The study of cytomegalovirus infection in vascular smooth muscle cells and in an organ culture of the saphenous vein:

Potential role in atherogenesis.

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By

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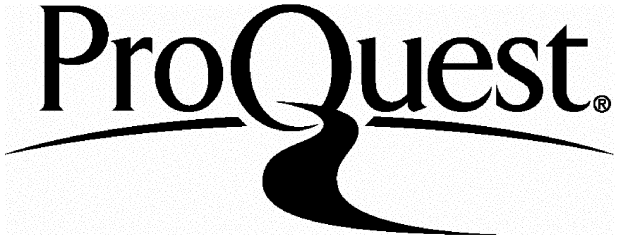

To my family, especially my parents

ABSTRACT

Studies in human beings suggest that infectious agents such as cytomegalovirus might be involved in the pathogenesis of vascular diseases such as atherosclerosis, restenosis and transplant-associated atherosclerosis. Smooth muscle cells in the blood vessel wall are involved in the development of these vascular diseases, and CMV infection of these cells may play significant roles in the disease processes. In the present study, an investigation into the potential role of CMV infection of smooth muscle cells in the pathogenesis of atherosclerosis was reported. Smooth muscle cells were isolated and cultured from human saphenous veins and from the veins of the umbilical cord. Aortic smooth muscle cells were also obtained from a commercial source, and served as an arterial alternative to smooth muscle cells isolated from the blood vessel wall of the venous vasculature. Using a high passage CMV strain, AD169, and a low passage CMV strain, C1F, it was demonstrated that CMV was capable of undergoing a full cycle of replication in smooth muscle cells of venous and arterial origin. Viral replication was demonstrated by the immunofluorescent staining of CMV viral antigens, which are induced following CMV infection of a permissive cell. The replication of CMV in these smooth muscle cell cultures led to the production of infectious virus particles. These studies demonstrated that CMV infection of smooth muscle cells proceeded in a fashion comparable to CMV infection in fully permissive fibroblasts.

The changes in the expression of adhesion molecules and MHC antigens following CMV infection of smooth muscle cells were investigated. It was shown that infection with CMV strains AD169 and C1F significantly increased the constitutive expression of ICAM-1. It was also shown that there was an increase in the expression of LFA-3 molecules on smooth muscle cells following their inoculation with CMV strain AD169. However the expression of LFA-3 was not altered following the infection of smooth muscle cells with the low passage CMV strain C1F. Increased adhesion molecule expression could potentially increase the infiltration of inflammatory cells, thereby contributing to the development and progression of atherosclerotic lesions. Furthermore, it was demonstrated that CMV potentially interfered with the adaptive immune response, by altering the expression of class I MHC, but not class II MHC molecules on the surface of smooth muscle cells. The infection of smooth muscle cells with CMV strain AD169 and a low passage CMV strain, C1F, resulted in the down-regulation of class I MHC antigens, suggesting that these cells might be able to evade recognition and killing by cytotoxic T cells and escape immune surveillance, thereby helping CMV to persist in the blood vessel wall.

Recently, an *in vitro* organ culture system of the human saphenous vein has been described where saphenous veins were cultured for extended periods of time in highly serum supplemented media. This system provides the useful advantage over the isolated cell culture technique in that it is possible to maintain the integrity and architecture of the vein wall, and thus the overall effects of CMV infection on the vessel wall can be assessed. Using the organ culture system, whole segments of saphenous veins were cultured *in vitro*, with the preservation of cell viability and vessel structure. Studies into the infection of these vein segments with CMV strain

AD169 and strain C1F were undertaken. At various stages post-infection, CMV replication was observed in the neointimal, intimal and adventitial layers of the vessel wall. This was evident by the detection of the expression of CMV-specific IE and early antigens by immunofluorescent staining in these layers of the vein segment. The medial portion of the vessel wall was apparently not infected with CMV. The number of CMV-infected cells in the neointima and adventitia significantly increased with time post-culture, suggesting that the infection was fully permissive, with the resultant spread of the virus in the cells of the vessel wall. CMV infection led to alterations in the structure of the vessel wall, with an increased deposition of extracellular matrix and the detachment of endothelial cells from the basement membrane. The results presented in this thesis provide further support to the premise that CMV infection of vascular smooth muscle cells may play a pathogenic role in the development and progression of atherosclerosis and/or vascular disease. Furthermore, this study has successfully set up important models which could be used in future studies to determine exactly how CMV could contribute to atherogenesis.

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ABBREVIATIONS USED IN THE TEXT

AIDS	Acquired immunodeficiency syndrome
BMT	Bone marrow transplantation
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic acid
CMV	Cytomegalovirus
crs	cis-represion sequence
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
FACS	Fluorescence activated cell sorter
FcγR	Fc gamma receptor
gB	Glycoprotein B
gH	Glycoprotein H
gp	Glycoprotein
HIV	Human immunodeficiency virus
HLA	Human Leukocyte antigen
HSV	Herpes Simplex virus
ICAM-1	Intracellular adhesion molecule-1
IE	Immediate early
LFA-3	Leukocyte function antigen-3
p72	Immediate early protein 72
p86	Immediate early protein 86
IgG	Immunoglobulin G
kD	Kilodalton
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
pp	Phosphoprotein
RNA	Ribonucleic acid

CHAPTER 1:

General Introduction

Cytomegalovirus (CMV), a member of the herpesvirus family, is a major pathogen in immunocompromised or immunodeficient individuals. The betaherpesviruses, to which CMV belongs, are highly species specific, and therefore *homo sapiens* are thought to be the only reservoir for human CMV (Roizman, 1996a). Cytomegaloviruses were generally known as salivary gland viruses until their common name, CMV, was proposed by Weller in 1960 to reflect the virus-induced cytopathic effects, and the virus's role in congenitally acquired cytomegalic inclusion disease (Weller et al., 1957; Weller et al., 1962). CMV infection is naturally acquired, and is widespread throughout most of the populations in developed and developing countries. Over the course of a lifetime, an individual is likely to be exposed to CMV infection, as demonstrated by the fact that the prevalence of seropositivity for antibodies to CMV in western countries is 10-15% in adolescents, rising to about 40-50% by age 35, and is estimated to be over 60% in individuals over 65 years (Bodurtha et al., 1988). Following primary CMV infection in an immunocompetent host, a persistent infection is established in the infected individual for life. CMV infection of immunocompetent individuals usually goes unnoticed, with the occasional individual developing a mild, mononucleosis-like illness (Britt & Alford, 1996). However, CMV infection of the immunologically immature foetus or immunocompromised individuals, such as transplant recipients or individuals with the acquired immunodeficiency syndrome (AIDS), can cause severe disease, which can be followed by significant mortality in some patient groups (Meyers, 1989; Mutimer, 1996; Avery, 1998). Individuals infected with CMV may shed the virus in bodily secretions and in the urine. Transmission of the virus occurs between infected and uninfected persons by close or intimate contact, transplacental transfer of the virus from mother to foetus (congenital infection), contact from the newborn with infectious secretions in the birth canal or breast milk (perinatal infection), or the transfusion of blood products or the transplantation of bone marrow or solid-organs from a seropositive donor (Demmler, 1996). The exogenous sources of CMV such as oropharyngeal secretions, urine, cervical and vaginal secretions, semen, breast milk, tears and faeces account for the predominant routes of horizontal (extrauterine) viral acquisition through the general population (Ho, 1991; Britt & Alford, 1996). Although CMV is known to be present in the blood of acutely and persistently infected individuals, this is not a major source of viral transmission in the general population (Stanier et al., 1989).

1.1. THE BIOLOGY OF CMV

1.1.1 Virion classification, structure and genomic arrangement

Herpesviruses have been divided into 3 subfamilies on the basis of their biological properties. The alphaherpesviruses are rapidly growing, highly cytolytic viruses, characterised by having a variable host range *in vitro*, and a short replication cycle with a rapid spread of infection that results in the mass destruction of susceptible cells. They possess a tropism for establishing a latent infection in sensory ganglia, and include varicella-zoster virus, herpes simplex virus (HSV) type 1 and 2 (Roizman, 1996a). The betaherpesviruses have a restricted host range *in vivo*, and a replication cycle which progresses more slowly in cultured cells *in vitro*. Infected cells frequently become enlarged (cytomegaly) both *in vitro* and *in vivo*. Inclusions containing viral DNA are present in both the nucleus and the cytoplasm of the infected cells. Beta-herpesviruses include CMV, human herpesvirus 6 and human herpesvirus 7 (Roizman, 1996a). The gammaherpesviruses have a restricted host range, with *in vitro* replication mainly confined to lymphoblastoid cells (they can infect either B or T lymphocytes), where infection does not reach the final stage of the production of viral progeny. Viruses belonging to this group include Epstein-Barr virus and human herpes virus 8 (Roizman, 1996a).

CMV has a virion structure typical of the betaherpesvirus subfamily (Roizman, 1996b). The virus has a large double stranded, linear deoxyribonucleic acid (DNA) genome, which is located in the central core of the viral particle (Lycke, 1985). The CMV genome is composed of 230 kilobases, and has more sequence complexity than most other herpesviruses (Chee et al., 1990). It has the capacity to encode more than 200 polypeptides. Human CMV is the only betaherpesvirus known to have a class E genome structure, that is, a genome with two components with direct and inverted repeat elements that invert to give rise to four sequence isomers. The genome contains sequences of repeated genetic information, and unique sequences. The unique sequences are contained in two portions of the genome, termed long, L, and short, S. Inversion of the L and S genome components is mediated by repeated sequences located at the genomic termini and the L-S junction (Kemble & Mocarski, 1989). The inverted repeats of the CMV genome, called b (alternatively, TR_L and IR_L) and c (alternatively IR_S and TR_S) sequences respectively, flank unique sequences of the genomic L and S components (U_L and U_S). A direct repeat, which is called the *a* sequence (Mocarski & Roizman, 1982), is present at

both ends of the viral genome, and this sequence is in an inverted orientation at the L-S junction. Figure 1.1 illustrates the organisation of the human CMV genome.

The entire CMV genome is enclosed in a protein capsid, which is constructed from 162 pentameric and hexagonal capsomere subunits. The capsid measures 116nm in diameter, and is surrounded by a poorly defined area, the tegument (Irmiere & Gibson, 1983). A lipid bilayer envelope carrying a number of virally encoded glycoproteins encloses the capsid and tegument region, constituting the entire virion structure. The assembly of the CMV virion can be strategically divided into stages. This involves the packaging of CMV DNA into preformed capsids, which then acquire the tegument proteins, and subsequently become enveloped (Mocarski, 1996; Roizman, 1996b). The diameter of the virion, as measured from electron micrographs of thin sections of CMV-infected cells, or from negatively stained extracellular particles, is approximately 230nm (Irmiere & Gibson, 1983). Figure 1.2 shows a schematic diagram of the virion structure of CMV.

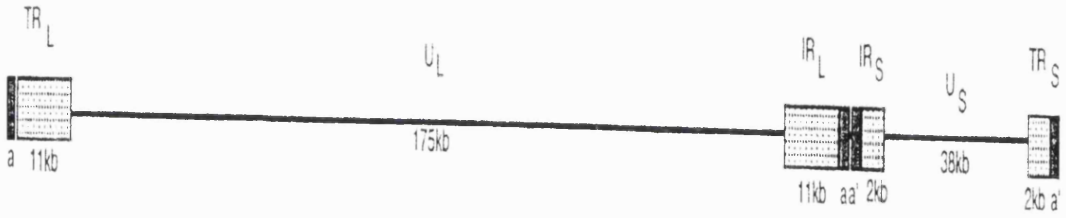

Figure 1.1. A schematic representation of the organisation of the human CMV genome.

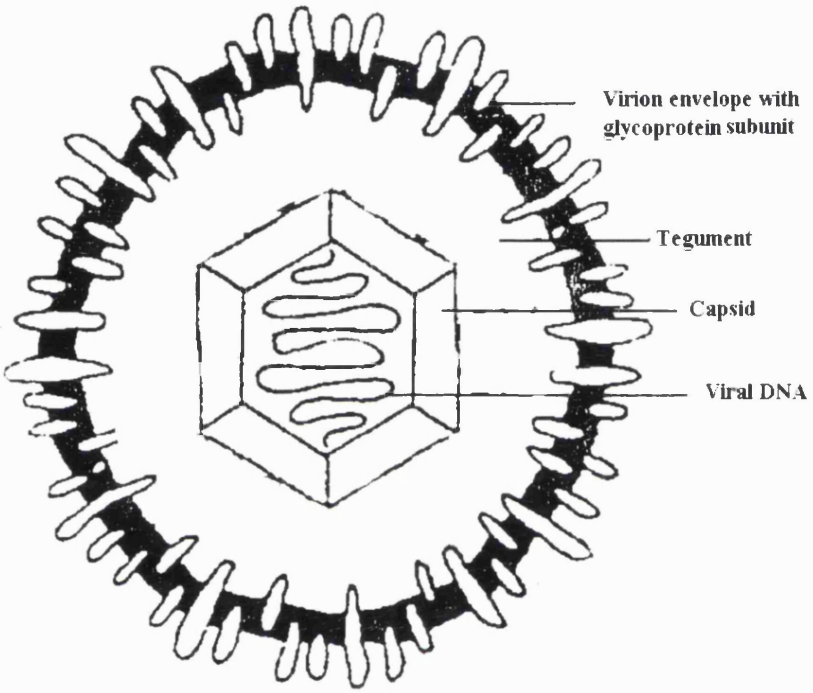

Figure 1.2. A schematic diagram of the virion structure of CMV.

