143:219-21.
- Barnes PD, Grundy JE (1992) Down-regulation of the class I HLA heterodimer and beta 2-microglobulin on the surface of cells infected with cytomegalovirus. *J Gen Virol* 73:2395-2403.
- Bender JR, Pardi R, Karasek MA, Engleman EG (1987) Phenotypic and functional characterization of lymphocytes that bind human microvascular endothelial cells in vitro. Evidence for preferential binding of natural killer cells. *J Clin Invest* 79:1679-1688.
- Berman ME, Xie Y, Muller WA (1996) Roles of platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) in natural killer cell transendothelial migration and beta 2 integrin activation. *J Immunol* 156:1515-1524.
- Betts RF, Freeman RB, Douglas RG, Jr., Talley TE, Rundell B (1975) Transmission of cytomegalovirus infection with renal allograft. *Kidney Int* 8:385-392.
- Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA, Jr. (1985) Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J Clin Invest* 76:2003-2011.
- Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA, Jr. (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci U S A* 84:9238-9242.
- Bevilacqua MP, Stengelin S, Gimbrone MA, Jr., Seed B (1989) Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* 243:1160-1165.
- Billingham ME (1994) Pathology and etiology of chronic rejection of the heart. *Clin Transplant* 8:289-292.
- Biron CA, Byron KS, Sullivan JL (1989) Severe herpesvirus infections in an adolescent without natural killer cells [see comments]. *N Engl J Med* 320:1731-1735.
- Boeckh M, Hoy C, Torok Storb B (1998) Occult cytomegalovirus infection of marrow stroma. *Clin Infect Dis* 26:209-210.
- Boppana SB, Polis MA, Kramer AA, Britt WJ, 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.
- Bostrom L, Ringden O, Jacobsen N, Zwaan F, Nilsson B (1990) A European multicenter study of chronic graft-versus-host disease. The role of cytomegalovirus serology in recipients and donors--acute graft-versus-host disease, and splenectomy. *Transplantation* 49:1100-1105.
- Bowden RA, Mori M, Dobbs S, Hackman R, Kopecky K, Crawford S (1993) Mononuclear cell reconstitution in the lung after marrow transplantation. Lack of influence of cytomegalovirus pneumonia, irradiation, and graft-versus-host disease. *Transplantation* 55:557-561.
- Brankin B, Hart MN, Cosby SL, Fabry Z, Allen IV (1995) Adhesion molecule expression and lymphocyte adhesion to cerebral endothelium: effects of measles virus and herpes simplex 1 virus. *J Neuroimmunol* 56:1-8.

Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober JS (1991) Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation* 51:537-539.

Briscoe DM, Cotran RS, Pober JS (1992) Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 in vivo. Correlation with CD3+ T cell infiltration. *J Immunol* 149:2954-2960.

Briscoe DM, Yeung AC, Schoen FJ, Allred EN, Stavrakis G, Ganz P, Cotran RS, Pober JS, Schoen ELJ (1995) Predictive value of inducible endothelial cell adhesion molecule expression for acute rejection of human cardiac allografts [published erratum appears in *Transplantation* 1995 Mar 27;59(6): 928]. *Transplantation* 59:204-211.

Britt WJ (1994) Infections associated with human cytomegalovirus. In: *Herpesvirus Infections* (Glaser R, Jones JF eds), pp 59-117. New York/Hong Kong: Marcel Dekker.

Britt WJ (1996) Human cytomegalovirus infections during pregnancy. In: *Bailliere's Clinical Infectious Disease. International practice and research* (Arvin AM ed), pp 327-371. London/Philadelphia: Bailliere Tindall/W.B.Saunders Co.Ltd.

Britt WJ, Alford CA (1996) Cytomegalovirus. In: *Fields Virology* (Fields BN, Knipe DM, Howley PM eds), pp 2493-2523. Philadelphia: Lippincott-Raven Publishers.

Brockmeyer C, Ulbrecht M, Schendel DJ, Weiss EH, Hillebrand G, Burkhardt K, Land W, Gokel MJ, Riethmuller G, Feucht HE (1993) Distribution of cell adhesion molecules (ICAM-1, VCAM-1, ELAM-1) in renal tissue during allograft rejection. *Transplantation* 55:610-615.

Bronsther O, Makowka L, Jaffe R, Demetris AJ, Breinig MK, Ho M, Esquivel CO, Gordon RD, Iwatsuki S, Tzakis A, et al (1988) Occurrence of cytomegalovirus hepatitis in liver transplant patients. *J Med Virol* 24:423-434.

Brown JM, Kaneshima H, Mocarski ES (1995) Dramatic interstrain differences in the replication of human cytomegalovirus in SCID-hu mice. *J Infect Dis* 171:1599-1603.

Bruggeman CA, Reinders R, Debie WM, Frederik PM, Engels W, van Boven CP (1984) Demonstration of an IgG-Fc receptor in rat cytomegalovirus infected cells. *Arch Virol* 81:79-89.

Buchmeier NA, Cooper NR (1989) Suppression of monocyte functions by human cytomegalovirus. *Immunology* 66:278-283.

Bukowski JF, Warner JF, Dennert G, Welsh RM (1985) Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. *J Exp Med* 161:40-52.

Burns LJ, Waring JF, Reuter JJ, Stinski MF, Ginder GD (1993) Only the HLA class I gene minimal promoter elements are required for transactivation by human cytomegalovirus immediate early genes. *Blood* 81:1558-1566.

Burton DR, Woof JM (1992) Human antibody effector functions. In: *Advances in Immunology* (Dixon FJ ed), pp 1-84. San Diego CA: Academic Press.

Butcher EC (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* 67:1033-6.

- Campbell AE, Slater JS (1994) Down-regulation of major histocompatibility complex class I synthesis by murine cytomegalovirus early gene expression. *J Virol* 68:1805-1811.
- Carney WP, Hirsch MS (1981) Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. *J Infect Dis* 144:47-54.
- Carveth HJ, Bohnsack JF, McIntyre TM, Baggolini M, Prescott SM, Zimmerman GA (1989) Neutrophil activating factor (NAF) induces polymorphonuclear leukocyte adherence to endothelial cells and to subendothelial matrix proteins. *Biochem Biophys Res Commun* 162:387-393.
- Cauda R, Prasthofer EF, Grossi CE, Whitley RJ, Pass RF (1987) Congenital cytomegalovirus: immunological alterations. *J Med Virol* 23:41-49.
- Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR (1996) Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J Virol* 70:78-83.
- Chatterjee SN, Fiala M, Weiner J, Stewart JA, Stacey B, Warner N (1978) Primary cytomegalovirus and opportunistic infections. Incidence in renal transplant recipients. *JAMA* 240:2446-2449.
- Cheung KS, Lang DJ (1977) Transmission and activation of cytomegalovirus with blood transfusion: a mouse model. *J Infect Dis* 135:841-845.
- Chou S, Kim DY, Norman DJ (1987) Transmission of cytomegalovirus by pretransplant leukocyte transfusions in renal transplant candidates. *J Infect Dis* 155:565-7.
- Chou SW (1989) Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors. *J Infect Dis* 160:11-15.
- Christofidou Solomidou M, Nakada MT, Williams J, Muller WA, DeLisser HM (1997) Neutrophil platelet endothelial cell adhesion molecule-1 participates in neutrophil recruitment at inflammatory sites and is down-regulated after leukocyte extravasation. *J Immunol* 158:4872-4878.
- Churchill MA, Zaia JA, Forman SJ, Sheibani K, Azumi N, Blume KG (1987) Quantitation of human cytomegalovirus DNA in lungs from bone marrow transplant recipients with interstitial pneumonia. *J Infect Dis* 155:501-509.
- Cines DB, Lyss AP, Bina M, Corkey R, Kefalides NA, Friedman HM (1982) Fc and C3 receptors induced by herpes simplex virus on cultured human endothelial cells. *J Clin Invest* 69:123-8No.
- Colden-Stanfield M, Ratcliffe D, Cramer EB, Gallin EK (1993) Characterization of influenza virus-induced leukocyte adherence to human umbilical vein endothelial cell monolayers. *J Immunol* 151:310-321.
- Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. [Review] [72 refs]. *FASEB J* 9:899-909.

- Colvin RB (1990) Cellular and molecular mechanisms of allograft rejection. *Ann Rev Med* 41:361-375.
- Compton T, Nepomuceno RR, Nowlin DM (1992) Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virology* 191:387-395.
- Compton T, Nowlin DM, Cooper NR (1993) Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology* 193:834-841.
- Cooper DK, Novitzky D, Schlegel V, Muchmore JS, Cucchiara A, Zuhdi N (1991) Successful management of symptomatic cytomegalovirus disease with ganciclovir after heart transplantation. *J Heart Lung Transplant* 10:656-662.
- Craigien JL, Yong KL, Jordan NJ, MacCormac LP, Westwick J, Akbar AN, Grundy JE (1997) Human cytomegalovirus infection up-regulates interleukin-8 gene expression and stimulates neutrophil transendothelial migration. *Immunology* 92:138-145.
- Craigien JL, Grundy JE (1996) Cytomegalovirus induced up-regulation of LFA-3 (CD58) and ICAM-1 (CD54) is a direct viral effect that is not prevented by ganciclovir or foscarnet treatment. *Transplantation* 62:1102-1108.
- Craighead JE (1971) Pulmonary cytomegalovirus infection in the adult. *Am J Pathol* 63:487-504.
- Creutz CE (1992) The annexins and exocytosis. *Science* 258:924-931.
- Crnkovic-Mertens I, Messerle M, Milotic I, Szepan U, Kucic N, Krmpotic A, Jonjic S, Koszinowski U, Koszinowski UH (1998) Virus Attenuation after Deletion of the Cytomegalovirus Fc Receptor Gene is Not due to Antibody Control. *Journal of Virology* 72:1377-1382.
- Crumpacker CS (1992) Mechanism of action of foscarnet against viral polymerases. *Am J Med* 92:3S-7S.
- Crumpacker CS (1996) Ganciclovir. *N Engl J Med* 335:721-9.
- Dankner WM, McCutchan JA, Richman DD, Hirata K, Spector SA (1990) Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. *J Infect Dis* 161:31-36.
- Davis MM, Bjorkman PJ (1988) T-cell antigen receptor genes and T-cell recognition [published erratum appears in *Nature* 1988 Oct 20;335(6192):744]. *Nature* 334:395-402.
- Davis-Poynter NJ, Farrell HE (1998) Human and Murine Cytomegalovirus Evasion of Cytotoxic T Lymphocyte and Natural Killer Cell-Mediated Immune Responses. *Seminars in Virology* 8:369-376.
- de Fougerolles AR, Springer TA (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.
- Del Val M, Munch K, Reddehase MJ, Koszinowski UH (1989) Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. *Cell* 58:305-315.