The viral envelope is depicted as a complex structure consisting of a lipid envelope bilayer, in which are embedded virus-specified glycoprotein subunits. The viral DNA is located in the central core of the viral particle, and is enclosed by a protein capsid. Between the capsid and the viral envelope lies the tegument region.

1.1.2 The structural proteins of CMV

Purified virions of CMV consist of at least 30 polypeptides with molecular weights ranging from 20 to 200kD (Spaete et al., 1994; Baldick & Shenk, 1996). Most of the prominent virion proteins have been identified as the products of specific viral genes, and have been correlated with either the capsid, tegument or envelope regions of the virus (Gibson, 1981; Irmiere & Gibson, 1983). A systematic nomenclature for CMV proteins has been introduced (Landini & Spaete, 1993), such that a protein mapped to an open reading frame on the viral genome should be given a name that corresponds to the open reading frame proceeded by the prefix, p. Thus, the protein encoded by the UL1 open reading frame, when identified, should be referred to as pUL1. The prefix pp and gp should be used to further define the known characteristics of the protein, if it is shown to be a phosphoprotein or glycoprotein, respectively.

The capsid proteins of CMV are composed of a relatively simple set of proteins that have homologues in HSV and other herpesviruses. (Gibson et al., 1996a, b). At least four predominant capsid proteins are present in CMV virions. They include the 150kD major capsid protein (pUL86), the 34-37kD minor capsid protein (pUL85), the 33kD minor capsid protein-binding protein (pUL46), and the 8.5kD smallest capsid protein (pUL48/49) (Gibson et al., 1996a, b). The major capsid protein has been reported to exhibit low immunogenicity, while the minor capsid protein is believed to play a role in anchoring viral DNA (Jahn & Mach, 1990).

The viral tegument is the region located between the capsid and the envelope. A large number of proteins, particularly phosphoproteins, are located in the tegument, and constitute approximately 40% of the virion protein mass (Irmiere & Gibson, 1983). They include the high molecular weight protein (ppUL48), the high molecular weight protein-binding protein (ppUL47), the basic phosphoprotein (pp150 or ppUL32), the upper matrix protein (ppUL82) and the lower matrix protein (pp65 or ppUL83) (Jahn et al., 1987; Ruger et al., 1987; Somogi et al., 1990; Bradshaw et al., 1994). The lower matrix protein, pp65, is the most abundant tegument protein, constituting approximately 18% of the virion protein mass (Irmiere & Gibson, 1983; Somogi et al., 1990). It is transported to the nucleus of CMV-infected cells immediately after infection, and can be detected in significant amounts before 24 hours post-infection (Grefte et al., 1992; Revello et al., 1992). It is likely that transportation of pp65 to the nucleus also occurs *in vivo*, as pp65 can be found in up to 1% of the nuclei of polymorphonuclear leucocytes from immunocompromised patients who have CMV viremia (Ehrnst et al., 1993; Francisci et al., 1997). pp150 appears to be one of the most immunogenic structural antigens of CMV

(Landini et al., 1986; Jahn et al., 1987). The function of the tegument proteins remains unclear, although comparison with other herpesviruses predicts that they may play important roles in viral gene regulation, in modification of host cell metabolism, and in the envelopment process (Roizman, 1996b).

The envelope of CMV contains viral proteins and host lipids that are derived from either the nuclear membrane or cytoplasmic membrane (Mocarski, 1996). Analysis of the sequence of the AD169 strain of human CMV has identified approximately 55 open reading frames encoding potential glycoproteins, although the extent of transcription and translation of most of these open reading frames is unknown (Chee et al., 1990). Of these, only two glycoproteins have been moderately well characterised in the envelopes of virions, CMV glycoprotein B (gB or gpUL55) and CMV glycoprotein H (gH or gpUL75). Several additional glycoproteins have been mapped to viral open reading frames. These include gpUL115 (gL), gpUL4 (gp48), gpUL16, gpUS10, gpUS11, and the gcII complex. There is no direct genetic evidence of specific functions for any CMV glycoprotein, even for those that share some homology with better defined glycoproteins of HSV. The glycoproteins gB, gH, gL and the gcII complex have been demonstrated to play roles in the early steps of virion attachment, fusion and penetration of the host cell (Cranage et al., 1988; Gretch et al., 1988; Kari et al., 1988; Kaye et al., 1992). gB and gH elicit strong host immune responses, including the production of virus neutralising antibodies (Rasmussen et al. 1991). This host response is thought to be a key component of host immunity and represents a goal for vaccine development.

Several additional glycoproteins are associated with the envelope of the human CMV virion. The US6 family members are a highly homologous family of open reading frames in the S component consisting of US6, US7, US8, US9, US10 AND US11, that are transcribed at early and late stages in infection (Chee et al., 1990; Jones & Muzithras, 1991). The members of this family have been demonstrated to encode functions that down-regulate class I major histocompatibility antigen (MHC) expression in CMV-infected cells (Wiertz et al., 1997; Davis-Poynter, 1998). The open reading frame designated UL18, exhibits significant nucleotide sequence identity with the class I α-chain genes of both HLA in man and H-2 in mouse (Beck & Barrel, 1988). Initial studies suggested that the class I MHC-like protein, encoded by UL18, was a prime candidate for disrupting cytotoxic T lymphocyte recognition of virally infected cells (Becks & Barrel, 1988; Wiley, 1988).

1.1.3 The entry of CMV into host cells

The entry of viruses into host cells comprises a series of steps that the virus must successfully complete in order to establish a productive infection. In the case of CMV, these steps include the attachment of the virion to the cell surface, the penetration, via the fusion of the viral envelope with the plasma membrane of the host cell, the uncoating of the viral nuclei acid, and the transport of the viral DNA to the nucleus of the host cell (Kielian & Jungerwirth, 1990). Virion attachment or viral adsorption is the first step of the entry process, whereby virus particles become loosely bound to the cell surface (Compton et al., 1993a). This occurs when viral envelope proteins (or viral receptors) with binding affinity for specific cell surface molecules are in close proximity to one another. The viral constituents that have been implicated to have roles in CMV entry into cells include the envelope glycoproteins gB, gH, gL and the gcII complex (Cranage et al., 1988; Gretch et al., 1988; Kaye et al., 1992). Although the cellular receptors for CMV entry have not been unequivocally identified, heparan sulfate proteoglycans, CD13 (aminopeptidase N), and annexin II have been reported to be associated with CMV entry into human fibroblast cells (Neyts et al., 1992; Compton et al., 1993b). However, the relatively broad *in vivo* tropism of CMV suggests that the virus might have multiple mechanisms to gain access to the various cell types.

It has been demonstrated that the initiation of CMV infection requires the initial reaction of virus envelope constituents with cell surface heparan sulphate. This virus-cell interaction is loose, and can be dissociated by moderate salt washes or competition with soluble heparin. In addition, the enzymatic digestion of cells with heparinase blocks virus attachment (Compton et al., 1993a; Compton et al., 1995). gB and a component of the gcII complex have been demonstrated to have heparin binding activity (Neyts et al., 1992; Compton et al., 1993a). This initial heparin-dissociable binding transits rapidly to a high affinity, heparin-resistant, CMV attachment. High affinity binding is believed to be regulated by the CD13 molecule (Soderberg et al., 1993b). It has been demonstrated that antibodies directed against human CD13 not only inhibit infection, but also block the binding of CMV virions to susceptible cells (Soderberg et al., 1993a). This molecule appears to confer binding activity for CMV to the surface of mouse cells when the human CD13 molecule is expressed in these cells (Soderberg et al., 1993a). Thus, human CD13 appears to mediate CMV infection by a process that increases binding. In addition, a glycoprotein membrane receptor having a molecular weight of approximately 30kD has also been implicated in mediating the binding of CMV to target cells. This protein has been suggested to be the principal CMV receptor, since it has been observed in a wide

variety of cultured cell lines, including non-human cells such as Chinese hamster ovary and mouse L cells (Nowlin et al., 1991). Recent evidence suggests that this 30kD protein is annexin II, a member of the lipocortin family of proteins (Wright et al., 1994). The precise role of annexin II in CMV infection is unknown, but it is postulated to function at the level of penetration and the cell-to-cell spread of infection. A cellular 92.5kD protein, thought to be a receptor for gH, is also proposed to be involved in CMV attachment (Keay et al., 1989; Keay & Baldwin, 1992).

Following the attachment of CMV to the surface of cells, the pathway of entry for the virus is direct fusion between the viral envelope and the plasma membrane of cells, followed by penetration of the virus into the host cell. It has been demonstrated that CMV penetrates permissive fibroblasts and non-permissive Chinese hamster ovary cells by pH-independent fusion between the virus envelope and the host cell plasma membrane, and not by low pH-induced fusion within endosomes (Compton et al., 1992). Anti-gB neutralising monoclonal antibodies have been demonstrated to interfere with virus penetration and cell-to-cell spread of infection, suggesting a functional role of gB in the penetration step of the entry pathway (Navarro et al., 1993). Similar observations have been demonstrated with anti-gH neutralising monoclonal antibodies. On penetration of the virus particle and delivery of the nucleocapsid into the host cell cytoplasm, the replication of the CMV genome is initiated. Figure 1.3 illustrates the proposed model for the entry of CMV into fibroblast cells (Compton et al., 1993a; Compton, 1995).

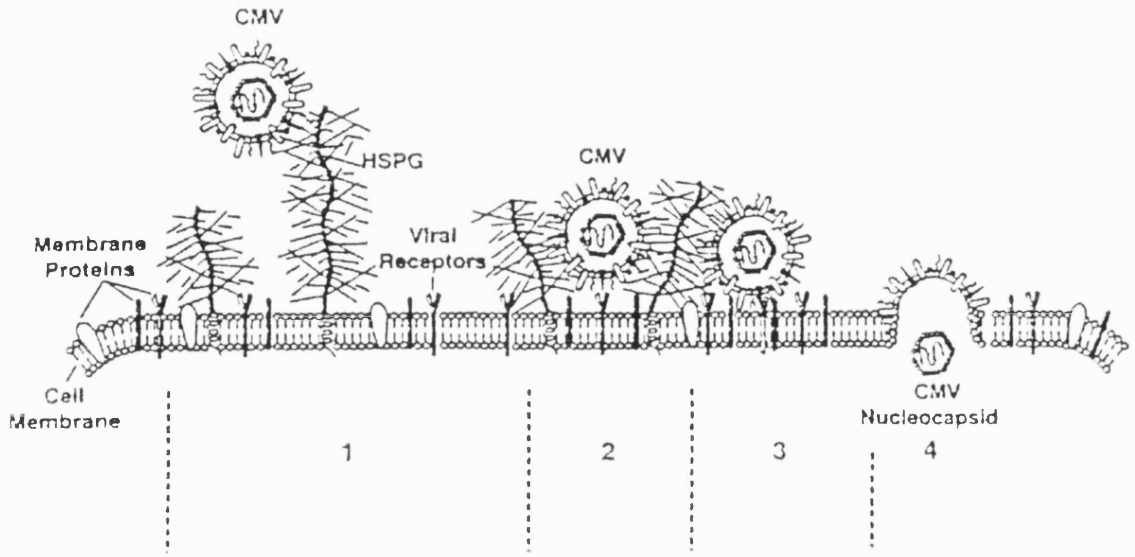

Figure 1.3. A model for the CMV entry pathway into cells.

(1) Initial low affinity virus attachment to extracellular heparan sulphate proteoglycan (HSPG) molecules. (2) High affinity (heparin-resistant) binding mediated by the CD13 cell surface molecule. (3) Alterations in viral structure permit interaction with annexin II cell surface molecules. (4). Envelope-plasma membrane fusion and release of the nucleocapsid in the cytoplasm. (Taken from Compton et al. 1995)

1.1.4 The regulation of gene expression

Once CMV has successfully penetrated the cellular membrane of the host cell, the delivery of the CMV nucleocapsid to the host cell nucleus is followed by the ordered replication of the CMV genome. The extent of CMV viral genome expression plays a crucial role in determining whether a permissive, abortive or latent infection is established in the host cell. Generally, when CMV infects a permissive cell, three broad categories of viral genes termed α (immediate-early), β(early) and γ (late) genes are transcribed progressively (Staprans et al., 1988; Stenberg et al., 1989). The immediate-early (IE) gene products play a pivotal role in regulating both cellular (Colberg-Poley et al., 1992; Hagemeier et al., 1992) and early viral (Malone et al., 1990) gene expression. In contrast, early viral gene products are intimately involved in viral DNA replication, and late genes encode the structural proteins of the virion (Chee et al., 1990).