- Denning SM, Dustin ML, Springer TA, Singer KH, Haynes BF (1988) Purified lymphocyte function-associated antigen-3 (LFA-3) activates human thymocytes via the CD2 pathway. *J Immunol* 141:2980-2985.
- Detmers PA, Powell DE, Walz A, Clark-Lewis I, Baggiolini M, Cohn ZA (1991) Differential effects of neutrophil-activating peptide 1/IL-8 and its homologues on leukocyte adhesion and phagocytosis. *J Immunol* 147:4211-4217.
- Dieterich DT, Chachoua A, Lafleur F, Worrell C (1988) Ganciclovir treatment of gastrointestinal infections caused by cytomegalovirus in patients with AIDS. *Rev Infect Dis* 10 Suppl 3:
- Dingwell KS, Brunetti CR, Hendricks RL, Tang Q, Tang M, Rainbow AJ, Johnson DC (1994) Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *J Virol* 68:834-845.
- Dingwell KS, Doering LC, Johnson DC (1995) Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. *J Virol* 69:7087-7098.
- Drew WL, Mintz L, Miner RC, Sands M, Ketterer B (1981) Prevalence of cytomegalovirus infection in homosexual men. *J Infect Dis* 143:188-92.
- Drew WL, Sweet ES, Miner RC, Mocarski ES (1984) Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: documentation by Southern blot hybridization. *J Infect Dis* 150:952-3.
- Dubin G, Frank I, Friedman HM (1990) Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. *J Virol* 64:2725-2731.
- Dubin G, Socolof E, Frank I, Friedman HM (1991) Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. *J Virol* 65:7046-7050.
- Duncan AJ, Dummer JS, Paradis IL, Dauber JH, Yousem SA, Zenati MA, Kormos RL, Griffith BP (1991) Cytomegalovirus infection and survival in lung transplant recipients. *J Heart Lung Transplant* 10:638-44; discussion 645-6.
- Duncan SR, Grgurich WF, Iacono AT, Burckart GJ, Yousem SA, Paradis, IL, Williams PA, Johnson BA, Griffith BP (1994) A comparison of ganciclovir and acyclovir to prevent cytomegalovirus after lung transplantation. *Am J Respir Crit Care Med* 150:146-152.
- Duncombe AS, Meager A, Prentice HG, Grundy JE, Heslop HE, Hoffbrand AV, Brenner MK (1990) Gamma-interferon and tumor necrosis factor production after bone marrow transplantation is augmented by exposure to marrow fibroblasts infected with cytomegalovirus. *Blood* 76:1046-1053.
- Dunn DL, Mayoral JL, Gillingham KJ, Loeffler CM, Brayman KL, Kramer MA, Erice A, Balfour HH, Jr., Fletcher CV, Bolman RM, 3d, et al (1991) Treatment of invasive cytomegalovirus disease in solid organ transplant patients with ganciclovir. *Transplantation* 51:98-106.
- Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA (1986) Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol* 137:245-254.

Egan JJ, Lomax J, Barber L, Lok SS, Martyszcuk R, Yonan N, Fox A, Deiraniya AK, Turner AJ, Woodcock AA (1998) Preemptive treatment for the prevention of cytomegalovirus disease: in lung and heart transplant recipients. *Transplantation* 65:747-752.

Egbert PR, Pollard RB, Gallagher JG, Merigan TC (1980) Cytomegalovirus retinitis in immunosuppressed hosts. II. Ocular manifestations. *Ann Intern Med* 93:664-670.

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

Emans N, Gorvel JP, Walter C, Gerke V, Kellner R, Griffiths G, Gruenberg J (1993) Annexin II is a major component of fusogenic endosomal vesicles. *J Cell Biol* 120:1357-1369.

Emanuel D, Cunningham I, Jules Elysee K, Brochstein JA, Kernan NA, Laver J, Stover D, White DA, Fels A, Polsky B, 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.

Fabricant CG, Fabricant J, Litrenta MM, Minick CR (1978) Virus-induced atherosclerosis. *J Exp Med* 148:335-340.

Fabricant CG, Fabricant J, Minick CR, Litrenta MM (1983) Herpesvirus-induced atherosclerosis in chickens. *Fed Proc* 42:2476-2479.

Fantone JC (1997) Cytokines and Neutrophils: Neutrophil -Derived Cytokines and the Inflammatory Response. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 373-380. New York: Marcel Dekker, Inc.

Farrell HE, Vally H, Lynch DM, Fleming P, Shellam GR, Scalzo AA, Davis Poynter NJ (1997) Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo [see comments]. *Nature* 386:510-514.

Fearon DT (1993) The CD19-CR2-TAPA-1 complex, CD45 and signaling by the antigen receptor of B lymphocytes. *Curr Opin Immunol* 5:341-8.

Fernando S, Booth J, Boriskin Y, Butcher P, Carrington D, Steel H, Tryhorn Y, Corbishley C, Keeling P, Murday A, et al (1994) Association of cytomegalovirus infection with post-transplantation cardiac rejection as studied using the polymerase chain reaction. *J Med Virol* 42:396-404.

Ferran C, Peuchmaur M, Desruennes M, Ghousoub JJ, Cabrol A, Brousse N, Cabrol C, Bach JF, Chatenoud L (1993) Implications of de novo ELAM-1 and VCAM-1 expression in human cardiac allograft rejection. *Transplantation* 55:605-609.

Fletcher JM, Prentice HG, Grundy JE (1998) Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV induced down-regulation of cell surface class I HLA. *The Journal of Immunology* (In Press)

Forbes R, Guttmann R, Gomersall M, Hibberd J (1983) A Controlled Serial Ultrastructural tracer Study of First-Set Cardiac Allograft Rejection in the Rat. Evidence that the Microvascular Endothelium is the Primary Target of Graft Destruction. *American Journal of Pathology* 111:184-196.

Foucar E, Mukai K, Foucar K, Sutherland DE, Van Buren CT (1981) Colon ulceration in lethal cytomegalovirus infection. *Am J Clin Pathol* 76:788-801.

Fowler KB, Stagno S, Pass RF, Britt WJ, Boll TJ, Alford CA (1992) The outcome of congenital cytomegalovirus infection in relation to maternal antibody status [see comments]. *N Engl J Med* 326:663-667.

Frank I, Friedman HM (1989) A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. *J Virol* 63:4479-4488.

Frazier DP, Cox D, Godshalk EM, Schaffer PA (1996a) Identification of cis-acting sequences in the promoter of the herpes simplex virus type 1 latency-associated transcripts required for activation by nerve growth factor and sodium butyrate in PC12 cells. *J Virol* 70:7433-7444.

Frazier DP, Cox D, Godshalk EM, Schaffer PA (1996b) The herpes simplex virus type 1 latency-associated transcript promoter is activated through Ras and Raf by nerve growth factor and sodium butyrate in PC12 cells. *J Virol* 70:7424-7432.

Friedman HM, Macarak EJ, MacGregor RR, Wolfe J, Kefalides NA (1981) Virus infection of endothelial cells. *J Infect Dis* 143:266-273.

Fujinami RS, Nelson JA, Walker L, Oldstone MB (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.

Furukawa T, Hornberger E, Sakuma S, Plotkin SA (1975) Demonstration of immunoglobulin G receptors induced by human cytomegalovirus. *J Clin Microbiol* 2:332-336.

Furukawa T, Jisaki F, Sakamuro D, Takegami T, Murayama T (1994) Detection of human cytomegalovirus genome in uterus tissue. *Arch Virol* 135:265-277.

Gallo D (1986) Elimination of Fc receptor binding of human immunoglobulin G in immunofluorescence assays for herpes simplex virus antibodies. *J Clin Microbiol* 24:672-674.

Gerna G, Zipeto D, Percivalle E, Parea M, Revello MG, Maccario R, Peri G, Milanese G (1992) Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. *J Infect Dis* 166:1236-1244.

Ghersa P, Hooft van Huijsduijnen R, Whelan J, Cambet Y, Pescini R, DeLamarter JF (1994) Inhibition of E-selectin gene transcription through a cAMP-dependent protein kinase pathway. *J Biol Chem* 269:29129-29137.

Gilbert MJ, Riddell SR, Plachter B, Greenberg PD (1996) Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* 383:720-722.

Gimbrone MA, Jr., Obin MS, Brock AF, Luis EA, Hass PE, Hebert CA, Yip YK, Leung DW, Lowe DG, Kohr WJ, et al (1989) Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. *Science* 246:1601-1603.

Gnann JW, Jr., Ahlmen J, Svalander C, Olding L, Oldstone MB, Nelson JA (1988) Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. *Am J Pathol* 132:239-48.

Gonczol E, Andrews PW, Plotkin SA (1984) Cytomegalovirus replicates in differentiated but not in undifferentiated human embryonal carcinoma cells. *Science* 224:159-161.

Goodman MD, Porter DD (1973) Cytomegalovirus vasculitis with fatal colonic hemorrhage. *Arch Pathol* 96:281-4.

Goodrich JM, Mori M, Gleaves CA, Du Mond C, Cays M, Ebeling DF, Buhles WC, DeArmond B, Meyers JD (1991) Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. *N Engl J Med* 325:1601-7.

Goral S, Helderman JH (1994) Cytomegalovirus and rejection. *Transplant Proc* 26:5-6.

Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A (1981) *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* 305:1425-1431.

Gouw AS, Huitema S, Grond J, Slooff MJ, Klompmaier IJ, Gips CH, Poppema S (1988) Early induction of MHC antigens in human liver grafts. An immunohistologic study. *Am J Pathol* 133:82-94.

Gratama JW (1989) Graft-versus-host disease and viral infections. *Curr Opin Immunol* 1:1221-4.

Grattan MT, Moreno Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE (1989) Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *JAMA* 261:3561-3566.

Greenberg P, Goodrich J, Riddell S (1991) Adoptive immunotherapy of human cytomegalovirus infection: potential role in protection from disease progression. *Transplant Proc* 23:97-101.

Grefte A, Blom N, van der Giessen M, van Son W, The TH (1993) Ultrastructural analysis of circulating cytomegalic cells in patients with active cytomegalovirus infection: evidence for virus production and endothelial origin. *J Infect Dis* 168:1110-1118.

Grefte JM, van der Gun BT, Schmolke S, van der Giessen M, van Son WJ, Plachter B, Jahn G, The TH (1992a) Cytomegalovirus antigenemia assay: identification of the viral antigen as the lower matrix protein pp65 [letter]. *J Infect Dis* 166:683-4.

Grefte JM, van der Gun BT, Schmolke S, van der Giessen M, van Son WJ, Plachter B, Jahn G, The TH (1992b) The lower matrix protein pp65 is the principal viral antigen present in peripheral blood leukocytes during an active cytomegalovirus infection. *J Gen Virol* 73:2923-2932.

Grefte JM, van der Giessen M, Blom N, The TH, van Son WJ (1995) Circulating cytomegalovirus-infected endothelial cells after renal transplantation: possible clue to pathophysiology? *Transplant Proc* 27:939-942.

Gregory CD, Murray RJ, Edwards CF, Rickinson AB (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 DR, Kari B, Rasmussen L, Gehrz RC, Stinski MF (1988) Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. *J Virol* 62:875-81.

Griffith RL, Virella GT, Stevenson HC, Lopes Virella MF (1988) Low density lipoprotein metabolism by human macrophages activated with low density lipoprotein immune complexes. A possible mechanism of foam cell formation. *J Exp Med* 168:1041-1059.

Grundy JE, Shanley JD, Griffiths PD (1987) Is cytomegalovirus interstitial pneumonitis in transplant recipients an immunopathological condition? *Lancet* 2:996-999.

Grundy JE, Ayles HM, McKeating JA, Butcher RG, Griffiths PD, Poulter LW (1988) Enhancement of class I HLA antigen expression by cytomegalovirus: role in amplification of virus infection. *J Med Virol* 25:483-495.

Grundy JE, Pahal GS, Akbar AN (1993) Increased adherence of CD2 peripheral blood lymphocytes to cytomegalovirus-infected fibroblasts is blocked by anti-LFA-3 antibody. *Immunology* 78:413-420.

Grundy JE (1998) Current antiviral therapy fails to prevent the pro-inflammatory effects of cytomegalovirus infection, whilst rendering infected cells relatively resistant to immune attack. *Monogr Virol* 21:67-89.

Grundy JE, Lawson KM, MacCormac LP, Fletcher JM, Yong KL (1998) Cytomegalovirus-infected endothelial cells recruit neutrophils by the secretion of C-X-C chemokines and transmit virus by direct neutrophil-endothelial cell contact and during neutrophil transendothelial migration. *J Infect Dis* 177:1465-1474.

Grundy JE, Downes KL (1993) Up-regulation of LFA-3 and ICAM-1 on the surface of fibroblasts infected with cytomegalovirus. *Immunology* 78:405-412.

Gyorkey F, Melnick JL, Guinn GA, Gyorkey P, DeBakey ME (1984) Herpesviridae in the endothelial and smooth muscle cells of the proximal aorta in arteriosclerotic patients. *Exp Mol Pathol* 40:328-39.

Hakkert BC, Kuijpers TW, Leeuwenberg JF, van Mourik JA, Roos D (1991) Neutrophil and monocyte adherence to and migration across monolayers of cytokine-activated endothelial cells: the contribution of CD18, ELAM-1, and VLA-4. *Blood* 78:2721-2726.

Hall BM, Bishop GA, Duggin GG, Horvath JS, Philips J, Tiller DJ (1984) Increased expression of HLA-DR antigens on renal tubular cells in renal transplants: relevance to the rejection response. *Lancet* 2:247-251.

Hancock WH, Whitley WD, Tullius SG, Heemann UW, Wasowska B, Baldwin WM, 3d, Tilney NL (1993) Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. *Transplantation* 56:643-650.

Hanshaw JB, Scheiner AP, Moxley AW, Gaev L, Abel V, Scheiner B (1976) School failure and deafness after "silent" congenital cytomegalovirus infection. *N Engl J Med* 295:468-70.

Harlan JM (1987) Consequences of leukocyte-vessel wall interactions in inflammatory and immune reactions. *Semin Thromb Hemost* 13:434-44.

Harper DR (1994) Herpesvirus assembly proteins. *Rev Med Virol* 4:119-128.

- Hayashi K, Suwa Y, Shimomura Y, Ohashi Y (1995) Pathogenesis of ocular cytomegalovirus infection in the immunocompromised host. *J Med Virol* 47:364-369.
- Hayry P, von Willebrand E, Parthenais E, Nemlander A, Soots A, Lautenschlager I, Alföldy P, Renkonen R (1984) The inflammatory mechanisms of allograft rejection. *Immunol Rev* 77:-1989.
- Hayry P, Mennander A, Yilmaz S, Ustinov J, Raisanen A, Lautenschlager I, Lemstrom K, Bruggeman CA, Paavonen T (1992a) Cellular and molecular mechanisms in allograft arteriosclerosis. *Transplant Proc* 24:2359-2361.
- Hayry P, Mennander A, Yilmaz S, Ustinov J, Raisanen A, Miettinen A, Lautenschlager I, Lemstrom K, Bruggeman CA, Paavonen T (1992b) Towards understanding the pathophysiology of chronic rejection. *Clin Investig* 70:780-790.
- Hayry P, Isoniemi H, Yilmaz S, Mennander A, Lemstrom K, Raisanen-Sokolowski A, Koskinen P, Ustinov J, Lautenschlager I, Taskinen E, Krogerus L, Aho P, Paavonen T (1993) Chronic Allograft Rejection. *Immunological Reviews* 134:33-81.
- Hayward AR, Herberger MJ, Groothuis J, Levin MR (1984) Specific immunity after congenital or neonatal infection with cytomegalovirus or herpes simplex virus. *J Immunol* 133:2469-2473.
- Hebert CA, Luscinskas FW, Kiely JM, Luis EA, Darbonne WC, Bennett GL, Liu CC, Obin MS, Gimbrone MA, Jr., Baker JB (1990) Endothelial and leukocyte forms of IL-8. Conversion by thrombin and interactions with neutrophils. *J Immunol* 145:3033-3040.
- Hechtman DH, Cybulsky MI, Fuchs HJ, Baker JB, Gimbrone MA, Jr. (1991) Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. *J Immunol* 147:883-892.
- Hedley-White ET, Craighead JE (1965) Generalised cytomegalic inclusion disease after renal transplantation. Report of a case with isolation of virus. *N Eng J Med* 272:473-475.
- Heemann UW, Tullius SG, Azuma H, Kupiec Weglinsky J, Tilney NL (1994) Adhesion molecules and transplantation [see comments]. *Ann Surg* 219:4-12.
- Heise MT, Connick M, Virgin HW, 4th (1998a) Murine cytomegalovirus inhibits interferon gamma-induced antigen presentation to CD4 T cells by macrophages via regulation of expression of major histocompatibility complex class II-associated genes. *J Exp Med* 187:1037-1046.
- Heise MT, Pollock JL, O'Guin A, Barkon ML, Bormley S, Virgin HV, 4th (1998b) Murine cytomegalovirus infection inhibits IFN gamma-induced MHC class II expression on macrophages: the role of type I interferon. *Virology* 241:331-344.
- Hendrix RM, Wagenaar M, Slobbe RL, Bruggeman CA (1997) Widespread presence of cytomegalovirus DNA in tissues of healthy trauma victims. *J Clin Pathol* 50:59-63.
- Hengel H, Lucin P, Jonjic S, Ruppert T, Koszinowski UH (1994) Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. *J Virol* 68:289-297.
- Herberman RB, Ortaldo JR (1981) Natural killer cells: their roles in defenses against disease. *Science* 214:24-30.

- Herrera GA, Alexander RW, Cooley CF, Luke RG, Kelly DR, Curtis JJ, Gockerman JP (1986) Cytomegalovirus glomerulopathy: a controversial lesion. *Kidney Int* 29:725-733.
- Hersman J, Meyers JD, Thomas ED, Buckner CD, Clift R (1982) The effect of granulocyte transfusions on the incidence of cytomegalovirus infection after allogeneic marrow transplantation. *Ann Intern Med* 96:149-152.
- Ho DD, Rota TR, Andrews CA, Hirsch MS (1984) Replication of human cytomegalovirus in endothelial cells. *J Infect Dis* 150:956-7.
- Ho M, Suwansirikul S, Dowling JN, Youngblood LA, Armstrong JA (1975) The transplanted kidney as a source of cytomegalovirus infection. *N Engl J Med* 293:1109-1112.
- Ho M (1977) Virus infections after transplantation in man. Brief review. *Arch Virol* 55:1-24.
- Ho M (1991a) Congenital and perinatal human cytomegalovirus infections. In: *Cytomegalovirus: Biology and infection* (Ho M ed), pp 205-227. New York/London: Plenum Medical Book Co.
- Ho M (1991b) Epidemiology of cytomegalovirus infection in man. In: *Cytomegalovirus: Biology and infection* (Ho M ed), pp 155-187. New York/London: Plenum Medical Book Co.
- Ho WZ, Cherukuri R, Ge SD, Cutilli JR, Song L, Whitko S, Douglas SD (1993) Centrifugal enhancement of human immunodeficiency virus type 1 infection and human cytomegalovirus gene expression in human primary monocyte/macrophages in vitro. *J Leukoc Biol* 53:208-212.
- Hodgkin PD, Scalzo AA, Swaminathan N, Price P, Shellam GR (1988) Murine cytomegalovirus binds reversibly to mouse embryo fibroblasts: implications for quantitation and explanation of centrifugal enhancement. *J Virol Methods* 22:215-230.
- Hogg N (1992) Roll, roll, roll your leukocyte gently down the vein... *Immunology Today* 13:113-115.
- Horwitz CA, Henle W, Henle G, Polesky H, Balfour HH, Jr., Siem RA, Borken S, Ward PC (1977) Heterophil-negative infectious mononucleosis and mononucleosis-like illnesses. Laboratory confirmation of 43 cases. *Am J Med* 63:947-957.
- Hosenpud JD, Chou SW, Wagner CR (1991) Cytomegalovirus-induced regulation of major histocompatibility complex class I antigen expression in human aortic smooth muscle cells. *Transplantation* 52:896-903.
- Hruban RH, Beschoner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM (1990) Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. *Am J Pathol* 137:871-882.
- Hu KQ, Yu CH, Vierling JM (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 AR, Kunkel SL, Todd RF, 3d, Weiss SJ (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8 [published errata appear in *Science* 1991 Nov 1;254(5032):631 and 1991 Dec 6;254(5037):1435]. *Science* 254:99-102.

- Hullett MD, Hogarth PM (1998) Molecular basis of Fc receptor function. In: *Advances in Immunology* (Dixon FJ ed), pp 1-127. Sandiego CA: Academic Press.
- Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.
- Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA (1991) Human cytomegalovirus productively infects primary differentiated macrophages. *J Virol* 65:6581-6588.
- Ibrahim L, Dominguez M, Yacoub M (1993) Primary human adult lung epithelial cells in vitro: response to interferon-gamma and cytomegalovirus. *Immunology* 79:119-124.
- Irmiere A, Gibson W (1985) Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. *J Virol* 56:277-83.
- Isobe M, Yagita H, Okumura K, Ihara A (1992) Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 255:1125-1127.
- Issekutz AC, Chuluyan HE, Lopes N (1995) CD11/CD18-independent transendothelial migration of human polymorphonuclear leukocytes and monocytes: involvement of distinct and unique mechanisms. *J Leukoc Biol* 57:553-561.
- Ito M, Watanabe M, Ihara T, Kamiya H, Sakurai M (1995) Increased expression of adhesion molecules (CD54, CD29 and CD44) on fibroblasts infected with cytomegalovirus. *Microbiol Immunol* 39:129-133.
- Iwamoto GK, Monick MM, Clark BD, Auron PE, Stinski MF, Hunninghake GW (1990) Modulation of interleukin 1 beta gene expression by the immediate early genes of human cytomegalovirus. *J Clin Invest* 85:1853-1857.
- Jacobson MA, Mills J (1988) Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. *Ann Intern Med* 108:585-594.
- Janeway CA, Travers P (1996a) The humoral immune response. In: *Immunobiology: The immune system in health and disease* (Janeway CA, Travers P eds), pp 8.1-8.52. London/New York: Current Biology Ltd/Garland Publishing Inc.
- Janeway CA, Travers P (1996b) Host Defence Against Infection. In: *Immunobiology: The immune system in health and disease* (Janeway CA., Travers P eds), pp 9.1-9.46. London/New York: Current Biology Inc/Garland Publishing Inc.
- Janeway CA, Travers P (1996c) T-cell mediated immunity. In: *Immunobiology: The immune system in health and disease* (Janeway CA, Travers P eds), pp 7.1-7.41. London/New York: Current Biology Inc/Garland Publishing Inc.
- Janeway CA, Jr. (1992) The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu Rev Immunol* 10:
- Johansson PJ, Myhre EB, Blomberg J (1985) Specificity of Fc receptors induced by herpes simplex virus type 1: comparison of immunoglobulin G from different animal species. *J Virol* 56:489-494.

Johnston RB, Jr. (1988) Current concepts: immunology. Monocytes and macrophages. *N Engl J Med* 318:747-52.

Jones DA, Smith CW, Picker LJ, McIntire LV (1996) Neutrophil adhesion to 24-hour IL-1-stimulated endothelial cells under flow conditions. *J Immunol* 157:858-863.

Jones TR, Hanson LK, Sun L, Slater JS, Stenberg RM, Campbell AE (1995) Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J Virol* 69:4830-4841.

Jones TR, Wiertz EJ, Sun L, Fish KN, Nelson JA, Ploegh HL (1996) Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc Natl Acad Sci U S A* 93:11327-11333.