The major IE gene region of CMV, located within the unique long component of the viral genome, is abundantly expressed from a strong and complex enhancer/promoter element. Its expression results in the generation of differentially spliced mRNA, which encode a series of protein isoforms. Two subregions of this gene exist, and these are commonly referred to as IE1 and IE2. Using recent nomenclature, these regions are also referred to as UL123 (IE1) and UL122 (IE2). p72 (pUL123) and p86 (pUL122) are the most predominant proteins from the IE1 and IE2 gene regions respectively, and are expressed during a productive infection of human fibroblast cells *in vitro*. p72 and p86 are synthesized during the first hour of infection, and their synthesis reaches a maximum by 3-4 hours post- infection (Stenberg et al., 1989). p72 is apparently synthesized throughout the course of infection. In contrast, levels of p86 decrease through early times after infection, but dramatically increase late in infection. p72 and p86 are capable of influencing the expression of their own promoter, the major IE promoter, on which they have profoundly opposite effects. p72 is an activator of the major IE promoter (Stenberg et al., 1990), and appears to act through nuclear factor-κB sites (Cherrington & Mocarski, 1989). In contrast, p86 is a repressor of the major IE promoter, and causes repression via a sequence, called the cis-repression sequence (Cherrington et al., 1991). p86-mediated repression is thought to occur by direct DNA binding to the cis-repression sequence. Mutations that disrupt DNA binding also disrupt p86-mediated repression of the major IE promoter (Lang & Stamminger, 1994). In combination, p72 and p86 are antagonistic, and can overcome the effect of each other in a dose-responsive manner. This suggests that the level of individual proteins can dramatically influence major IE promoter expression, and thus influence the course of viral infection. Because, the abundance of the major IE

promoter is dictated by alternative splicing, post-transcriptional replication of IE gene products must certainly play a critical role in regulating CMV replication (Stenberg et al., 1990).

Several of the viral IE proteins mentioned above serve as essential transactivators of the next class of gene products, termed the early (β) genes. The early gene products by definition are induced prior to viral DNA replication, and exemplify differential patterns of expression as the infection progresses. The class of viral early products includes a vast array of proteins, including enzymes involved in viral DNA replication, non-structural proteins, some structural proteins, and glycoproteins. There are 3 known transcription units, which are all activated at early times. They include the major 2.7kb and 1.2kb transcripts encoded by the repeat bounding the long unique segment of the genome (Klucher et al, 1990; Scully et al., 1995), and a family of differentially spliced transcripts (originally designated the 2.2kb ribonucleic acid-open reading frame UL112-113), which encode four nuclear phosphoproteins (Klucher et al., 1993; Iwayama et al., 1994). The steady-state concentration of the 2.7kb RNA increases greatly between 8 and 14 hours post-infection, while the steady-state concentration of the 1.2 kb RNA exhibits a major increase between 28 and 72 hours. These patterns are in contrast to those of the 2.2kb family of transcripts, whose steady state levels remain about the same after 8 hours post-infection. The promoter for these three early genes are all specified by the major IE region of the human CMV region, with p86 serving as the major viral transactivator.

Finally, late genes, which constitute the majority of the viral genome, are transcribed in abundance only after viral DNA replication, and encode primarily virion structural proteins. The initiation of viral DNA replication originates at a site within or near the large and structural complex lytic-phase replicator, ori-Lyt, near the centre of U_L (Hamzeh et al., 1990). Current models of CMV replication were formed by extrapolation from similarities to HSV, although novel features are emerging. Six HSV-group common genes encode proteins that are likely to constitute the replicative fork-machinery. They include a two-subunit DNA polymerase, a helicase-primase complex, and a single-stranded DNA-binding protein (Huang et al., 1975; Hirai et al., 1976; Anders & Gibson, 1988).

Understanding the regulation of gene expression in CMV is crucial in attempts to develop adequate antiviral strategies. As described above, CMV expresses a battery of protein enzymes that perform essential functions in DNA metabolism and synthesis. Because these enzymes are essential to viral replication and pathogenesis, it is likely that they can be exploited as targets for the development of less toxic, effective anti-CMV

drugs. Antiviral drugs currently licensed for the treatment of CMV infections include ganciclovir, foscarnet and cidofovir (Erice, 1999). Ganciclovir is a nucleoside analogue of guanosine, a homologue of acyclovir, and the first antiviral drug to be effective in the treatment of CMV infection in humans (Martin et al., 1983; Ashton et al., 1982). It inhibits the viral DNA polymerase, by competitively inhibiting the incorporation of deoxyguanosine triphosphate into elongating viral DNA. After the release of pyrophosphate, ganciclovir monophosphate is incorporated into the end of a growing chain of viral DNA, slowing replication (Cheng et al., 1983). Normally in cells infected with HSV, the viral thymidine kinase phosphorylates ganciclovir to the monophosphate form, and cellular enzymes convert the monophosphate to ganciclovir triphosphate, the actual inhibitor of viral DNA synthesis (Martin et al., 1983). CMV, however, does not have a thymidine kinase homologue. Instead, a CMV enzyme with putative protein kinase activity, the product of the CMV UL97 gene, is able to phosphorylate ganciclovir (Littler et al., 1992; Sullivan et al., 1993). CMV strains with mutations or deletions in the UL97 gene do not phosphorylate gangiclovir efficiently, conferring resistant to this antiviral drug. For CMV strains that are resistant to ganciclovir, forscarnet is an alternative antiviral agent. Foscarnet binds directly to viral polymerases and inhibits the polymerases of many viruses, including CMV, human immunodeficiency virus (HIV) type 1 and 2, varicella-zoster virus, and human herpesvirus 6 (Crumpacker, 1996). Another antiviral agent, Cidofovir, is a nucleotide with a similar mechanism of action to ganciclovir, which, in contrast to ganciclovir, requires phosphorylation by a cellular enzyme to its active intracellular metabolite (Ho et al., 1992). Ganciclovir and these other two drugs offer the first and alternative therapies to combat serious and life-threatening CMV infections in immunocompromised patients (Holland & Saral, 1993; Griffiths, 1993; Stocchi et al., 1999).

1.1.5 The strains of CMV

The attenuated CMV strains that are widely used in the laboratory include the Towne and AD169 strains. They were initially developed as vaccine candidates and used in clinical trials. The Towne strain of CMV was adapted for growth in human diploid fibroblasts designated WI-38, and attenuated by propagation in this cell type for 129 consecutive passages. The AD169 strain was also attenuated by a high number of passages in fibroblasts, and was shown to be well tolerated and immunogenic in normal seronegative volunteers, as judged by the seroconversion of the vaccinees without

producing disease (Elek & Stern, 1974). The Towne strain of CMV also induced seroconversion in normal volunteers without producing disease (Plotkin et al., 1976; Quinnan et al., 1984). In addition, the Towne vaccine protected immunosuppressed renal transplant recipients against CMV disease, and was not shed in body secretions (Plotkin et al., 1976). On the other hand, a low-passage human CMV strain, Toledo, produced clinically apparent disease when administered to healthy adult volunteers (Quinnan et al., 1984). These clinical observations suggested that human CMV strains exhibited different levels of virulence, depending on their passage history in cell culture.

High and low-passage human CMV strains have also been demonstrated to exhibit different competencies in their replication in a range of cell types. When the growth of the CMV strains Toledo, Towne, and AD169 was compared in the SCID-hu mouse, the low passage Toledo strain was observed to replicate to levels at least 3 orders of magnitude higher than did either of the high passage strains. The difference between these viruses was attributed to the possible loss of genetic information by the Towne and AD169 strains during propagation in cell culture (Brown et al., 1995). It was later demonstrated that the low passage strains of CMV have segments of DNA of approximately 13-15 kilobase pairs that are not present in the genome of the high passage CMV strains (Cha et al., 1996). Hence, comparisons in the competency of infection between the high-passaged strains and the low-passaged strains of human CMV in various cell types are important.

1.1.6 CMV in other species

For the study of viral pathogenesis *in vivo*, well-defined animal models are of great value. Animal models offer the opportunity to study the interaction of the virus and the host, and to unravel the mechanisms involved in various processes, such as the persistence of the virus, and virus-induced pathology. Due to the restricted host range of CMV, human CMV infection cannot be studied in other species. However, many animal species have cytomegalovirus related to human CMV, and these viruses together with the appropriate animal species can be used as animal models for the human infection. The mouse, guinea pig and rat are the three animal species most frequently used as models for the pathogenesis of human CMV infection. In general, murine CMV is used for studies of generalized infections, guinea-pig CMV for intrauterine infections and rat CMV for transplant-associated complications (Bruggeman et al., 1995).

1.1.7 The cell tropism of CMV

After the adsorption of CMV to the cell surface, viral penetration and the subsequent delivery of the viral DNA to the cell nucleus, a number of factors associated with the host cell determine the outcome of CMV infection. These cellular factors determine the ability of CMV to undergo an abortive or a full cycle of viral replication, or to become latent in the infected cell.

In cell culture, a variety of different cell types have been reported to be permissive for CMV infection. These cell types include fibroblasts, epithelial cells, endothelial cells, haematopoietic cells and smooth muscle cells. Cultured human skin fibroblasts and embryonic lung fibroblasts have most commonly been used to propagate high passage strains of CMV, as well as low passage strains, to high titres. As a result, most of the studies on the replication cycle and the molecular biology of CMV have been performed in the fibroblast system (Mocarski, 1993). Apart from skin fibroblasts and lung fibroblasts, permissive infection by CMV has also been demonstrated in bone marrow fibroblasts (Apperely et al., 1989) and in an immortalized fibroblast line (Compton, 1993b). The infection of epithelial cells *in vitro* by CMV has rarely been reported, probably due to the difficulties with culturing primary epithelial cells. There are only a few reports on permissive infection of retinal pigment epithelial cells (Miceli et al., 1989; Detrick et al., 1996), renal tubular epithelium and renal glomerular epithelium (Heieren et al., 1988). There have been very few studies on the replication of CMV in smooth muscle cells *in vitro*. However, the results of these studies are controversial, with CMV replication reported to be delayed and prolonged in one study (Tumilowicz et al., 1990), and capable of achieving near 100% infection as determined by quantitating the number of cells expressing IE antigen by immunofluorescence in another study (Hosepud et al., 1991). The highly fibroblast-passaged CMV strains AD169 and Towne have been observed to exhibit a restricted capacity to infect endothelial cells *in vitro* (Friedman et al., 1981; MacCormac & Grundy, 1999). In contrast, the low passage Toledo strain and other low passage strains of CMV replicate well in endothelial cells, but these cells are still much less permissive than fibroblasts (MacCormac & Grundy, 1999). There are two main explanations for these observations. The first suggests that the extensive propagation in fibroblasts selects out variants from the original virus population that are not endothelial cell-trophic (MacCormac & Grundy, 1999). The second, which is supported by the demonstration that the highly passaged laboratory strains of CMV have large scale deletions of the viral genome when compared with recent clinical isolates, suggests that *in vitro* passage may lead to the loss of viral genes (Cha et al., 1996).

It is well established that CMV can be transmitted by blood transfusion. The reduction in the rate of CMV transmission following leukocyte depletion of peripheral blood has indicated that infectious virus might be predominantly cell associated, suggesting one or more of the leukocyte populations are likely to be the carrier cells of the virus (Winston et al., 1980). Consequently, circulating leukocytes are regarded as targets of CMV infection. Except for one report (Maciejewski et al., 1993), most *in vitro* studies have consistently found it difficult to productively infect fresh peripheral blood cells, including monocytes and polymorphonuclear leukocytes with either high or low passage strains of CMV (Einhorn & Ost, 1984). However, the extended culture of peripheral blood monocytes with (Lathey & Spector, 1991; Ibanez et al., 1991) or without (Soderberg et al., 1993) specific differentiation signals, resulting in a shift towards a macrophage phenotype, have been reported to increase the number of cells expressing IE-antigen and subsequently becoming productively infected. In support of these observations, are results obtained by applying polymerase chain reaction (PCR) technology to the study of CMV infection of monocytes. These studies revealed that the differentiation of the monocytes of healthy carriers to a macrophage phenotype *in vitro* results in the induction of endogenous IE gene expression (Taylor-Wiedeman et al., 1994; Maciejewski & St Jeor, 1999). In addition, it has been reported that in tissue macrophages, viral genes from all three phases of the CMV replication cycle are detectable (Ibanez et al., 1991; Lathey & Spector, 1991; Sinzger et al., 1996). This suggests that CMV can replicate and produce progeny virus in this cell type, and that the state of differentiation of a CMV-infected cell may have some influence in supporting productive viral replication.