Jonjic S, Mutter W, Weiland F, Reddehase MJ, Koszinowski UH (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, Koszinowski UH (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, Koszinowski UH (1994) Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J Exp Med* 179:1713-1717.

Jordan ML, Hrebinko RL, Jr., Dummer JS, Hickey DP, Shapiro R, Vivas CA, Simmons RL, Starzl TE, Hakala TR (1992) Therapeutic use of ganciclovir for invasive cytomegalovirus infection in cadaveric renal allograft recipients. *J Urol* 148:1388-92.

Kapasi K, Rice GP (1988) Cytomegalovirus infection of peripheral blood mononuclear cells: effects on interleukin-1 and -2 production and responsiveness. *J Virol* 62:3603-3607.

Kari B, 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, Gehrz R (1993) Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II. *J Gen Virol* 74:255-264.

Karupiah G, Harris N (1997) Gamma Interferon-Induced Nitric Oxide in Antiviral Defence and Immunopathology. In: *Gamma Interferon in Antiviral Defence* (Karupiah G ed), pp 119-142. R.G. Landes Company.

Kavanaugh AF, Lightfoot E, Lipsky PE, Oppenheimer Marks N (1991) Role of CD11/CD18 in adhesion and transendothelial migration of T cells. Analysis utilizing CD18-deficient T cell clones. *J Immunol* 146:4149-4156.

Keay S, Merigan TC, 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, Baldwin B (1991) Anti-idiotypic antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. *J Virol* 65:5124-5128.

Keay S, Baldwin B (1992) The human fibroblast receptor for gp86 of human cytomegalovirus is a phosphorylated glycoprotein. *J Virol* 66:4834-4838.

Keller R, Peitchel R, Goldman JN, Goldman M (1976) An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts. *J Immunol* 116:772-777.

Khoury EL, Pereira L, Greenspan FS (1991) Induction of HLA-DR expression on thyroid follicular cells by cytomegalovirus infection in vitro. Evidence for a dual mechanism of induction. *Am J Pathol* 138:1209-1223.

Kirby JA, Rajasekar MR, Lin Y, Proud G, Taylor RM (1993) Interaction between T lymphocytes and kidney epithelial cells during renal allograft rejection. *Kidney Int Suppl* 39:S124-8.

Kirman N, Ginn RK, Mittal KK, Manischewitz JF, Quinnan GV, Jr. (1981) Cytomegalovirus-specific cytotoxicity mediated by non-T lymphocytes from peripheral blood of normal volunteers. *Infect Immun* 34:441-447.

Kline JN, Geist LJ, Monick MM, Stinski MF, Hunninghake GW (1994) Regulation of expression of the IL-1 receptor antagonist (IL-1ra) gene by products of the human cytomegalovirus immediate early genes. *J Immunol* 152:2351-2357.

Knight DA, Waldman WJ, Sedmak DD (1997) Human cytomegalovirus does not induce human leukocyte antigen class II expression on arterial endothelial cells. *Transplantation* 63:1366-1369.

Kobayashi T, Yokoyama I, Nishiyama Y, Hayashi S, Uchida K, Takagi H (1995) Strong enhancement of HLA class I surface expression by incomplete cytomegalovirus particles: analysis by dual-color flow cytometry using saponin. *Transplant Proc* 27:966-969.

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis [see comments]. *Science* 258:1798-801.

Kondo K, Kaneshima H, Mocarski ES (1994) Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proc Natl Acad Sci U S A* 91:11879-11883.

Koskinen P, Nieminen M, Mattila S, Lautenschlager I (1993) Quantitative immunodetection of cytomegalovirus antigenemia in the clinical diagnosis of CMV infection after heart transplantation. *Transplant Proc* 25:1426.

Koskinen P, Lemstrom K, Bruggeman C, Lautenschlager I, 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.

Koskinen PK (1993) The association of the induction of vascular cell adhesion molecule-1 with cytomegalovirus antigenemia in human heart allografts. *Transplantation* 56:1103-1108.

Koskinen PK, Krogerus LA, Nieminen MS, Mattila SP, Hayry PJ, Lautenschlager IT (1993a) Quantitation of cytomegalovirus infection-associated histologic findings in endomyocardial biopsies of heart allografts. *J Heart Lung Transplant* 12:343-354.

Koskinen PK, Nieminen MS, Krogerus LA, Lemstrom KB, Mattila SP, Hayry PJ, Lautenschlager IT (1993b) Cytomegalovirus infection and accelerated cardiac allograft vasculopathy in human cardiac allografts. *J Heart Lung Transplant* 12:724-729.

Koskinen PK, Nieminen MS, Mattila SP, Hayry PJ, Lautenschlager IT (1993c) The correlation between symptomatic CMV infection and CMV antigenemia in heart allograft recipients. *Transplantation* 55:547-551.

Koskinen PK, Krogerus LA, Nieminen MS, Mattila SP, Hayry PJ, Lautenschlager IT (1994) Cytomegalovirus infection-associated generalized immune activation in heart allograft recipients: a study of cellular events in peripheral blood and endomyocardial biopsy specimens. *Transpl Int* 7:163-171.

Koskinen PK, Yilmaz S, Kallio E, Bruggeman CA, Hayry PJ, Lemstrom K (1996) Rat cytomegalovirus infection and chronic kidney allograft rejection. *Transpl Int* 9 Suppl 1:S3-4.

Koszinowski UH, Reddehase MJ, Jonjic S (1991) The role of CD4 and CD8 T cells in viral infections. *Curr Opin Immunol* 3:471-475.

Kraus E, Schneider Schaulies S, Miyasaka M, Tamatani T, 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.

Kuijpers TW (1993) Terminal glycosyltransferase activity: a selective role in cell adhesion. *Blood* 81:873-882.

Kunkel SL, Lukacs NW, Chensue SW, Strieter RM (1997) Chemokines and the Inflammatory Response. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 121-132. New York: Marcel Dekker, Inc.

Labarrere CA, Pitts D, Nelson DR, Faulk WP (1995) Coronary artery disease in cardiac allografts: association with arteriolar endothelial HLA-DR and ICAM-1 antigens. *Transplant Proc* 27:1939-40.

Lafemina RL, Hayward GS (1988) Differences in cell-type-specific blocks to immediate early gene expression and DNA replication of human, simian and murine cytomegalovirus. *J Gen Virol* 69:355-374.

Landini MP, Spaete RR (1993) Human cytomegalovirus structural proteins: a report of the first nomenclature workshop. In: *Multidisciplinary approaches to understanding cytomegalovirus disease* (Michelson S, Plotkin SA eds), pp 65-74. Amsterdam: Elsevier.

Lanier LL, Corliss B, Phillips JH (1997) Arousal and Inhibition of human NK Cells. *Immunol Rev* 155:145-154.

Lapierre LA, Fiers W, Pober JS (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.

Lautenschlager I, Hockerstedt K, Taskinen E, von Willebrand E (1996) Expression of adhesion molecules and their ligands in liver allografts during cytomegalovirus (CMV) infection and acute rejection. *Transpl Int* 9 Suppl 1:S213-5.

Ledebur HC, Parks TP (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.

Leeuwenberg JF, Jeunhomme GM, Buurman WA (1990a) Adhesion of polymorphonuclear cells to human endothelial cells. Adhesion-molecule-dependent, and Fc receptor-mediated adhesion-molecule-independent mechanisms. *Clin Exp Immunol* 81:496-500.

Leeuwenberg JF, von Asmuth EJ, Jeunhomme TM, Buurman WA (1990b) IFN-gamma regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells in vitro. *J Immunol* 145:2110-2114.

Lemstrom K, Sihvola R, Bruggeman C, Hayry P, Koskinen P (1997) Cytomegalovirus infection-enhanced cardiac allograft vasculopathy is abolished by DHPG prophylaxis in the rat. *Circulation* 95:2614-2616.

Lemstrom KB, Bruning JH, Bruggeman CA, Koskinen PK, Aho PT, Yilmaz S, Lautenschlager IT, Hayry PJ (1994) Cytomegalovirus infection-enhanced allograft arteriosclerosis is prevented by DHPG prophylaxis in the rat. *Circulation* 90:1969-1978.

Ley K, Baker JB, Cybulsky MI, Gimbrone MA, Jr., Luscinskas FW (1993) Intravenous interleukin-8 inhibits granulocyte emigration from rabbit mesenteric venules without altering L-selectin expression or leukocyte rolling. *J Immunol* 151:6347-6357.

Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR (1994) Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. *Blood* 83:1971-9.

Li FL, Grauls G, Yin M, Bruggeman CA (1996) Correlation between the intensity of cytomegalovirus infection and the amount of perivascularitis in aortic allografts. *Transpl Int* 9 Suppl 1:S340-4.

Li R, Nortamo P, Valmu L, Tolvanen M, Huuskonen J, Kantor C, Gahmberg CG (1993) A peptide from ICAM-2 binds to the leukocyte integrin CD11a/CD18 and inhibits endothelial cell adhesion. *J Biol Chem* 268:17513-17518.

Lin CS, Penha PD, Krishnan MN, Zak FG (1981) Cytomegalic inclusion disease of the skin. *Arch Dermatol* 117:282-284.

Lindsley MD, Torpey DJ, 3d, Rinaldo CR, Jr. (1986) HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by Leu-3-positive T cells. *J Immunol* 136:3045-3051.

Littler E, Stuart AD, Chee MS (1992) Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature* 358:160-2.

Litwin V, Sandor M, Grose C (1990) Cell surface expression of the varicella-zoster virus glycoproteins and Fc receptor. *Virology* 178:263-272.

Litwin V, Jackson W, Grose C (1992) Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *J Virol* 66:3643-3651.

- Liu B, Stinski MF (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.
- Loebe M, Schuler S, Zais O, Warnecke H, Fleck E, Hetzer R (1990) Role of cytomegalovirus infection in the development of coronary artery disease in the transplanted heart. *J Heart Transplant* 9:707-711.
- Long EO, Burshtyn DN, Clark WP, Peruzzi M, Rajagopalan S, Rojo S, Wagtmann N, Winter CC (1997) Killer cell inhibitory receptors: diversity, specificity, and function. *Immunol Rev* 155:135-144.
- Lucin P, Pavic I, Polic B, Jonjic S, Koszinowski UH (1992) Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. *J Virol* 66:1977-1984.
- Luscinskas FW, Cybulsky MI, Kiely JM, Peckins CS, Davis VM, Gimbrone MA, Jr. (1991) Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. *J Immunol* 146:1617-1625.
- Luscinskas FW, Kiely JM, Ding H, Obin MS, Hebert CA, Baker JB, Gimbrone MA, Jr. (1992) In vitro inhibitory effect of IL-8 and other chemoattractants on neutrophil-endothelial adhesive interactions. *J Immunol* 149:2163-2171.
- Luscinskas FW, Gimbrone MA, Jr. (1996) Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. *Annu Rev Med* 47:413-421.
- Maciejewski JP, Bruening EE, Donahue RE, Mocarski ES, Young NS, St Jeor SC (1992) Infection of hematopoietic progenitor cells by human cytomegalovirus. *Blood* 80:170-178.
- Mahe Y, Mukaida N, Kuno K, Akiyama M, Ikeda N, Matsushima K, Murakami S (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.
- Maidji E, Tugizov S, Jones T, Zheng Z, Pereira L (1996) Accessory human cytomegalovirus glycoprotein US9 in the unique short component of the viral genome promotes cell-to-cell transmission of virus in polarized epithelial cells. *J Virol* 70:8402-8410.
- Manez R, White LT, Kusne S, Martin M, Demetris AJ, Starzl TE, Duquesnoy RJ (1993) Association between donor-recipient HLA-DR compatibility and cytomegalovirus hepatitis and chronic rejection in liver transplantation. *Transplant Proc* 25:908-9.
- Mantovani A, Dejana E, Introna M, Bussolino F (1997) Cytokines and endothelial cells. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 323-338. New York: Marcel Dekker, Inc.
- Mayer A, Podlech J, Kurz S, Steffens HP, Maiberger S, Thalmeier K, Angele P, Dreher L, Reddehase MJ (1997) Bone marrow failure by cytomegalovirus is associated with an in vivo deficiency in the expression of essential stromal hemopoietin genes. *J Virol* 71:4589-4598.

McDonald K, Rector TS, Braulin EA, Kubo SH, Olivari MT (1989) Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus infection [see comments]. *Am J Cardiol* 64:359-362.

McEver RP (1992) Leukocyte-Endothelial Cell Interactions. *Current Opinions in Cell biology* 4:840-849.

McTaggart SP, Burns WH, White DO, Jackson DC (1978) Fc receptors induced by herpes simplex virus. I. Biologic and biochemical properties. *J Immunol* 121:726-730.

Meier JL, Stinski MF (1997) Effect of a modulator deletion on transcription of the human cytomegalovirus major immediate-early genes in infected undifferentiated and differentiated cells. *J Virol* 71:1246-1255.

Meyers JD, Flournoy N, Thomas ED (1986) Risk factors for cytomegalovirus infection after human marrow transplantation. *J Infect Dis* 153:478-488.

Miller DM, Rahill BM, Boss JM, Lairmore MD, Durbin JE, Waldman JW, Sedmak DD (1998) Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. *J Exp Med* 187:675-83.

Miller W, Flynn P, McCullough J, Balfour HH, Jr., Goldman A, Haake R, McGlave P, Ramsay N, Kersey J (1986) Cytomegalovirus infection after bone marrow transplantation: an association with acute graft-v-host disease. *Blood* 67:1162-1167.

Milton AD, Fabre JW (1985) Massive induction of donor-type class I and class II major histocompatibility complex antigens in rejecting cardiac allografts in the rat. *J Exp Med* 161:98-112.

Minton EJ, Tysoe C, Sinclair JH, Sissons JG (1994) Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. *J Virol* 68:4017-4021.

Miossec P, Cavender D, Ziff M (1986) Production of interleukin 1 by human endothelial cells. *J Immunol* 136:2486-2491.

Mocarski ES, Liu AC, Spaete RR (1987) Structure and variability of the a sequence in the genome of human cytomegalovirus (Towne strain). *J Gen Virol* 68:2223-30.

Mocarski ES, Bonyhadi M, Salimi S, McCune JM, Kaneshima H (1993) Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. *Proc Natl Acad Sci U S A* 90:104-108.

Mocarski ES (1996) Cytomegaloviruses and their replication. In: *Fields Virology* (Fields BN, Knipe DM, Howley PM eds), pp 2447-2492. Philadelphia: Lippincott-Raven Publishers.

Mouthon L, Kaveri SV, Spalter SH, Lacroix-Desmazes S, Lefranc C, Desai R, Kazatchkine MD (1996) Mechanisms of action of intravenous immune globulin in immune-mediated diseases. [Review] [63 refs]. *Clin Exp Immunol* 104 Suppl 1:3-9.

Muller WA, Weigl SA, Deng X, Phillips DM (1993) PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* 178:449-460.

Murayama T, Shimokawa K, Furukawa T (1987) Inhibition of antibody-dependent cell-mediated cytolysis of tumor cells by Fc receptor of human cytomegalovirus. Brief report. Arch Virol 97:145-150.

Murayama T, Kuno K, Jisaki F, Obuchi M, Sakamuro D, Furukawa T, Mukaida N, Matsushima K (1994) Enhancement human cytomegalovirus replication in a human lung fibroblast cell line by interleukin-8. J Virol 68:7582-7585.

Murayama T, Ohara Y, Obuchi M, Khabar KS, Higashi H, Mukaida N, Matsushima K (1997) Human cytomegalovirus induces interleukin-8 production by a human monocytic cell line, THP-1, through acting concurrently on AP-1- and NF-kappaB-binding sites of the interleukin-8 gene. J Virol 71:5692-5695.

Mutter W, Reddehase MJ, Busch FW, Buhring HJ, Koszinowski UH (1988) Failure in generating hemopoietic stem cells is the primary cause of death from cytomegalovirus disease in the immunocompromised host. J Exp Med 167:1645-1658.

Naraqi S (1990) Cytomegaloviruses. In: Textbook of Human Virology (Belshe RB ed), pp 889-924. St. Louis, MO: Mosby Year Book, Inc.

Navarro D, Paz P, Tugizov S, Topp K, La Vail J, 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.

Neeffes JJ, Stollorz V, Peters PJ, Geuze HJ, Ploegh HL (1990) The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. Cell 61:171-83.

Neiman PE, Reeves W, Ray G, Flournoy N, Lerner KG, Sale GE, Thomas ED (1977) A prospective analysis of interstitial pneumonia and opportunistic viral infection among recipients of allogeneic bone marrow grafts. J Infect Dis 136:754-767.

Neish AS, Read MA, Thanos D, Pine R, Maniatis T, Collins T (1995) Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. Mol Cell Biol 15:2558-2569.

Nemlander A, Soots A, von Willebrand E, Husberg B, Hayry P (1982) Redistribution of renal allograft-responding leukocytes during rejection. II. Kinetics and specificity. J Exp Med 156:1087-1100.

Newman PJ (1997) The biology of PECAM-1. J Clin Invest 99:3-8.

Neyts J, Snoeck R, Schols D, Balzarini J, Esko JD, Van Schepdael A, De Clercq E (1992) Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. Virology 189:48-58.

Ng Bautista CL, Sedmak DD (1995) Cytomegalovirus infection is associated with absence of alveolar epithelial cell HLA class II antigen expression. J Infect Dis 171:39-44.

Niubo J, Perez JL, Martinez Lacasa JT, Garcia A, Roca J, Fabregat J, Gil Vernet S, Martin R (1996) Association of quantitative cytomegalovirus antigenemia with symptomatic infection in solid organ transplant patients. Diagn Microbiol Infect Dis 24:19-24.

- Normann SJ, Salomon DR, Leelachaikul P, Khan SR, Staples ED, Alexander JA, Mayfield WR, Knauf DG, Sadler LA, Selman S (1991) Acute vascular rejection of the coronary arteries in human heart transplantation: pathology and correlations with immunosuppression and cytomegalovirus infection. *J Heart Lung Transplant* 10:674-87.
- Nortamo P, Li R, Renkonen R, Timonen T, Prieto J, Patarroyo M, Gahmberg CG (1991) The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. *Eur J Immunol* 21:2629-2632.
- Nowlin DM, Cooper NR, Compton T (1991) Expression of a human cytomegalovirus receptor correlates with infectibility of cells. *J Virol* 65:3114-3121.
- O'Grady JG, Sutherland S, Harvey F, Calne RY, Alexander GJM, Donaldson PT, Portmann B, Williams R (1988) Cytomegalovirus Infection and Donor/Recipient HLA Antigens: Interdependent Co-factors in Pathogenesis of Vanishing Bile Duct Syndrome After Liver Transplantation. *The Lancet* ii:302-305.
- Oldstone MB (1975) Virus neutralization and virus-induced immune complex disease. Virus-antibody union resulting in immunoprotection or immunologic injury--two sides of the same coin. *Prog Med Virol* 19:84-119.
- Onorato M, Pollard RB (1996) Human cytomegalovirus infections in patients with HIV. In: Bailliere's Clinical Infectious Diseases. International practice and research (Arvin AM ed), pp 345-355. London/Philadelphia: Bailliere Tindall/W.B.Saunders Co.Ltd.
- Oppenheimer Marks N, Davis LS, Lipsky PE (1990) Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. *J Immunol* 145:140-148.
- Oppenheimer Marks N, Davis LS, Bogue DT, Ramberg J, Lipsky PE (1991) Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. *J Immunol* 147:2913-2921.
- Ouwehand AJ, Balk AH, Baan CC, Vaessen LM, Jutte NH, Bos E, Claas FH, Weimar W (1994) Cytomegalovirus infection and allospecific cytotoxic activity of graft-infiltrating cells after heart transplantation. *J Med Virol* 42:175-181.
- Paavonen T, Mennander A, Lautenschlager I, Hayry P (1992) Endothelialitis in accelerated allograft arteriosclerosis in human cardiac transplant recipients. *Transplant Proc* 24:342-3.
- Para MF, Baucke RB, Spear PG (1980) Immunoglobulin G(Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. *J Virol* 34:512-520.
- Para MF, Goldstein L, Spear PG (1982) Similarities and differences in the Fc-binding glycoprotein (gE) of herpes simplex virus types 1 and 2 and tentative mapping of the viral gene for this glycoprotein. *J Virol* 41:137-144.
- Parham P (1992) CD8 and CD4. The box and the rod [news]. *Nature* 357:538-9.
- Parra E, Wingren AG, Hedlund G, Sjogren HO, Kalland T, Sansom D, Dohlsten M (1993) Human naive and memory T-helper cells display distinct adhesion properties to ICAM-1, LFA-3 and B7 molecules. *Scand J Immunol* 38:508-514.

- Pass RF, Stagno S, Myers GJ, Alford CA (1980) Outcome of symptomatic congenital cytomegalovirus infection: results of long-term longitudinal follow-up. *Pediatrics* 66:758-62.
- Patel JA, Kunimoto M, Sim TC, Garofalo R, Elliott T, Baron S, Ruuskanen O, Chonmaitree T, Ogra PL, 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 Respir Cell Mol Biol* 13:602-609.
- Patel KD, Moore KL, Nollert MU, McEver RP (1995) Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. *J Clin Invest* 96:1887-1896.
- Paya CV, Hermans PE, Wiesner RH, Ludwig J, Smith TF, Rakela J, Krom RA (1989) Cytomegalovirus hepatitis in liver transplantation: prospective analysis of 93 consecutive orthotopic liver transplantations. *J Infect Dis* 160:752-758.
- Percivalle E, Revello MG, Vago L, Morini F, Gerna G (1993) Circulating endothelial giant cells permissive for human cytomegalovirus (HCMV) are detected in disseminated HCMV infections with organ involvement. *J Clin Invest* 92:663-670.
- Petrie BL, Melnick JL, Adam E, Burek J, McCollum CH, DeBakey ME (1987) Nucleic acid sequences of cytomegalovirus in cells cultured from human arterial tissue [letter]. *J Infect Dis* 155:158-159.
- Pietropaolo RL, Compton T (1997) Direct interaction between human cytomegalovirus glycoprotein B and cellular annexin II. *J Virol* 71:9803-7.
- Pinola M, Saksela E, Tiisala S, Renkonen R (1994) Human NK cells expressing alpha 4 beta 1/beta 7 adhere to VCAM-1 without preactivation. *Scand J Immunol* 39:131-136.
- Plotkin SA, Weibel RE, Alpert G, Starr SE, Friedman HM, Preblud SR, Hoxie J (1985) Resistance of seropositive volunteers to subcutaneous challenge with low-passage human cytomegalovirus. *J Infect Dis* 151:737-9.
- Plotkin SA, Starr SE, Friedman HM, Gonczol E, Weibel RE (1989) Protective effects of Towne cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge. *J Infect Dis* 159:860-865.
- Pober JS, Slowik MR, De Luca LG, Ritchie AJ (1993) Elevated cyclic AMP inhibits endothelial cell synthesis and expression of TNF-induced endothelial leukocyte adhesion molecule-1, and vascular cell adhesion molecule-1, but not intercellular adhesion molecule-1. *J Immunol* 150:5114-5123.
- Podzamczar D, Ferrer E, Garcia A, Ramon JM, Niubo J, Santin M, Ruffi G, Perez JL, Martin R, Gudiol F (1997) pp65 antigenemia as a marker of future CMV disease and mortality in HIV-infected patients. *Scand J Infect Dis* 29:223-227.
- Polis MA, deSmet MD, Baird BF, Mellow S, Falloon J, Davey RT, Jr., Kovacs JA, Palestine AG, Nussenblatt RB, Masur H, et al (1993) Increased survival of a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis who received sodium phosphonoformate (foscarnet) [see comments]. *Am J Med* 94:175-80.