Several cell culture systems have been described which support CMV viral gene expression and replication upon cell differentiation. The induction of cell differentiation by phorbol esters and thymidine analogs appears to promote productive infection in non-differentiated teratocarcinoma cells, which are otherwise non-permissive for CMV infection (Gonczol et al., 1984). In addition, the treatment of endothelial and epithelial cells 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 (Tanaka et al., 1991; Wu et al., 1994).

Histopathological analyses of autopsy material from CMV-infected patients have shown that virtually any organ can be infected by CMV (Toorkey & Carrigan, 1989). During acute infection *in vivo*, CMV nucleic acids and antigens can be detected in a variety of organs of the infected host such as the kidney (Gnann et al., 1988), liver (Theise

et al., 1993), brain (Wiley & Nelson, 1988), retina (DiLoreto et al., 1994; Rummelt et al., 1994), all parts of the gastrointestinal tract (Sinzger et al., 1993a; Sinzger et al., 1995), and the lungs (Schmidt et al., 1993; Wilkens et al., 1994). The various cell types involved in active CMV infection in various organs have been identified by simultaneously staining cell marker proteins and viral antigens in double immunohistochemical staining procedures (Toorkey & Carrigan, 1989; Sinzger et al., 1993a, b; Sinzger et al., 1995). Such procedures have revealed that the major cell targets of CMV infection *in vivo* include, ubiquitously distributed cell types such as epithelial cells, endothelial cells and fibroblasts (Roberts et al., 1989; Sinzger et al., 1993a; Sinzger et al., 1995), leukocytes circulating in the peripheral blood (Grefte et al., 1992; Grefte et al., 1994) and specialised parenchymal cells such as neurons in the brain and the retina, smooth muscle cells in the gastrointestinal tract, and hepatocytes (Wiley & Nelson, 1988; Rummelt et al., 1994). Late-stage CMV-infected cells in histological sections can be recognized by the appearance of typical giant cells, so called cytomegalic cells (hence the name of the virus, cyto = cell, megalo = large), with intranuclear inclusions presenting an "owl's eye" appearance. These cells represent microscopically visible sites of virus assembly or cellular damage, and are collectively referred to as the cytopathic effects of CMV (Briitt & Alford, 1996). This is in contrast to cells, which are morphologically unaltered and have been demonstrated to express IE antigens, but to lack the expression of early and late gene products of CMV replication (Sinzger et al., 1993a, b; Sinzger et al., 1995; Grefte et al., 1994). The cells which have CMV replication restricted to IE gene expression are said to be abortively infected by CMV.

1.1.8 The dissemination of CMV

A number of observations clearly demonstrate that CMV is disseminated haematogenously. The strategic position of endothelial cells at the interface between organ tissues and the blood circulation, suggests that CMV-infected endothelium might be involved in the haematogenous dissemination of the virus. CMV infection is transmitted by blood transfusion from asymptomatic seropositive donors (Choudhury et al., 1996; Landaw et al., 1996). During CMV viremia, CMV DNA is detectable in monocytes, lymphocytes and neutrophils (Bruggeman et al., 1993). Polymorphonuclear leukocytes, in particular neutrophils, are more frequently positive for viral DNA than mononuclear cells (Turtinen et al., 1987; Dankner et al., 1990). In a recent study, Grundy et al. (1998) demonstrated that neutrophils can acquire the CMV tegument protein pp65 and infectious

virus, following either their co-culture with endothelial cells infected with an endothelial cell-trophic clinical isolate of CMV, or by transmigration through infected endothelium. Furthermore, the infectious virus acquired by the neutrophils was shown to be transmissible to fibroblasts. It was concluded that CMV-infected endothelial cells could recruit neutrophils by the secretion of several chemokines, and transmit the virus to them by direct cell-to-cell contact and during neutrophil transmigration. This potential mechanism suggested that the neutrophil-endothelial cell interaction could play an important role in virus dissemination *in vivo*. It has also been demonstrated that there is a bi-directional transfer of infectious virus between endothelial cells and monocytes, suggesting that monocytes may also be a vehicle for the dissemination of infectious virus to distant locations within the body (Waldman et al., 1995). Further evidence implicating the monocyte as a vector for the dissemination of CMV during active infection is provided by the observation that at late stages of viral replication in monocyte-derived macrophages, CMV appears to be sequestered within vacuoles which are frequently seen at cellular contact points (Fish et al., 1995). The compartmentization of CMV was thought to accommodate the viral replication cycle without cell lysis, allowing the macrophage to function as a vehicle for cell-to-cell transmission of CMV. Furthermore, the presence of late stage infected-endothelial cells in the peripheral blood of patients with an active CMV infection has been reported (Grefte et al., 1993; Percivalle et al., 1993; Sinzger et al., 1995). These cells are believed to have detached from the basal membrane of the vessel wall as a result of CMV infection. It is suggested that these circulating cytomegalic endothelial cells, due to their size, might become trapped in tissue capillaries and initiate organ infection by transmitting CMV to capillary endothelial cell at that site.

In summary, a wide variety of cells are preferentially infected in solid-organ tissues *in vivo*. From these sites of replication, the virus can possibly be carried by peripheral blood monocytes, neutrophils and circulating endothelial cells to reach distant sites of the body.

1.1.9 The types of CMV infection

The classifications and definitions of the resultant CMV infection and disease have varied greatly, prompting attempts to establish standard terminology (Ljungman et al., 1994). Acute CMV infection is defined as a laboratory-based diagnosis of viral replication, characterized by the detectable presence of CMV in clinical specimens such as blood or other body fluids, or organ biopsies. The type of infection is an important factor

in the pathogenesis of infection and the expression of symptomatic disease (Grundy, 1990; Alford et al., 1990). In transplant recipients, CMV infection can arise from either an exogenous source usually via donor organs or blood products administered to the patient from a seropositive donor, or from the reactivation of latent endogenous virus in the recipient. Thus, an acute infection may be primary or secondary. A primary infection occurs when a previously seronegative individual is infected with exogenous virus. Secondary infections could either be the result of reactivation of latent endogenous virus in a seropositive patient, or reinfection of a seropositive patient, with a new strain of CMV from an exogenous source (Grundy et al., 1988). The clinical presentation of an acute infection may be symptomatic or asymptomatic. Symptomatic infections usually begin with fever and malaise, and may progress to pneumonitis, hepatitis, gastrointestinal disease or retinitis. This is discussed in detail in section 1.4.

1.2 THE HOST IMMUNE RESPONSE TO CMV

An important biological property of CMV is its ability to persist in the infected host for life, despite an ongoing immune response (Rook, 1988). Infection of the normal host with CMV and subsequent viral DNA replication is followed by both cellular and humoral immune responses against the virus (Rasmussen, 1991). The importance of cell-mediated immunity against CMV is exemplified by the occurrence of severe and prolonged CMV infection in individuals with congenital, or acquired deficiencies of this arm of host immunity. In contrast, CMV infections in most individuals with primary B cell disorders are usually not severe (Rook, 1988). The acquisition of CMV-specific immune responses appears to be essential for limiting primary infection, and for the maintenance of a clinical state of latency.

An essential part of the cellular immune response to CMV infection is the recognition and elimination of infected cells by cytotoxic T lymphocytes. To achieve this, a display mechanism has evolved which is present in almost all nucleated cells. This mechanism is referred to as the class I MHC antigen-processing pathway (Heemels & Ploegh, 1995). The recognition of virus-infected cells by $CD8^+$ cytotoxic T lymphocytes requires that the viral proteins be processed into peptides. The generation of peptides largely occurs by proteasome-mediated degradation of cytosolic proteins. The transporter associated with antigen processing translocates peptides of 8-12 amino acids from the cytosol into the endoplasmic reticulum (Androlewicz et al., 1993). Endoplasmic-resident chaperone proteins facilitate the folding of class I heavy chains, and the loading of free

peptides onto the binding grove of the class I MHC molecule (Neefjes et al., 1993). Peptide-loaded class I MHC antigenic complexes are then exported through the Golgi apparatus to the cell surface, where the antigenic complexes display virally derived peptide to $CD8^+$ cytotoxic T lymphocytes (Heemels & Ploegh, 1995). Once this occurs, virus-specific $CD8^+$ cytotoxic T lymphocytes proliferate, and may lyse virally infected cells, or release cytokines that inhibit viral replication and activate the immune system.

The murine CMV-infected mouse model has provided a vast amount of information on host defence mechanisms against CMV infection. Reddehase et al. (1985) demonstrated that mice immunosuppressed by γ-irradiation showed enhanced virus growth, and an increased mortality rate, compared to their non-irradiated counterparts. However, the adoptive transfer of spleen cells from immune donors restored protection from murine CMV infection. The cells responsible for this protective response were found to be $CD8^+$ cytotoxic T-lymphocytes (Reddehase et al., 1987). It has also been shown in T-cell depleted mice, that the adoptive transfer of murine CMV-immune lymph node cells could prevent the development of retinitis (Lu et al., 1997). Recently, Steffen et al. (1998) demonstrated that 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, the adrenal glands and the salivary glands. This finding is of particular importance, since it has 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).

CMV-reactive cytotoxic T cells have also been observed in humans (Reusser et al., 1996). Their presence correlates with protection from CMV disease. Studies of immune reconstitution after allogeneic bone marrow transplantation have identified a role for the recovery of $CD8^+$ T-cell class I MHC restricted cytotoxic T lymphocyte responses in preventing the development of CMV disease (Reusser et al., 1991: Riddell et al., 1992). Adoptive transfer protocols using homologous CMV-specific cytotoxic T lymphocytes hold promise for the treatment of post-transplant CMV disease (Greenberg et al., 1991; Walter et al., 1995; Smyth & Kershaw et al., 1995).

Several structural (pp65, pp150 (ppUL132), gB, gH) and non-structural (IE) CMV proteins, synthesised during the IE, early and late periods of virus replication, have been identified as eliciting cytotoxic cellular immune responses (Alps et al., 1991; van Zanten et al., 1995; Wills et al., 1996). IE-specific cytotoxic T lymphocytes efficiently lyse autologous cells infected with recombinant vaccinia virus encoding the p72 protein, but not cells infected with CMV, although both target cells express the protein (Jonjic et al., 1988; Gilbert et al., 1993). These cytotoxic T lymphocytes fail to lyse permissively

infected target cells even if viral replication is expressed at the IE stage, suggesting that CMV may have a unique mechanism to interfere with the presentation of IE antigens. Gilbert et al. (1996) has recently shown that the structural protein pp65 released into the cytosol after virus entry can modify the presentation of IE antigens. The modification induced by pp65 is the phosphorylation of a threonine residue in the p72 protein. This interferes with IE antigen presentation without detectable changes in either the stability of the IE protein, or its ability to activate transcription (Schmolke et al., 1995). These results suggest that, in contrast to murine CMV infection in BALB/c mice, in which IE-specific cytotoxic T lymphocytes lyse permissively infected cells and confer protective immunity (Reddehase et al., 1987), cytotoxic T lymphocytes specific for the human IE counterpart would be less effective in promoting the clearance of CMV infection.

Target cells infected with CMV under complete transcriptional blockade with actinomycin D are lysed by polyclonal CMV-specific cytotoxic T lymphocytes from all seropositive individuals. These results demonstrate that viral gene expression is not required to provide antigens for T-cell recognition, and that the majority of the response is directed against structural virion proteins, which enter the cell with the virion in sufficient amounts for the recognition by $CD8^+$ cytotoxic T lymphocytes (Riddell et al., 1991). pp65 has been identified as the major target for the cytotoxic T lymphocyte mediated response during primary infection (Boppana & Britt, 1996; Wills et al., 1996). Three major T-cell epitopes within the C-terminus of pp65 have been identified as targets for cytotoxic T lymphocyte responses (Khattab et al., 1997). Besides pp65, pp150 or ppUL132 is particularly immunodominant, with up to 90% of all CMV specific cytotoxic T lymphocytes directed against one or both of these viral antigens in individual patients (Wills et al., 1996). Cytotoxic T lymphocytes specific for pp65 and pp150 effectively destroy permissively infected cells, because epitopes derived from these antigens remain at the cell surface to permit recognition by cytotoxic T lymphocytes at all stages of virus replication. However, such T cells may not be effective against cells reactivating latent virus, because the relevant proteins would not be available for processing in these cells until the genes encoding them are expressed at early and late times of virus replication (Khattab et al., 1997). Also, at early and late times of virus replication, class I MHC expression is reduced, and antigen presentation is inhibited by the combined effects of the US gene products (Jones et al., 1996; Jones et al., 1997). This phenomenon is dealt with in more detail in the next section.

Natural killer cells play a protective role in the host immune response to viruses, prior to the induction of a more specific T cell response (Welsh & Vargas-Cortes, 1992).

Natural killer cells play a role in the innate immune response, both as effector cells, and as a source of cytokines that modulate the activities of other cell types, including T-cells and macrophages. As effector cytolytic cells, natural killer cells lyse transformed cell lines as well as virus-infected cells. Unlike T-cells, which recognise antigens as peptide fragments bound to an MHC molecule, natural killer cells become functional in the absence of class I MHC proteins on target cells. This missing-self hypothesis proposes that T-cell and natural killer cell immunity represent complimentary arms of the cellular immune response (Kos & Engleman, 1996).

Selective adoptive transfer experiments in the murine CMV model have demonstrated that natural killer cells protect against lethal CMV infection (Bukowski et al., 1984; Bukowski et al., 1985). In a more recent study, Lathbury et al. (1996) demonstrated that in the absence of T cells, natural killer cells were responsible for controlling murine CMV replication in visceral organs early in infection. In man, isolated natural killer cell deficiencies are associated with severe primary CMV infection (Biron et al., 1989). van den Berg et al. (1992) demonstrated that the spontaneous recovery from primary CMV infection coincided with a marked increase in the number of natural killer cells in the peripheral blood of renal transplant recipients with CMV infection. In addition, Venema et al. (1994) has reported a positive correlation between increased levels of activated natural killer cells and recovery from CMV infection in renal transplant patients.

The humoral immune response to CMV is important in modifying the clinical manifestations associated with subsequent CMV infections, particularly in the neonate (Donner et al., 1993; Lipitz et al., 1997) and in the immunocompromised host (Adler, 1996). Antibody responses with effector activity include the induction of virus neutralizing antibodies, and antibodies that mediate antibody-dependent cellular cytotoxicity. Clinical data have provided evidence for an important role of antibody in the prevention of severe CMV disease. Such studies have shown that newborns who have acquired CMV transplacentally, and are therefore at risk of developing congenital CMV disease, are protected if their mothers had antibodies to CMV prior to pregnancy (Adler, 1995). Numerous studies have provided evidence that serum antibodies to CMV obtained before transplantation give relative protection from severe CMV disease (Marshall et al., 1992; Alberola et al., 1998; Alberola et al., 2000). Passive immunization procedures using hyperimmune γ–globulin have given some protection against CMV-associated syndromes (Snydman et al., 1987). Standard intravenous administration of γ–globulin in seronegative

recipients of seropositive organs has led to a reduced incidence of CMV associated fever, leucopenia, and mortality (Metselaar et al., 1990), providing immunity comparable to that obtained through natural immunity (Steinmuller et al., 1990).

The murine CMV-infected mouse model has also being used to study the parameters that influence the protection afforded by adoptive humoral immunity. It has been demonstrated in both immunocompetent and immunosuppressed mice that the administration of either early (≤ day 7 post-infection) or late (≥ day 47 post-infection) immune sera 24 hours prior to murine CMV infection reduces virus replication and dissemination to below detectable levels (Lawson et al., 1988). However, this treatment does not prevent the establishment of a latent infection (Balthesen et al., 1994). In contrast, protection against a lethal murine CMV infection was not observed if antibody was administered 24 hours after murine CMV infection in these mice, suggesting that specific antibody may only be effective if administered prophylactically. Furthermore, it has been reported that the passive transfer of anti-gB antibodies and immunization with vaccinia virus expressing murine CMV gB protects mice against a lethal challenge (Rapp et al., 1993).

Overall, the observations in man and the mouse illustrate the central importance of the cellular and humoral immune response in controlling CMV infection and preventing or reducing CMV disease.

1.3 CMV IMMUNE ESCAPE MECHANISMS

A common biological property of herpesviruses is their ability to persist lifelong within the infected host, despite an ongoing immune response. In addition to the establishment of latent infections, herpersviruses have acquired an impressive array of immunomodulatory mechanisms, which contribute to their success as long-term parasites. Recent studies have revealed that CMV has evolved a variety of distinct mechanisms for the evasion of cellular immunity mediated by CD8+ cytotoxic T lymphocytes and natural killer cells, two of the major cellular effectors for the identification and eradication of virally-infected cells (Hengel et al., 1998). CMV primarily avoids the cytotoxic T cell responses by interfering with the surface expression of class I MHC molecules and thus antigen presentation by CD8+ T lymphocytes (Del-Val et al., 1989; Barnes & Grundy, 1992). Since cytotoxic T lymphocytes recognize foreign peptides in association with class I MHC, the down-regulation of cell surface expression of class I MHC molecules at the surface of infected cells would appear to provide a powerful means of inhibiting viral

clearance by cytotoxic T lymphocytes. Several *in vitro* studies have confirmed that reduced cell surface expression of class I MHC molecules correlates with increased resistance to lysis of CMV-infected cells by cytotoxic T lymphocytes (Del-Val et al., 1992; Beersma et al., 1993; Warren et al., 1994).

As mentioned earlier, CMV has been demonstrated to specifically block the presentation of peptides derived from the IE protein, p72. Studies utilizing vaccinia virus recombinants have demonstrated that in the presence of pp65, the recognition of cells infected with the vaccinia virus expressing the p72 protein, by p72-specific cytotoxic T lymphocytes are inhibited, whereas pp65 had no effect upon the recognition by cytotoxic T lymphocytes directed against vaccinia virus peptides (Gilbert et al., 1996).

The US region of the CMV genome encodes at least five to six different glycoproteins, each potentially interfering in a different way with the elimination of the virus by cytotoxic T lymphocytes. It is the believed that the concerted actions of these glycoproteins allow CMV to escape from elimination by the host immune system during acute and perhaps also latent infection (Wiertz et al., 1997; Farrell & Davis-Poynter, 1998). CMV has been demonstrated to inhibit the first step in class I MHC antigen presentation namely the proteolysis of cytosolic proteins. Thus, in addition to the selective inhibition of peptide presentation mentioned above, peptide translocation into the endoplasmic reticulum via the transporter associated with antigen processing is also blocked following CMV infection (Hengel et al., 1996). Two independent studies have identified a glycoprotein encoded by US6 to be responsible for this action (Hengel et al., 1996; Ahn et al., 1997). Using deletion mutants, the early gene product US11 was first identified as a major cause of the instability of class I MHC molecules in CMV-infected cells (Jones et al., 1995). Another gene product US2 has also been found to cause class I degradation in a manner similar to that caused by US11 (Wiertz et al., 1996). Whereas US2 and US11 induce the rapid breakdown of class I heavy chains, US3 retains the class I complexes in the endoplasmic reticulum (Ahn et al., 1996; Jones et al., 1996; Jones et al., 1997; Wiertz et al., 1997). Table 1.1 summarises the mechanisms by which CMV evades immune recognition by disrupting MHC/peptide presentation in the host cell (Farrell & David-Poynter, 1998).

Table 1.1. The inhibition of class I MHC antigen presentation by CMV gene products.

Gene product[a]	Expression	Proposed function
pp65	Pre-IE	To block IE-1 peptide presentation
gpUS6	L	Inhibits TAP[c]-dependent peptide translocation
gpUS2	E	To dislocate class I to cytosol
gpUS11	E	To dislocate class I to cytosol
gpUS3	IE	Retains class I in ER[d]

[a]Proteins are named according to their open reading frame designations, with prefixes denoting glycoproteins (gp) or phosphoproteins (pp).

[b]The expression kinetics of the proteins described according to the immediate early (IE), early (E) , and late (L) phases of CMV gene expression.

[c]TAP-transporter associated with antigen presentation.

[d]ER-endoplasmic reticulum.

Whilst cytotoxic T cells recognize and are activated by target cells that express novel MHC/peptide complexes, natural killer cells, are activated by and kill target cells which have lost the expression of class I MHC proteins (Leong et al., 1998). Thus, viral-infected cells with reduced class I MHC surface expression are inherently susceptible to attack from natural killer cells. However, there is evidence that CMV may have evolved a mechanism to protect infected cells with little or no surface class I MHC from lysis by natural killer cells. This idea is supported by the observation that CMV-infected endothelial cells that lack surface class I MHC show a marked resistance to lysis by allogeneic natural killer cells (Waldman et al., 1998). When initially identified, the class I MHC-like protein of CMV, encoded by UL18, was considered to be a prime candidate for disrupting cytotoxic T lymphocyte recognition of virally infected cells (Beck & Barrell, 1988; Wiley, 1988). The UL18 protein was described as a classical class I MHC heavy chain with regard to forming a complex with β2-microglobulin, and raised the possibility that UL18 may down-regulate cell surface class I MHC by the sequestration of endogenous β2-microglobulin (Browne et al., 1990). However, a later study has discounted the role of the UL18 gene product in the down-regulation of class I MHC antigen expression (Browne et al., 1992). It was demonstrated in the latter study using a CMV mutant in which the UL18 open reading frame had been disrupted, that the synthesis of cellular class I MHC in infected human fibroblasts was down-regulated to the same extent as was achieved with a wild-type CMV strain. This suggested that the UL18 gene product was not directly involved in the disruption of class I assembly. Furthermore, the UL18 homologue has been implicated to act as a molecular "decoy", which may mimic the ability of cellular class I MHC to inhibit the functions of natural killer cells. Natural killer cells carry triggering receptors and inhibitory receptors. Activation of the triggering receptor by a target cell will result in its destruction unless the inhibitory receptor detects a class I MHC molecule. The results from *in vitro* studies are not uniform, but in general they support the notion that the UL18 homologue engages inhibitory receptors from natural killer cells that normally interact with cellular class I MHC and are known to preferentially attack cells displaying low levels of class I MHC complexes (Raulet, 1996; Karre, 1995; Farrell et al., 1999).

CMV also induces the expression of a receptor for the Fc portion of human immunoglobulin G in several cell types (Furukawa et al., 1975; Westmoreland et al., 1976; Stannard & Hardie, 1991; MacCormac & Grundy, 1996). This receptor (FcγR) has been suggested to be a glycoprotein of Mr 42000 (Sakuma et al., 1977) which appears at early times post-infection. and is found in the perinuclear region of the infected cell (Keller et

al., 1976). The function of the FcγR is unknown, but it may allow CMV to evade host antibody responses (MacCormac & Grundy, 1996). It is assumed that these FcγRs protect 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). Thus, this phenomenon may represent an important strategy of the virus to evade host responses.

In conclusion, CMV possesses a diverse array of mechanisms for the disruption of cytotoxic T lymphocyte recognition and natural killer lysis of virus-infected cells, ensuring their survival in the infected host.

1.4 CLINICAL SYNDROMES ASSOCIATED WITH CMV INFECTION

CMV infection is the most common congenital viral infection in humans, with an incidence in the United States of approximately 0.4%-2.3% of all live born infants infected with CMV (Demmler, 1991; Hirota et al., 1992; Nelson & Demmler, 1997). This translates into about 40, 000 infected infants born each year in the United States. Primary infection with CMV during pregnancy occurs in approximately 0.7% to 4.1% of pregnancies, with reported transmission rates to the foetus of between 24% and 75% (Alford et al., 1990; Nelson & Demmler, 1997). Young infants and children with subclinical infection appear to be a major source of virus for primary infection in pregnant women. Day care centres and similar nursery settings where women are in daily contact with children, especially with toddlers, pose a high risk setting for acquiring primary infection in pregnant women (Pass & Kinney, 1985; de Mello et al., 1996; Shen et al., 1996). Oral and respiratory spread appears to be the predominant routes of virus transmission in these populations (Nelson & Demmler, 1997). In certain groups, especially in pregnant women attending clinics for sexually transmitted diseases, an important form of transmission is through sexual contact (Demmler et al., 1986). CMV may also be transmitted to a pregnant woman by blood transfusion. However, the risk of CMV transmission is considerably reduced by the use of leukocyte-depleted blood products or blood products from seronegative donors (Demmler et al., 1986). The transmission of CMV from the mother to the foetus may occur in all three trimesters of pregnancy with apparent equal frequency, and it is assumed to occur by transplacental transmission of the virus after maternal viremia (Griffiths & Baboonia, 1984; Stagno et al., 1986; Yow et al., 1988). The latter proposal is supported by studies in guinea pigs showing that intrauterine infection occurred when acute primary infection with guinea pig

CMV was initiated during pregnancy (Griffith et al., 1986). Whilst 90% of newborns congenitally infected with CMV are asymptomatic at delivery, 10% will have a variety of signs, symptoms or laboratory abnormalities apparent at the time of birth, including smallness for gestational age, microcephaly, intracranial calcifications, retinitis, sensorineural hearing loss, jaundice, hepatomegaly and thrombocytopenia (Demmler, 1991, Fakhry & Khoury, 1991, Nelson & Demmler, 1997). 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 (Nelson & Demmler, 1997).