- Przepiorka D, LeParc GF, Werch J, Lichtiger B (1996) Prevention of transfusion-associated cytomegalovirus infection. Practice parameter. American Society of Clinical Pathologists. *Am J Clin Pathol* 106:163-169.
- Qin S, La Rosa G, Campbell JJ, Smith-Heath H, Kassam N, Shi X, Zeng L, Buthcher EC, 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 GV, Jr., Kirmani N, Esber E, Saral R, Manischewitz JF, Rogers JL, Rook AH, Santos GW, Burns WH (1981) HLA-restricted cytotoxic T lymphocyte and nonthymic cytotoxic lymphocyte responses to cytomegalovirus infection of bone marrow transplant recipients. *J Immunol* 126:2036-41.
- Quinnan GV, Jr., Kirmani N, Rook AH, Manischewitz JF, Jackson L, Moreschi G, Santos GW, Saral R, Burns WH (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.
- Quinnan GV, Jr., Delery M, Rook AH, Frederick WR, Epstein JS, Manischewitz JF, Jackson L, Ramsey KM, Mittal K, Plotkin SA, et al (1984) Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. *Ann Intern Med* 101:478-483.
- Radsak K, Fuhrmann R, Franke RP, Schneider D, Kollert A, Brucher KH, Drenckhahn D (1989) Induction by sodium butyrate of cytomegalovirus replication in human endothelial cells. *Arch Virol* 107:151-158.
- Rager Zisman B, Grose C, Bloom BR (1976) Mechanism of selective nonspecific cell-mediated cytotoxicity of virus-infected cells. *Nature* 260:369-70.
- Raines EW, Ross R (1993) Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *Br Heart J* 69:S30-7.
- Rasmussen L, Kelsall D, Nelson R, Carney W, Hirsch M, Winston D, Preiksaitis J, Merigan TC (1982) Virus-specific IgG and IgM antibodies in normal and immunocompromised subjects infected with cytomegalovirus. *J Infect Dis* 145:191-199.
- Rasmussen L, Hong C, Zipeto D, Morris S, Sherman D, Chou S, Miner R, Drew WL, Wolitz R, Dowling A, Warford A, Merigan TC (1997) Cytomegalovirus gB genotype distribution differs in human immunodeficiency virus-infected patients and immunocompromised allograft recipients. *J Infect Dis* 175:179-184.
- Reddehase MJ, Weiland F, Munch K, Jonjic S, Luske A, Koszinowski UH (1985) Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. *J Virol* 55:264-273.
- Reddehase MJ, Balthesen M, Rapp M, Jonjic S, Pavic I, Koszinowski UH (1994) The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. *J Exp Med* 179:185-193.
- Reed EC, Bowden RA, Dandliker PS, Lilleby KE, Meyers JD (1988a) Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. *Ann Intern Med* 109:783-788.

Reed EC, Shepp DH, Dandliker PS, Meyers JD (1988b) Ganciclovir treatment of cytomegalovirus infection of the gastrointestinal tract after marrow transplantation. *Bone Marrow Transplant* 3:199-206.

Reichert CM, O'Leary TJ, Levens DL, Simrell CR, Macher AM (1983) Autopsy pathology in the acquired immune deficiency syndrome. *Am J Pathol* 112:357-82.

Reiser H, Kuhn J, Doerr HW, Kirchner H, Munk K, Braun R (1986) Human cytomegalovirus replicates in primary human bone marrow cells. *J Gen Virol* 67:2595-2604.

Reth M (1994) B cell antigen receptors. *Curr Opin Immunol* 6:3-8.

Reusser P, Riddell SR, Meyers JD, Greenberg PD (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.

Reusser P (1996) Human cytomegalovirus infection and disease after bone marrow and solid organ transplantation. In: *Baillieres Clinincal Infectious Diseases. International practice and research* (Arvin AM ed), pp 357-371. London/Philadelphia: Baillere Tindall/W.B. Saunders Co.Ltd.

Revello MG, Percivalle E, Di Matteo A, Morini F, Gerna G (1992) Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viraemic patients. *J Gen Virol* 73:437-42.

Reyburn HT, Mandelboim O, Vales Gomez M, Davis DM, Pazmany L, Strominger JL (1997) The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells [see comments]. *Nature* 386:514-517.

Rice GE, Munro JM, Bevilacqua MP (1990) Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *J Exp Med* 171:1369-1374.

Rice GP, Schrier RD, Oldstone MB (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 WP, Colvin RB, Cheeseman SH, Tolkoff Rubin NE, Herrin JT, Cosimi AB, Collins AB, Hirsch MS, McCluskey RT, Russell PS, Rubin RH (1981) Glomerulopathy associated with cytomegalovirus viremia in renal allografts. *N Engl J Med* 305:57-63.

Rinaldo CR, Jr., Black PH, Hirsch MS (1977) Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. *J Infect Dis* 136:667-678.

Rinaldo CR, Jr., Carney WP, Richter BS, Black PH, Hirsch MS (1980) Mechanisms of immunosuppression in cytomegaloviral mononucleosis. *J Infect Dis* 141:488-495.

Rinaldo CR, Jr. (1994) Modulation of major histocompatibility complex antigen expression by viral infection [comment]. *Am J Pathol* 144:637-50.

Rival Y, Del Maschio A, Rabiet MJ, Dejana E, Duperray A (1996) Inhibition of platelet endothelial cell adhesion molecule-1 synthesis and leukocyte transmigration in endothelial cells by the combined action of TNF-alpha and IFN-gamma. *J Immunol* 157:1233-1241.

Roberts WH, Sneddon JM, Waldman J, Stephens RE (1989) Cytomegalovirus infection of gastrointestinal endothelium demonstrated by simultaneous nucleic acid hybridization and immunohistochemistry. *Arch Pathol Lab Med* 113:461-464.

Roizman B (1996) Herpesviridae. In: *Fields Virology* (Fields BN, Knipe DM, Howley PM eds), pp 2221-2230. Philadelphia: Lippencott-Raven Publishers.

Romer LH, McLean NV, Yan HC, Daise M, Sun J, DeLisser HM (1995) IFN-gamma and TNF-alpha induce redistribution of PECAM-1 (CD31) on human endothelial cells. *J Immunol* 154:6582-6592.

Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-809.

Rouse BT, Grewal AS, Babiuk LA, Fujimiya Y (1977) Enhancement of antibody-dependent cell-mediated cytotoxicity of herpesvirus-infected cells by complement. *Infect Immun* 18:660-665.

Rousset F, Billaud M, Blanchard D, Figdor C, Lenoir GM, Spits H, De Vries JE (1989) IL-4 induces LFA-1 and LFA-3 expression on Burkitt's lymphoma cell lines. Requirement of additional activation by phorbol myristate acetate for induction of homotypic cell adhesions. *J Immunol* 143:1490-1498.

Ruby J (1997) Antiviral Activity of Gamma Interferon. In: *Gamma Interferon in Antiviral Defence* (Karupiah G ed), pp 86-100. R.G. Landes Company.

Ryan US, Schultz DR, Ruan JW (1981) Fc and C3b receptors on pulmonary endothelial cells: induction by injury. *Science* 214:557-558.

Salomon RN, Hughes CC, Schoen FJ, Payne DD, Pober JS, Libby P (1991) Human coronary transplantation-associated arteriosclerosis. Evidence for a chronic immune reaction to activated graft endothelial cells. *Am J Pathol* 138:791-798.

Saltzman RL, Quirk MR, Jordan MC (1988) Disseminated cytomegalovirus infection. Molecular analysis of virus and leukocyte interactions in viremia. *J Clin Invest* 81:75-81.

Salzberger B, Myerson D, Boeckh M (1997) Circulating cytomegalovirus (CMV)-infected endothelial cells in marrow transplant patients with CMV disease and CMV infection. *J Infect Dis* 176:778-781.

Sasseville VG, Newman W, Brodie SJ, Hesterberg P, Pauley D, Ringler, DJ. (1994) Monocyte adhesion to endothelium in simian immunodeficiency virus-induced AIDS encephalitis is mediated by vascular cell adhesion molecule-1/alpha 4 beta 1 integrin interactions. *American Journal of Pathology* 144:27-40.

Scholz M, Hamann A, Blaheta RA, Auth MK, Encke A, Markus BH (1992) Cytomegalovirus- and interferon-related effects on human endothelial cells. Cytomegalovirus infection reduces upregulation of HLA class II antigen expression after treatment with interferon-gamma. *Hum Immunol* 35:230-238.

Schrier RD, Oldstone MB (1986) Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. *J Virol* 59:127-131.

Sedmak DD, Roberts WH, Stephens RE, Buesching WJ, Morgan LA, Davis DH, Waldman WJ (1990) Inability of cytomegalovirus infection of cultured endothelial cells to induce HLA class II antigen expression. *Transplantation* 49:458-462.

Sedmak DD, Guglielmo AM, Knight DA, Birmingham DJ, Huang EH, Waldman WJ (1994a) Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells [see comments]. *Am J Pathol* 144:683-692.

Sedmak DD, Knight DA, Vook NC, Waldman JW (1994b) Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. *Transplantation* 58:1379-1385.

Sedmak DD, Chaiwiriyaikul S, Knight DA, Waldmann WJ (1995) The role of interferon beta in human cytomegalovirus-mediated inhibition of HLA DR induction on endothelial cells. *Arch Virol* 140:111-126.

Sen GC (1997) The interferons. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 199-208. New York: Marcel Dekker, Inc.

Shahgasempour S, Woodroffe SB, Garnett HM (1997a) Alterations in the expression of ELAM-1, ICAM-1 and VCAM-1 after in vitro infection of endothelial cells with a clinical isolate of human cytomegalovirus. *Microbiol Immunol* 41:121-129.

Shahgasempour S, Woodroffe SB, Sullivan Tailyour G, Garnett HM (1997b) Alteration in the expression of endothelial cell integrin receptors alpha 5 beta 1 and alpha 2 beta 1 and alpha 6 beta 1 after in vitro infection with a clinical isolate of human cytomegalovirus. *Arch Virol* 142:125-138.

Shanley JD, Jordan MC, Stevens JG (1981) Modification by adoptive humoral immunity of murine cytomegalovirus infection. *J Infect Dis* 143:231-237.

Shen J, Devery JM, King NJ (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.

Shen J, T-To S, Schrieber L, King N (1997) Early E-Selectin, VCAM-1, ICAM-1, and late major histocompatibility complex antigen induction on human endothelial cells by flavivirus and modulation of adhesion molecule expression by immune cytokines. *J Virol* 71:9323-9332.

Shepp DH, Dandliker PS, de Miranda P, Burnette TC, Cederberg DM, Kirk, LE, Meyers JD (1985) Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine in the treatment of cytomegalovirus pneumonia. *Ann Intern Med* 103:368-373.

Shimizu Y, Newman W, Gopal TV, Horgan KJ, Graber N, Beall LD, van Seventer GA, Shaw S (1991) Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J Cell Biol* 113:1203-1212.

- Shimizu Y, Newman W, Tanaka Y, Shaw S (1992) Lymphocyte Interactions with Endothelial Cells. *Immunology Today* 13:106-112.
- Shoskes DA, Wood KJ (1994) Indirect presentation of MHC antigens in transplantation. *Immunol Today* 15:32-38.
- Simmons P, Kaushansky K, Torok Storb B (1990) Mechanisms of cytomegalovirus-mediated myelosuppression: perturbation of stromal cell function versus direct infection of myeloid cells. *Proc Natl Acad Sci U S A* 87:1386-1390.
- Sing GK, Ruscetti FW (1990) Preferential suppression of myelopoiesis in normal human bone marrow cells after in vitro challenge with human cytomegalovirus. *Blood* 75:1965-1973.
- Sinzger C, Grefte A, Plachter B, Gouw AS, The TH, 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 TH, Jahn G (1996) Tissue macrophages are infected by human cytomegalovirus in vivo. *J Infect Dis* 173:240-245.
- Slater JS, Campbell AE (1997) Down-regulation of MHC class I synthesis by murine cytomegalovirus occurs in immortalized but not primary fibroblasts. *Virology* 229:221-227.
- Slavin MA, Bindra RR, Gleaves CA, Pettinger MB, Bowden RA (1993) Ganciclovir sensitivity of cytomegalovirus at diagnosis and during treatment of cytomegalovirus pneumonia in marrow transplant recipients. *Antimicrob Agents Chemother* 37:1360-1363.
- Smiley ML, Wlodaver CG, Grossman RA, Barker CF, Perloff LJ, Tustin NB, Starr SE, Plotkin SA, Friedman HM (1985) The role of pretransplant immunity in protection from cytomegalovirus disease following renal transplantation. *Transplantation* 40:157-61.
- Smiley ML, Mar EC, Huang ES (1988) Cytomegalovirus infection and viral-induced transformation of human endothelial cells. *J Med Virol* 25:213-226.
- Smith CW, Rothlein R, Hughes BJ, Mariscalco MM, Rudloff HE, Schmalstieg FC, Anderson DC (1988) Recognition of an endothelial determinant for CD 18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest* 82:1746-1756.
- Smith CW, Kishimoto TK, Abbassi O, Hughes B, Rothlein R, McIntire LV, Butcher E, Anderson DC, Abbass O, corrected to Abbassi-O] (1991) Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro [published erratum appears in *J Clin Invest* 1991 May;87(5):1873]. *J Clin Invest* 87:609-618.
- Smyth S, Joneckis CC, Parise LV (1993) Regulation of vascular integrins. *Blood* 81:2827-2843.
- Snydman DR, Werner BG, Heinze Lacey B, Berardi VP, Tilney NL, Kirkman RL, Milford EL, Cho SI, Bush HL, Jr., Levey AS, et al (1987) Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. *N Engl J Med* 317:1049-54No.
- Snydman DR, Werner BG, Dougherty NN, Griffith J, Rubin RH, Dienstag JL, Rohrer RH, Freeman R, Jenkins R, Lewis WD, 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* 119:984-91.

Soderberg Naucier C, Fish KN, Nelson JA (1997) Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* 91:119-26.

Somersalo K, Carpen O, Saksela E, Gahmberg CG, Nortamo P, Timonen T (1995) Activation of natural killer cell migration by leukocyte integrin-binding peptide from intracellular adhesion molecule-2 (ICAM-2). *J Biol Chem* 270:8629-8636.

Sonnabend J, Witkin SS, Purtilo DT (1983) Acquired immunodeficiency syndrome, opportunistic infections, and malignancies in male homosexuals. A hypothesis of etiologic factors in pathogenesis. *JAMA* 249:2370-2374.

Span AH, van Boven CP, Bruggeman CA (1989) The effect of cytomegalovirus infection on the adherence of polymorphonuclear leucocytes to endothelial cells. *Eur J Clin Invest* 19:542-548.

Span AH, Mullers W, Miltenburg AM, Bruggeman CA (1991a) Cytomegalovirus induced PMN adherence in relation to an ELAM-1 antigen present on infected endothelial cell monolayers. *Immunology* 72:355-360.

Span AH, van Dam Mieras MC, Mullers W, Endert J, Muller AD, Bruggeman CA (1991b) The effect of virus infection on the adherence of leukocytes or platelets to endothelial cells. *Eur J Clin Invest* 21:331-338.

Springer TA, Dustin ML, Kishimoto TK, Marlin SD (1987) The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. [Review] [145 refs]. *Annu Rev Immunol* 5:223-252.

Springer TA (1990) Adhesion Molecules of the Immune System. *Nature* 346:425-434.

St.Jeor S, Rapp F (1973) Cytomegalovirus: conversion of nonpermissive cells to a permissive state for virus replication. *Science* 181:1060-1.

Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS (1989) Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors [see comments]. *BMJ* 299:897-8.

Steffens HP, Kurz S, Holtappels R, Reddehase MJ (1998) Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence. *J Virol* 72:1797-1804.

Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K (1993) Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia. Lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2, and LFA-3. *Am J Pathol* 142:481-488.

Steinmassl M, 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.

Stinski MF (1976) Human cytomegalovirus: glycoproteins associated with virions and dense bodies. *J Virol* 19:594-609.

- Stinski MF (1990) Cytomegalovirus and its replication. In: *Virology* (Fields BN, Knipe DM eds), pp 1959-1980. New York: Raven Press Ltd.
- Stovin PG, Wreghitt TG, English TA, Wallwork J (1989) Lack of association between cytomegalovirus infection of heart and rejection-like inflammation. *J Clin Pathol* 42:81-83.
- Tamashiro JC, Filpula D, Friedmann T, Spector DH (1984) Structure of the heterogeneous L-S junction region of human cytomegalovirus strain AD169 DNA. *J Virol* 52:541-8.
- Tanaka J, Sadanari H, Sato H, Fukuda S (1991) Sodium butyrate-inducible replication of human cytomegalovirus in a human epithelial cell line. *Virology* 185:271-280.
- Tanaka S, Toh Y, Mori R, Komori K, Okadome K, Sugimachi K (1992) Possible role of cytomegalovirus in the pathogenesis of inflammatory aortic diseases: a preliminary report. *J Vasc Surg* 16:274-279.
- Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S (1993a) T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta [see comments]. *Nature* 361:79-82.
- Tanaka Y, Adams DH, Shaw S (1993b) Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunology Today* 14:111-115.
- Tanaka Y, Fukudome K, Hayashi M, Takagi S, 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.
- Tang Q, Hendricks RL (1996) Interferon gamma regulates platelet endothelial cell adhesion molecule 1 expression and neutrophil infiltration into herpes simplex virus-infected mouse corneas. *J Exp Med* 184:1435-1447.
- Tanio JW, Basu CB, Albelda SM, Eisen HJ (1994) Differential expression of the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin in normal and posttransplantation myocardium. Cell adhesion molecule expression in human cardiac allografts. *Circulation* 89:1760-1768.
- Taylor Wiedeman J, Sissons P, Sinclair J (1994) Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J Virol* 68:1597-1604.
- Taylor HP, Cooper NR (1990) The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. *J Virol* 64:2484-2490.
- Taylor PM, Rose ML, Yacoub MH, Pigott R (1992) Induction of vascular adhesion molecules during rejection of human cardiac allografts. *Transplantation* 54:451-457.
- Thale R, Lucin P, Schneider K, Eggers M, Koszinowski UH (1994) Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. *J Virol* 68:7757-7765.
- Tocci MJ, Schmidt JA (1997) Interleukin-1: Structure and Function. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 1-29. New York: Marcel Dekker,inc.
- Toorkey CB, Carrigan DR (1989) Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J Infect Dis* 160:741-751.

Tooze J, Hollinshead M, Reis B, Radsak K, Kern H (1993) Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol* 60:163-78.

Torok Storb B, Fries B, Stachel D, Khaira D (1993) Cytomegalovirus: variations in tropism and disease. *Leukemia* 7 Suppl 2:S83-S85.

Torok Storb B, Boeckh M, Hoy C, Leisenring W, Myerson D, Gooley T (1997) Association of specific cytomegalovirus genotypes with death from myelosuppression after marrow transplantation. *Blood* 90:2097-2102.

Tosi MF, Stark JM, Hamedani A, Smith CW, Gruenert DC, Huang YT (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 HG, Foster L, Karre K (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides [see comments]. *Nature* 340:443-8No.

Tracey KJ (1997) Tumor Necrosis Factor. In: *Cytokines in health and disease* (Remick D, Friedland J eds), pp 223-240. New York: Marcel Dekker, Inc.

Tuder RM, Weinberg A, Panajotopoulos N, Kalil J (1991) Cytomegalovirus enhances PBMC binding and HLA class I expression on cultured endothelial cells. *Transplant Proc* 23:91-92.

Tuder RM, Weinberg A, Panajotopoulos N, Kalil J (1994) Cytomegalovirus infection amplifies class I major histocompatibility complex expression on cultured human endothelial cells. *J Heart Lung Transplant* 13:129-138.

Tugizov S, Navarro D, Paz P, Wang Y, Qadri I, Pereira L (1994) Function of human cytomegalovirus glycoprotein B: syncytium formation in cells constitutively expressing gB is blocked by virus-neutralizing antibodies. *Virology* 201:263-276.

Turtinen LW, Saltzman R, Jordan MC, Haase AT (1987) Interactions of human cytomegalovirus with leukocytes in vivo: analysis by in situ hybridization. *Microb Pathog* 3:287-297.

Ustinov J, Loginov R, Bruggeman C, van der Meide P, Hayry P, Lautenschlager I (1993) Direct induction of class II antigens by cytomegalovirus in rat heart endothelial cells. *Transplant Proc* 25:1143-4.

Ustinov J, Loginov R, Bruggeman C, Suni J, Hayry P, Lautenschlager I (1994a) CMV-induced class II antigen expression in various rat organs. *Transpl Int* 7:302-308.

Ustinov JA, Loginov RJ, Mattila PM, Nieminen VK, Suni JI, Hayry PJ, Lautenschlager IT (1991) Cytomegalovirus infection of human kidney cells in vitro. *Kidney Int* 40:954-960.

Ustinov JA, Lahtinen TT, Bruggeman CA, Hayry PJ, Lautenschlager IT (1994) Direct induction of class II molecules by cytomegalovirus in rat heart microvascular endothelial cells is inhibited by ganciclovir (DHPG). *Transplantation* 58:1027-1031.

van den Berg AP, van Son WJ, Tegzess AM, The TH (1993) Cellular immune activation reflects antiviral immunity and is a favorable prognostic marker in patients with cytomegalovirus infection. *Transplant Proc* 25:1419-20.

van den Berg AP, Klompmaker IJ, Hepkema BG, Gouw AS, Haagsma EB, Lems SP, The TH, Slooff MJ (1996) Cytomegalovirus infection does not increase the risk of vanishing bile duct syndrome after liver transplantation. *Transpl Int* 9 Suppl 1:S171-3.

van Dorp WT, Jonges E, Bruggeman CA, Daha MR, van Es LA, van Der Woude FJ (1989) Direct induction of MHC class I, but not class II, expression on endothelial cells by cytomegalovirus infection. *Transplantation* 48:469-472.

van Dorp WT, van Wieringen PA, Marselis Jonges E, Bruggeman CA, Daha MR, van Es LA, van der Woude F (1993) Cytomegalovirus directly enhances MHC class I and intercellular adhesion molecule-1 expression on cultured proximal tubular epithelial cells. *Transplantation* 55:1367-1371.

van Kemenade FJ, Kuijpers KC, de Waal Malefijt R, van Lier RA, Miedema F (1993) Skewing to the LFA-3 adhesion pathway by influenza infection of antigen-presenting cells. *Eur J Immunol* 23:635-639.