CMV continues to be an important pathogen affecting solid-organ transplant recipients (Stratta, 1993; Falagas & Snydman, 1995). It is the most important cause of morbidity in the post-transplant period for patients undergoing kidney, renal, liver, heart and heart-lung transplantation (Smyth et al., 1991; Decoene et al., 1996; Andersen, 1997; Ducloux et al., 1997). The incidence of CMV infection is similar between groups, with 60-90% of patients showing evidence of the virus as judged by viral excretion (Britt, 1996). The onset of disease is dependent on several risk factors, which contribute to infection in the post-transplant period. These include the degree of immunosuppression, the donor and recipient serologic status, the source of the allograft (living-related versus cadaveric), human leukocyte antigen (HLA)-mismatching and the type and amount of blood products used (Dummer, 1990; Smyth et al., 1991). Seropositive blood products administered as part of the medical management have also been implicated as an important source of virus transmission in the allograft recipient. Multiple transfusions and/or the transfusion of large quantities of blood are associated with CMV infection (Choudhury et al., 1996; Landaw et al., 1996). Procedures that limit the quantity of leukocytes in transfused blood significantly reduce the incidence of CMV infection following transfusion, suggesting that leukocytes are responsible for most transfusion-associated CMV infections in allograft recipients (Miller & Mintz, 1995; Gunter, 1995). The wide variety of clinical disease manifestations observed in solid-organ transplant recipients typically appear within the first three months post-transplantation. Severe end organ complications include encephalitis, pneumonitis, retinitis, adrenalitis, enteritis and hepatitis, with other clinical complications in the absence of organ involvement being fever, leucopenia, thrombocytopenia and malaise (Britt & Alford, 1996).

There is evidence to suggest that CMV infection plays a role in the pathogenesis of solid-organ transplant rejection (Nakhleh et al., 1991; Arbustini et al., 1996; Martelius

et al., 1998). An association of viral infection with allograft rejection has been demonstrated in both clinical practice and experimental models of transplantation. In one of the first studies to report a relationship between CMV infection and allograft rejection, a high frequency of late acute rejection, associated with increased class II MHC expression was observed in CMV-infected individuals undergoing renal transplantation (von-Willebrand et al., 1986). Subsequently, several other studies have reported that CMV infection is associated with a significant risk for acute rejection in recipients of cardiac (Grattan et al, 1989; Loebe et al., 1990), pulmonary (Bando et al., 1995), renal (Reinke et al., 1994; Massy et al., 1998) and liver allografts (Fietze et al., 1994; Martelius et al., 1998). Additional evidence from a number of studies indicate that there is a significant association between CMV infection and the development of transplant-associated atherosclerosis (Loebe et al., 1990; Koskinen et al., 1993a; Koskinen et al., 1993b; Decoene et al., 1996; Koskinen et al., 1996a). Although, transplant-associated atherosclerosis was first extensively studied in cardiac recipients, it has also been shown to occur in renal, lung and liver allografts recipients, where it is referred to as glomerulopathy, obliterative bronchiolitis and the vanishing bile duct syndrome, respectively (Massy et al., 1998; Scott et al., 1997; Lautenschlager et al., 1997b). Transplant-associated atherosclerosis is characterised by the occurrence of a diffuse concentric intimal proliferation in all arteries and arterioles of the transplanted organ, which leads to the narrowing and eventually complete occlusion of the blood vessels serving the allograft (Duquesnoy & Demetris, 1995; Ventura et al., 1995; Dong et al., 1996; Hayry et al., 1998). The evidence implicating a pathogenic role of CMV infection in human cardiac recipients and in the rat model of transplant atherosclerosis is dealt with extensively in sections 1.6.4 and 1.6.5, respectively.

Until recently, CMV was a major cause of morbidity and mortality after allogeneic bone marrow transplantation (BMT), and among patients who were seropositive, approximately 15% to 20% of deaths occurring after transplantation could be attributed to CMV interstitial pneumonitis (Forman & Zaia, 1994; Reusser, 1996). Risk factors for CMV pneumonia include old age, conditioning with total-body irradiation, and severe graft-vs-host disease. CMV-associated pneumonitis in the allogeneic marrow recipient develops approximately 7 to 10 weeks after BMT, with a median onset of approximately 50 days, and with most episodes occurring in the first 100 days. An important predisposing risk factor for the occurrence of CMV-associated pneumonitis is the serological status of the patient and the donor (Meyers et al., 1989). In a seronegative patient who has a seropositive donor, the source of subsequent primary CMV infection is

the donor marrow itself and administered blood products. The relevance of administered blood products as a source of CMV infection is supported by studies where elimination of the leukocyte fraction from the transfused blood greatly reduces the incidence of transfusion acquired CMV infection (Bowden, 1991; Miller & Mintz, 1995). However, in a CMV seropositive recipient, the donor status is probably not relevant to the subsequent development of infection, although the donor marrow immune response could influence subsequent disease occurrence (Grob et al., 1987). In seropositive recipients, it is usually the reactivation of endogenous recipient CMV rather than infection with exogenous virus that leads to CMV disease progression (Winston et al., 1990). This suggests that the donor marrow does not present a high risk for transmission of CMV infection to the seropositive recipient. In allogeneic bone marrow transplant recipients, CMV-associated pneumonitis has been suggested to represent an immunopathological condition (Grundy et al., 1987). Evidence to support this hypothesis comes from observations in which a poor correlation was shown to exist between the titres of infectious virus in the lung tissue and bronchoalveolar lavage fluids and the severity and outcome of disease (Churchill et al., 1987; Slavin et al., 1992). A further stimulus was suggested to be required for the development of disease because CMV was detectable in bronchoalveolar lavage fluids at day 35 post bone marrow transplantation, but the peak of the onset of pneumonitis occurred between 70 and 120 days post transplantation (Wingard et al., 1988a; Schmidt et al., 1991). The initial trials with the antiviral drug, ganciclovir, showed that there was no improvement in survival rate in treated patients above untreated patients, despite a reduction of viral titres in the lungs (Shepp et al., 1985). In addition, the presence of extensive inflammatory infiltrates with only limited viral replication further suggested that the pathogenesis of CMV pneumonitis involves tissue damage caused by the immune system (Risse et al., 1997; Leonard et al., 2000). Furthermore, the association of CMV pneumonitis with allogeneic, but not autologous, bone marrow transplantation, suggests that the allogenicity of the graft is an important factor (Wingard et al., 1988b). The occurrence of graft versus host disease is a risk factor for CMV pneumonitis, further suggesting that the immune response may be involved (Miller et al., 1986)

Studies in the murine CMV mouse model of pneumonitis have formed the basis for the hypothesis that in allogeneic transplant recipients, CMV infection is an immunopathological condition. Analysis of murine CMV pneumonitis after bone marrow transplantation in mice indicate that murine CMV can replicate in the lungs of immunocompetent mice with minimal or no histological evidence of the disease (Shanley et al., 1987; Shanley, 1991). It has been demonstrated in several other models of murine

CMV pneumonitis that some alterations to the immune response is required to produce disease. In one such model, pneumonitis was shown to develop only in mice infected with murine CMV and given a graft-vs-host challenge (Grundy et al., 1985). In the absence of the graft-vs-host reaction, the virus replicated in the lung without causing pneumonitis (Grundy et al., 1985; Shanley et al., 1987). Ganciclovir treatment of these mice with pneumonitis reduced virus replication in the lung to undetectable levels, but did not reduce the histological characteristics of pneumonitis (Shanley et al., 1987). The depletion of T cells in the mice using antibodies, protected against the development of CMV pneumonitis, while reconstitution of the mice with syngeneic T cells resulted in pneumonitis (Erlich et al., 1989). Thus, in mice, as in man, the occurrence of graft-vs-host disease is a risk factor for CMV pneumonitis. In addition, the infiltration of T cells contributes to the pathology of CMV disease in mice (Mennander et al., 1991), supporting the hypothesis that the development of CMV-associated pneumonitis in man is immune-mediated (Grundy et al., 1987).

CMV is responsible for the most common viral opportunistic infections in persons with AIDS. Clinical disease due to CMV has been reported in up to 40% of patients with advanced human immunodeficiency virus (HIV) disease (Cheung & Teich, 1999). The clinical manifestations of reactivation of CMV infection in patients with AIDS (CD4 count $< 50/mm^3$) are widely recognised (Cheung & Teich, 1999). Most patients with AIDS are usually co-infected with CMV (75-98%) because both CMV and HIV are transmitted via haematologic and sexual routes (Peters et al., 1991). In addition, CMV seropositivity has been associated with an increased risk for AIDS, suggesting that CMV could serve as a cofactor in the progression of HIV infection (Schooley, 1990; Webster et al., 1992). CMV is believed to potentiate the cellular immunodeficiency observed in patients with HIV infection, either directly, or through the enhancement of HIV replication. CMV causes a number of clinical syndromes, including retinitis and gastroenteritis, with the occasional incidence of neurological CMV infection and encephalitis, in patients with AIDS (Jacobson, 1994; Bell, 1998). The most common clinical syndrome in AIDS patients is retinitis, occurring in 30% of long lived AIDS patients, and CMV infection is the most frequent cause of sight threatening disease or blindness in these patients (Verbraak et al., 1998). Gastrointestinal disorders are the second most common clinical syndrome, occurring in 5% to 12% of HIV infected individuals (Jacobson & Mills, 1988; Williams & Wilson, 1992). In CMV-infected gastrointestinal tissues, the most frequent primary lesions are ulcerations, possibly due to vasculitis resulting from the presence of infected endothelial cells in the wall of small vessels (Tatum et al., 1989; Kyriazis & Mitra, 1992).

Besides endothelial cells, epithelial cells and smooth muscle cells are the major infected cell types (Singzer et al., 1995). The destruction of the epithelial and smooth muscle cell layers of the gastrointestinal tract may lead to erosions and ulcerations. The damage is believed to result from direct viral cytopathogenicity, because the symptoms are resolved following anti-viral chemotherapy (Chachoua et al., 1987; Dieterich et al., 1988).

In conclusion, CMV is a major pathogen in the immunocompromised host, and infects a variety of organs during acute infection. The pathology of CMV in these organs can be caused either by direct viral damage of infected cells, or by indirect tissue damage caused by the host immune system, or by both mechanisms. Direct viral damage is common at sites where the absence of an active immune response allows for high levels of viral replication and systemic disease. The mechanism of virus-induced immunopathology remains to be definitively elucidated.

1.5 ATHEROSCLEROSIS

Epidemiological and clinical studies have implicated CMV infection, and its associated pathology in infected tissues, as being involved in the development of a type of coronary artery disease known as atherosclerosis. Atherosclerosis (sometimes called the "hardening of arteries") is a disease of large and medium-sized arteries characterized by the focal thickening of the inner portion of the artery wall in association with lipid deposits (Ross, 1999). Although atherosclerotic lesions can be observed throughout the body, certain areas of the arterial tree are particularly prone to the development of lesions, such as the aortic arch and the iliac, femoral, coronary and cerebral arteries (Ross, 1999). Advanced lesions of atherosclerosis can abruptly interfere with blood flow, particularly to strategic blood vessels supplying the heart and brain, and as such contribute to the pathogenesis of myocardial and cerebral infarction. The major cells comprising atherosclerotic plaques are phenotypically modified smooth muscle cells, monocyte derived macrophages and T lymphocytes (Ross, 1999). To appreciate the role of these cells in the pathogenesis of atherosclerosis, it is necessary to know something about the structure of normal arteries.

All blood vessels contain three concentric coats, or tunics: the tunica intima, the tunica media, and the tunica adventitia (Figure 1.4). The tunica intima, the innermost layer, is formed by a monolayer of endothelial cells located at the luminal side of the vessel wall and its underlying basal lamina, the internal elastic membrane. The middle coat, the tunica media, consists chiefly of circularly arranged smooth muscle cells, with varying amounts of elastic and collagenous fibres, and proteoglycans interspersed between the muscle cells. The outermost coat, the tunica adventitia, consists of connective tissue containing elastic and collagenous fibres that run parallel to the long arms of the vessel. In arteries, the tunica intima and the tunica media are separated by the internal elastic lamina, a thin layer of elastic fibres. Often a similar layer, the external elastic lamina, separates the tunica media and the tunica adventitia.