Van Vliet KE, De Graaf Miltenburg LA, Verhoef J, Van Strijp JA (1992) Direct evidence for antibody bipolar bridging on herpes simplex virus-infected cells. *Immunology* 77:109-115.

Visser MR, Jacob HS, Goodman JL, McCarthy JB, Furcht LT, Vercellotti GM (1989) Granulocyte-mediated injury to herpes simplex virus-infected human endothelium. *Lab Invest* 60:296-304.

Vogel FS (1958) Enhanced susceptibility of proliferating endothelium to salivary gland virus under naturally occurring and experimental conditions. *American Journal of Pathology* 34:1069-1079.

Volpes R, van den Oord JJ, Desmet VJ, Yap SH (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, Hayry P (1986) CMV infection, class II antigen expression, and human kidney allograft rejection. *Transplantation* 42:364-367.

von Willebrand E, Loginov R, Salmela K, Isoniemi H, Hayry P (1993) Relationship between intercellular adhesion molecule-1 and HLA class II expression in acute cellular rejection of human kidney allografts. *Transplant Proc* 25:870-1.

Waldman WJ, Sneddon JM, Stephens RE, Roberts WH (1989) Enhanced endothelial cytopathogenicity induced by a cytomegalovirus strain propagated in endothelial cells. *J Med Virol* 28:223-230.

Waldman WJ, Roberts WH, Davis DH, Williams MV, Sedmak DD, Stephens RE (1991) Preservation of natural endothelial cytopathogenicity of cytomegalovirus by propagation in endothelial cells. *Arch Virol* 117:143-164.

Waldman WJ, Adams PW, Orosz CG, Sedmak DD (1992) T lymphocyte activation by cytomegalovirus-infected, allogeneic cultured human endothelial cells. *Transplantation* 54:887-896.

Waldman WJ, Knight DA, Adams PW, Orosz CG, Sedmak DD (1995a) In vitro induction of endothelial adhesion molecule and MHC antigen expression by cytomegalovirus-activated CD4+ T cells. *Transplant Proc* 27:1269-1271.

Waldman WJ, Knight DA, Huang EH, Sedmak DD (1995b) Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: an in vitro model. *J Infect Dis* 171:263-272.

Waldman WJ, Adams PW, Knight DA, Sedmak DD (1997) CMV as an exacerbating agent in transplant vascular sclerosis: potential immune-mediated mechanisms modelled in vitro. *Transplant Proc* 29:1545-1546.

Waldman WJ, Knight DA (1996) Cytokine-mediated induction of endothelial adhesion molecule and histocompatibility leukocyte antigen expression by cytomegalovirus-activated T cells. *Am J Pathol* 148:105-119.

Wang D, Liebowitz D, Wang F, Gregory C, Rickinson A, Larson R, Springer T, Kieff E (1988) Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J Virol* 62:4173-4184.

Warren AP, Ducroq DH, Lehner PJ, Borysiewicz LK (1994a) 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.

Warren AP, Owens CN, Borysiewicz LK, Patel K (1994b) Down-regulation of integrin alpha 1/beta 1 expression and association with cell rounding in human cytomegalovirus-infected fibroblasts. *J Gen Virol* 75:3319-3325.

Weinshenker BG, Wilton S, Rice GP (1988) Phorbol ester-induced differentiation permits productive human cytomegalovirus infection in a monocytic cell line. *J Immunol* 140:1625-1631.

Weller TH (1971) The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. I. [Review] [127 refs]. *N Engl J Med* 285:203-214.

Wertheim P, Buurman C, Geelen J, van der Noordaa J (1983) Transmission of cytomegalovirus by renal allograft demonstrated by restriction enzyme analysis [letter]. *Lancet* i:980-1.

Westmoreland D, St.Jeor S, Rapp F (1976) The development by cytomegalovirus-infected cells of binding affinity for normal human immunoglobulin. *J Immunol* 116:1566-1570.

Wewers MD (1997) Cytokines and macrophages. In: *Cytokines in health and disease* (Remick, D; Friedland, J, eds) pp 339-356. New York: Marcel Dekker.

Wiertz E, Hill A, Tortorella D, Ploegh H (1997) Cytomegaloviruses use multiple mechanisms to elude the host immune response. *Immunol Lett* 57:213-216.

Wiertz EJ, Jones TR, Sun L, Bogoy M, Geuze HJ, Ploegh HL (1996) The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84:769-779.

Winston DJ, Ho WG, Howell CL, Miller MJ, Mickey R, Martin WJ, Lin CH, Gale RP (1980) Cytomegalovirus infections associated with leukocyte transfusions. *Ann Intern Med* 93:671-675.

- Winston DJ, Ho WG, Bartoni K, Du Mond C, Ebeling DF, Buhles WC, Champlin RE (1993) Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. *Ann Intern Med* 118:179-84.
- Woodroffe SB, Garnett HM, Danis VA (1993) Interleukin-1 production and cell-activation response to cytomegalovirus infection of vascular endothelial cells. *Arch Virol* 133:295-308.
- Woodruff JJ, Clarke LM, Chin YH (1987) Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. *Ann Rev Immunol* 5:201-222.
- Wright JF, Kurosky A, Wasi S (1994) An endothelial cell-surface form of annexin II binds human cytomegalovirus. *Biochem Biophys Res Commun* 198:983-989.
- Wright JF, Kurosky A, Prydzial EL, Wasi S (1995) Host cellular annexin II is associated with cytomegalovirus particles isolated from cultured human fibroblasts. *J Virol* 69:4784-4791.
- Wu QH, Ascensao J, Almeida G, Forman SJ, Shanley JD (1994) The effect of short-chain fatty acids on the susceptibility of human umbilical vein endothelial cells to human cytomegalovirus infection. *J Virol Methods* 47:37-50.
- Wu TC, Hruban RH, Ambinder RF, Pizzorno M, Cameron DE, Baumgartner WA, Reitz BA, Hayward GS, Hutchins GM (1992) Demonstration of cytomegalovirus nucleic acids in the coronary arteries of transplanted hearts. *Am J Pathol* 140:739-747.
- Xu L, Kelvin D, J., Ye G, Q., Taub D, D., Ben-Baruch A, Oppenheim J, J., Wang JM (1995) Modulation of IL-8 receptor expression on purified human T-lymphocytes is associated with changed chemotactic responses to IL-8. *J Leuk Biol* 57:335-342.
- Yamashiroya HM, Ghosh L, Yang R, Robertson AL, Jr. (1988) Herpesviridae in the coronary arteries and aorta of young trauma victims. *Am J Pathol* 130:71-79.
- Yamashita Y, Shimokata K, Mizuno S, Yamaguchi H, Nishiyama Y (1993) Down-regulation of the surface expression of class I MHC antigens by human cytomegalovirus. *Virology* 193:727-736.
- Yamauchi M, Nishiyama Y, Fujioka H, Isomura S, Maeno K (1985) On the intracellular transport and the nuclear association of human cytomegalovirus structural proteins. *J Gen Virol* 66:675-84.
- Yang HY, Dundon PL, Nahill SR, Welsh RM (1989) Virus-induced polyclonal cytotoxic T lymphocyte stimulation. *J Immunol* 142:1710-1718.
- Yilmaz S, Koskinen PK, Kallio E, Bruggeman CA, Hayry PJ, Lemstrom KB (1996) Cytomegalovirus infection-enhanced chronic kidney allograft rejection is linked with intercellular adhesion molecule-1 expression. *Kidney Int* 50:526-537.
- Yue TL, Wang X, Sung CP, Olson B, McKenna PJ, Gu JL, Feuerstein GZ (1994) Interleukin-8. A mitogen and chemoattractant for vascular smooth muscle cells. *Circ Res* 75:1-7.
- Zeng Y, Gage A, Montag A, Rothlein R, Thistlethwaite JR, Bluestone JA (1994) Inhibition of transplant rejection by pretreatment of xenogeneic pancreatic islet cells with anti-ICAM-1 antibodies. *Transplantation* 58:681-689.

Zhuravskaya T, Maciejewski JP, Netski DM, Bruening E, Mackintosh FR, St Jeor S (1997) Spread of human cytomegalovirus (HCMV) after infection of human hematopoietic progenitor cells: model of HCMV latency. *Blood* 90:2482-2491.

Zocchi MR, Ferrero E, Leone BE, Rovere P, Bianchi E, Toninelli E, Pardi R (1996) CD31/PECAM-1-driven chemokine-independent transmigration of human T lymphocytes. *Eur J Immunol* 26:759-767.

Zsak L, Zuckermann F, Sugg N, Ben Porat T (1992) Glycoprotein gl of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. *J Virol* 66:2316-2325.

Zuckermann FA, Mettenleiter TC, Schreurs C, Sugg N, Ben Porat T (1988) Complex between glycoproteins gl and gp63 of pseudorabies virus: its effect on virus replication. *J Virol* 62:4622-4626.

LIST OF ABSTRACTS AND PUBLICATIONS RELEVANT TO THE DATA PRESENTED IN THIS THESIS.

ABSTRACTS

L.P. MacCormac and J.E. Grundy. Alterations to Class I HLA and adhesion molecule expression following HCMV infection of endothelial cells.
5th International Cytomegalovirus Conference, Stockholm, May 1995.

PUBLICATIONS

1) **L.P. MacCormac** and J.E. Grundy.

Human Cytomegalovirus induces a Fc gamma receptor in endothelial cells and fibroblasts which is distinct from the human Fc gamma receptors.

Journal of Infectious Diseases. 1996. 174: 1151-61.

2) N.J. Borthwick, A.N. Akbar, **L.P. MacCormac**, M. Lowdell, J.L. Craigen, I. Hassan, J.E. Grundy, M. Salmon and K.L. Yong.

Preferential migration of highly differentiated primed T cells, defined by low expression of CD45RB, across human umbilical vein endothelial cells: effects of viral infection on transmigration.

Immunology. 1997. 90: 272-280.

3) J.L. Craigen, K.L. Yong, N. Jordan, **L.P. MacCormac**, J. Westwick, A.N. Akbar and J.E. Grundy.

Human cytomegalovirus increases IL-8 gene expression and enhances transendothelial neutrophil migration.

Immunology. 1997. 92:138-145.

4) J.E. Grundy, K. Lawson, **L.P. MacCormac**, J. Fletcher and K.L. Yong.

Cytomegalovirus-infected endothelial cells recruit neutrophils by the secretion of C-X-C chemokines and transmit virus by direct neutrophil-endothelial cell contact and during neutrophil transendothelial migration.

Journal of Infectious Diseases. 1998. 177:1465-1474.

5) **L.P. MacCormac** and J.E. Grundy.

Two clinical isolates and the Toledo strain of cytomegalovirus contain endothelial cell tropic variants which are not present in the AD169, Towne or Davis strains.

(Journal of Medical Virology, In Press, 1998).