The actual structure of the vascular wall is of course directly related to the function of the specific parts of the vascular tree. Arteries contain a rather thick tunica media, whilst veins have a rather thin tunica media but a relatively thick tunica adventitia with little elastic tissue. However, certain veins of the limb, exemplified by the superficial veins of the leg, possess a highly developed muscular media that counteracts the tendency to distension as a result of venous return against the force of gravity. This modification is particularly noticeable in the saphenous vein, which possesses an inner longitudinal as well as outer circular layer of smooth muscle in their media (Figure 1.5).

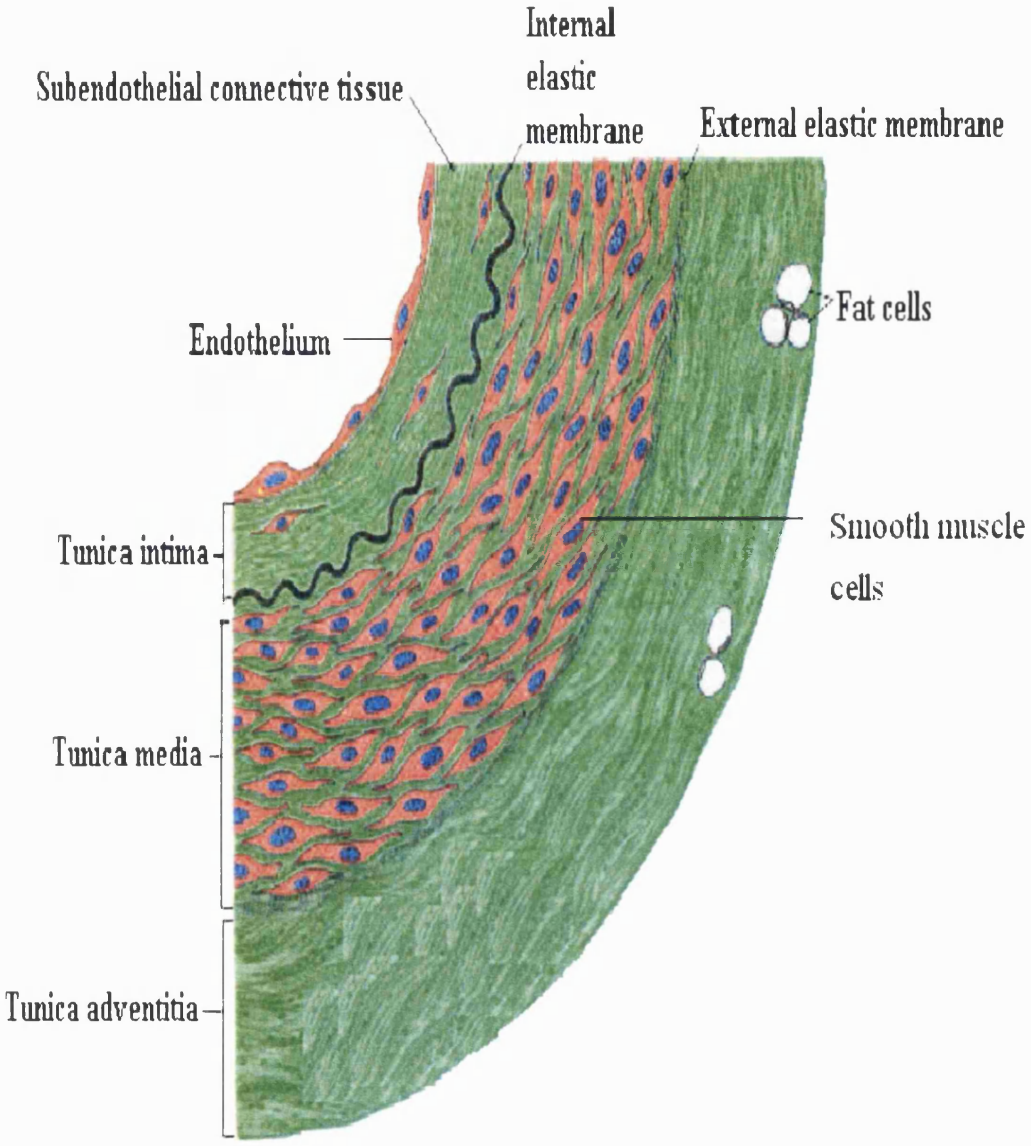

Figure 1.4. A schematic diagram illustrating the general structure and organization of blood vessels.

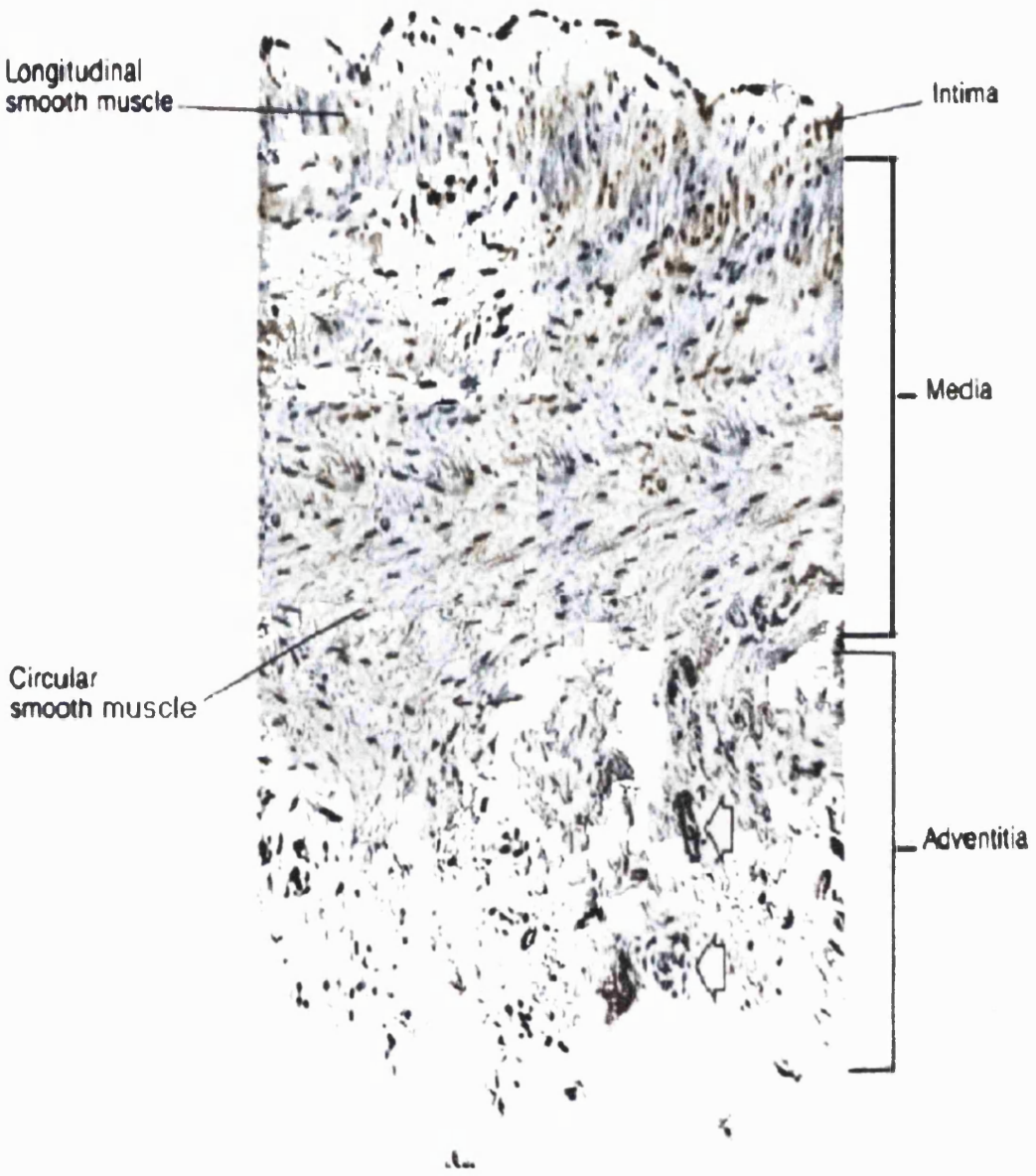

Figure 1.5. A photomicrograph showing the structure of the saphenous vein.

The muscular media contains an inner longitudinal layer of smooth muscle in addition to its thick outer circular layer of smooth muscle.

1.5.1 The lesions of atherosclerosis

Of the three layers mentioned, the principal changes that take place in the artery wall during the development of the lesions of atherosclerosis occur largely within the intima. The lesions of atherosclerosis that are clinically recognized include the fatty streak, the intermediate or fibrofatty plaque, and the advanced complicated lesion of atherosclerosis or fibrous plaque (Ross, 1995). It is questionable whether each lesion type acts as a precursor for the other. Observations in experimentally induced hypercholesterolemic animals, demonstrate that the adherence of leukocytes, predominately monocytes and small numbers of T lymphocytes, to the endothelium is the first step in the development of atherosclerotic lesions (Masuda & Ross, 1990). The adhered leukocytes then migrate into the subendothelial space in response to chemotactic proteins where they become filled with lipids, leading to the formation of the activated lipid-filled macrophage or "foam cell". The accumulation of lipid-filled macrophages in the subendothelial space constitutes the fatty streak, the earliest lesion of atherosclerosis. Fatty streak formations are observed in children in early infancy and in adolescents in autopsy material, depending on dietary habits and lifestyle (Raines & Ross, 1995). They also appear early after the initiation of an atherogenic diet in animal models of hypercholesterolemia (Masuda & Ross, 1990). The monocyte-derived macrophages and T lymphocytes produce specific matrix-degrading enzymes that initiate a smooth muscle phenotypic change to a state in which these smooth muscle cells become responsive to a vast array of mitogens released by cells within the artery wall. Cytokines are also released to mediate the inflammatory reaction. The result of this cascade of events is the development of the fibroproliferative lesion. The latter consists of layers of lipid-filled macrophages and T cells, that alternate with layers of varying numbers of smooth muscle cells, which are surrounded by a relatively poorly developed connective tissue matrix of fine collagen fibrils, elastic fibres and proteoglycans. Continued inflammation results in increased numbers of macrophages and lymphocytes, both of which migrate from the blood and multiply within the lesion. This leads to the formation of a fibrous tissue which enhances further enlargement and restructuring of the lesion, so that it becomes covered by a fibrous cap that overlies a core of lipid and necrotic tissue. The lesion at this stage of atherosclerotic development is referred to as an advanced complicated lesion of atherosclerosis. The fibrous plaques increase in size, and, by protruding into the arterial lumen, may impede the flow of blood. The sudden deaths from myocardial infarction are due to ruptures or fissures, particularly in macrophage-rich regions of the fibrous cap, resulting in haemorrhage into the plaque, thrombosis and eventually occlusion of the

artery (Ross, 1993; Schachter, 1997). From these observations, the lesions of atherosclerosis are regarded to represent a series of highly specific cellular and molecular responses that are cumulatively thought to constitute an inflammatory reaction. Thus, atherosclerosis is presently regarded as an inflammatory disease (Ross, 1999).

Adhesion molecules and cytokines play prominent roles in mediating the inflammatory response, and the cellular interactions that take place in the development of the fatty streak and the fibrous plaque. These adhesion molecules are not constitutively expressed on the surface of the normal endothelium, and their expression appears to be regulated, in part, by the risk factors for atherosclerosis. The increased surface expression of two classes of adhesion molecules, the selectins and the immunoglobulin superfamily, appear to be a common endothelial cell response to a variety of atherogenic stimuli. The initiation of "rolling", the first step in leukocyte recruitment, appears to depend on the interaction of P- and E-selectin on endothelial cells with a carbohydrate ligand on the leukocyte. Rolling appears to cause a conformational change in certain integrins that supports high-affinity binding with their respective ligands. Firm adhesion of the leukocytes on the surface of endothelial cell is then facilitated via the interaction between the integrin very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1), or the integrin leukocyte function antigen –1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) on the leukocyte and endothelial cell respectively. It has been observed in atherogenic animal models, that VCAM-1 binding to the VLA-4 integrin located on monocytes and T lymphocytes, results in the early accumulation of these cells at sites of vascular inflammation (Li et al., 1993). In addition, there is an increased expression of ICAM-1 adhesion molecules at the arterial luminal surface of atherosclerotic lesions, suggesting a role for this adhesion molecule in the recruitment and retention of leukocytes in atherosclerotic plaques (Poston et al., 1992; Printseva et al., 1992). Following firm adhesion of leukocytes to the arterial endothelium, diapedesis and transmigration of leukocytes to the subendothelial space is initiated.

Growth-regulatory molecules also mediate the cellular processes involved in the formation of atherosclerotic plaques. These molecules include transforming growth factor-β, basic fibroblast growth factor, tumour necrosis factor-α, platelet-derived growth factor, heparin-binding epidermal growth factor-like growth factor, insulin-like growth factor-1 and interleukin-1. These molecules can induce multiple and divergent effects, and may act as chemoattractants and cytokines, depending on the local environment and their target cells. Chemotaxis is necessary to bring leukocytes into the artery wall, and, at some sites, smooth muscle cells from the media into the intima of the artery. One of the most potent chemoattractants inducing monocyte chemotaxis and endothelial cell transmigration

is oxidized low density lipoprotein (Campbell & Campbell, 1994). Basic fibroblast growth factor is another chemoattractant that has been suggested to be involved in angiogenesis, which is the development of capillaries within the lesion (Campbell & Campbell, 1994). These capillaries are critical to the most advanced phases of atherosclerosis, when alterations in the structure of the plaques, such as fissuring, can lead to haemorrhage from the lumen or from these small vessels and set the stage for the terminal thrombus.

The cytokines interleukin-1, tumour necrosis factor-α and interferon-γ are modulators of the inflammatory response that occur once the endothelium has been exposed to injurious agents. The up-regulation of the secretion of these cytokines act like immunological mediators in atherosclerosis.

Thus, the cell-surface expression of adhesion molecules and the synthesis of growth-regulatory molecules and/or cytokines in response to pathophysiological stimuli mediates the interaction between the endothelium and leukocytes central to the development of atherosclerosis.

1.5.2 Cellular interactions in atherogenesis

The interaction between leukocytes and blood vessels are central to the development of the atherosclerotic process. Under normal circumstances, leukocytes do not associate with the blood vessel, but in response to inflammatory stimuli, mononuclear leukocytes, T lymphocytes and platelets interact with the vessel wall and induce changes in the vascular microenvironment that support atheroma formation. These three cellular components in the circulation, together with the endothelium and smooth muscle cells of the vessel wall, interact in multiple ways to generate the lesions of atherosclerosis.

The endothelium is not simply an inert cellular barrier, but plays numerous functional roles in the maintenance of vascular integrity. It provides a selective permeability barrier, which plays a role in regulating vascular tone and in maintaining homeostasis, and provides a non-thrombogenic lining to the artery wall. The removal of this barrier results in interactions between platelets and the underlying connective tissue, and exposes the underlying arterial cells to materials released from platelets, and to all plasma constituents. Endothelial cell activation in response to an atherogeneic stimulus is followed by an increase in the expression of adhesion molecules on the surface of the endothelial cell that binds to those on leukocytes. Additionally, endothelial cells can

participate actively in immune regulation, thrombosis and the secretion of growth factors for smooth muscle cells, monocytes and T cells (Ross, 1999). Alterations in any one or more of these functions may be important in the early stages of atherogenesis (Ross, 1993).

Monocyte-derived macrophages have been implicated in multiple aspects of atherosclerotic plaque development (Farugi & DiCorleto, 1993). They contribute to the formation of fatty streak lesions by accumulating lipids, and becoming foam cells. They have been implicated in the production of growth-regulatory molecules and cytokines, which can be chemotactic, growth agonists, or antagonists. The macrophage can produce agents that induce monocyte proliferation (macrophage colony stimulating factor, granulocyte colony stimulating factor), smooth muscle cell proliferation (platelet derived growth factor -AA, platelet derived growth factor-BB, heparin-binding epidermal growth factor, basic fibroblast growth factor, transforming growth factor β), and endothelial cell proliferation (vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor α) (Clinton & Libby, 1992). Activated macrophages can also produce a series of chemotactic molecules for other monocytes (macrophage colony stimulating factor, granulocyte colony stimulating factor, monocyte chemotactic protein-1, oxidized low density lipoprotein), for endothelial cells (vascular endothelial growth factor, basic fibroblast growth factor), and for smooth muscle cells (transforming growth factor β, platelet derived growth factor, fibroblast growth factor) (Ross, 1993). The latter have a proliferative effect on vascular smooth muscle cells, thereby contributing to plaque development. They can also generate cytotoxic factors for neighbouring cells, leading to smooth muscle cell and endothelial cell damage and death. Finally, lipid-filled macrophages can emigrate from the vessel wall, leading to further physical damage to the endothelium. In advanced lesions of atherosclerosis, studies of human coronary arteries suggest that lesions with macrophage-rich areas are more prone to rupture, leading to the formation of a terminal thrombus (Davies et al., 1993)

The presence of T lymphocytes and numerous macrophages in atherosclerotic tissue suggests that there is not only an inflammatory reaction, but also an immunologic reaction mediating the disease process (Wick et al., 1995; Lamb & Ferns, 1999; Witztum & Palinski, 1999). T cells and monocyte-derived macrophages are among the earliest cells infiltrating the vessel wall in the initial stages of atherosclerosis. Among the T cells, 70% are of the $CD4^+$ phenotype, the remainder being $CD8^+$. The majority of these T-cells express class II MHC HLA-DR and the interleukin-2 receptor, indicating an activated state

(van der Wal et al., 1992; Stemme et al., 1992; Schmitz et al., 1998). The presence of activated T lymphocytes in atherosclerotic tissue suggests a local immune response, possibly directed to local antigens (Nicoletti et al., 1998). There are also deposits of immunoglobulins and co-localised complement components, as well as high levels of expression of the C3b receptor (CR1) and C3bi receptor (CR3) on macrophages in atherosclerotic lesions, but not in non-atherosclerotic arteries (Seifert et al., 1991). Several adhesion molecules and immune cell-derived cytokines such as VCAM-1, ICAM-1, monocyte chemotactic protein-1, interferon- γ (IFN-γ), interleukin-1, and tumour necrosis factor-α, are expressed in atherosclerotic plaques (Libby & Galis, 1995; Libby & Li., 1993). Additionally, the demonstration of antibodies to oxidized low density lipoprotein and heat shock proteins in atherosclerotic patients argues for a possible role for an autoimmune process in the development of atherosclerotic lesions (Yla Herttuala et al., 1998; Xu et al., 1993).

Smooth muscle cells constitute an important component of the blood vessel wall. They are usually confined to the tunica media of the vessel wall, where their contractile function serves to maintain vascular tone in response to various hormonal and haemodynamic stimuli. The size, elasticity, and integrity of the large arteries is determined by the smooth muscle cells of the tunica media, and by the connective tissue matrix, including collagen, elastin and proteoglycans, which are synthesized and deposited by smooth muscle cells. The *in vivo* response to arterial injury in unbranched vessels, such as the rat common carotid artery, is sufficiently standardized, and is extensively used to study the regulation of smooth muscle cell proliferation. When a balloon catheter is used to remove the endothelium and subendothelial tissue of the rat carotid artery, medial smooth muscle cells start to proliferate within two days (Clowes et al., 1983a, b). Following the initial smooth muscle cell division in the media, smooth muscle cells start to migrate upwards, passing through the damaged internal elastic lamina and ending up in the intima, which rapidly increases in size due to the migratory process (Clowes et al., 1983a, b). Several growth factors are released from smooth muscle cells, including platelet derived growth factor, fibroblast growth factor, insulin growth factor, monocyte-colony stimulating factor, transforming growth factor β, and heparin-binding epidermal growth factor-like growth factor. The role of the release of growth factors in smooth muscle cell proliferation is supported by the observation that antibodies to basic fibroblast growth factor and platelet derived growth factor inhibit the division of medial smooth muscle cells, and the migration of smooth muscle cell from the media to the intima respectively

(Lindner et al., 1991; Ferns et al., 1991). It is known that platelet derived growth factor has chemotactic activity towards smooth muscle cells, and it appears likely that a chemotactic response to platelet derived growth factor released from platelets and monocytes on the vascular surface drives the migration of smooth muscle cells from the media to the intima (Jawien et al., 1992; Ross, 1999). As soon as the smooth muscle cells have migrated into the thin intimal layer, they start to divide rapidly. This can be seen at the end of the first week after injury and continues for several weeks. This process continues, and results in the formation of a thick neointimal lesion consisting of numerous layers of smooth muscle cells, surrounded by a connective-tissue matrix that is as thick as the media, and thereby reduces the lumen diameter in the afflicted arterial segment (Clowes et al., 1983a, b). Thus, the inappropriate proliferation of smooth muscle cells in the arterial intima is a major component of vascular diseases including atherosclerosis, vascular rejection, and restenosis following angioplasty.

The above observations in the rat carotid artery model have led to the description of at least two different phenotypes for smooth muscle cells in the arterial wall. This description is based on the distribution of myosin filaments, and the formation of large amounts of secretory protein apparatus, such as rough endoplasmic reticulum and the Golgi apparatus (Campbell et al., 1988; Thyberg et al., 1990). Medial smooth muscle cells (termed "contractile") are arranged in concentric layers, and are filled with myofilaments and dense bodies, but contain a relatively poorly developed Golgi apparatus and rough endoplasmic reticulum. In contrast, smooth muscle cells characteristic of the intimal lesions of atherosclerosis (termed "synthetic"), have lost this appearance, and are characterized by an abundance of Golgi apparatus and rough endoplasmic reticulum with few myofilaments. These synthetic smooth muscle cells are observed in blood vessels during embryonic development, and in the neointima that develops after the induction of intimal lesions by balloon catherization. It has recently been shown that matrix-degrading enzymes (particularly the heparan sulphate-degrading enzymes) produced by macrophages and T lymphocytes have profound effects on the phenotype, and thus the biology of smooth muscle cells (Campbell et al., 1992).

Smooth muscle cells are solely responsible for the synthesis and deposition of the extracellular matrix that accumulates in atherosclerotic lesions. Collagens constitute a major class of proteins within the arteries, and type I collagen accumulates during the formation of the plaque (Wight, 1989). The increased deposition of collagen in the lesions alters the mechanical properties of the vessel wall, making it more susceptible to changes in the flow characteristics of the blood, and more susceptible to fissuring or ulcerations. Proteoglycans also accumulate in lesions during the development of atherosclerosis.

Proteoglycans bind to several molecules that are involved in lipid accumulation, calcification and thrombosis.

Thus, the focal accumulation of smooth muscle cells is fundamental to the development of atherosclerosis. The accumulation of smooth muscle cells can be argued to precede or accompany both the deposition of lipid, and the accumulation of extracellular connective tissue matrix components, which are secretory products of smooth muscle cells.

1.5.3 The pathogenesis of atherosclerosis

Different hypotheses regarding the pathogenesis of atherosclerosis have been formulated by a great number of laboratories, from which two main concepts have emerged.

1. The response-to-injury-hypothesis.
2. The oxidized low density lipoprotein (LDL) hypothesis.

The response-to-injury hypothesis of atherosclerosis suggests injury or denudation of the arterial endothelium to be the first step (Ross, 1993). The most recent version of this hypothesis emphasizes endothelial dysfunction rather than denudation (Ross, 1999). Examples of potentially injurious atherogeneic agents include hypercholesterolemia, hyperhomocysteinemia, by-products of cigarette smoking, mechanical stress by blood pressure, immune reactions, toxins, and infection with viruses and bacteria. These injuries to the endothelium initiate a chronic inflammatory response, featuring an increase in the adherence of monocytes/macrophages and T lymphocytes to the endothelial layer (Joris et al., 1983; Masuda & Ross, 1990). The monocytes and T lymphocytes then migrate into the subendothelial space in response to chemotactic proteins, where they become filled with lipids, leading to the formation of foam cells. The accumulation of foam cells in the arterial intima, constitute the fatty streak, the earliest lesion of atherosclerosis. This lesion progresses to form a thick fibrous cap of proliferated phenotypically modified smooth muscle cells, and the extracellular matrix that they have produced, overlaying a laterally placed cellular region of macrophages and T lymphocytes. This ultimately processes to form the advanced lesion of atherosclerosis, as described above.

The second hypothesis, the oxidized low density lipoprotein hypothesis postulates that modified (oxidized) low density lipoprotein is taken up by endothelial cells, macrophages and smooth muscle cells, all of which possess the so-called low density lipoprotein-scavenger receptor that exclusively binds low density lipoprotein. The

modification of low density lipoprotein is thought to occur either in the serum or during the transgression process through endothelial cells. In the subendothelial space, modified low density lipoprotein can act as a chemoattractant for monocytes that adhere to the endothelium, inducing their migration into the subendothelial space, where they become macrophages, which subsequently transform into foam cells after the uptake of oxidized low density lipoprotein. This process ultimately results in the development of fatty streaks, and atherogenesis occurs as described above.

Another interesting hypothesis is the monoclonal origin of atherosclerosis hypothesis (Benditt and Benditt, 1973; Benditt, 1977; Murry et al., 1997), which proposes that the formation of atherosclerotic plaques is the result of smooth muscle cell proliferation derived from the transformation of, or a stable mutation in, smooth muscle cells. This hypothesis originated from the observation that the smooth muscle cells of many atherosclerotic plaques were monotypic, based on isozyme analysis (Benditt and Benditt, 1973). This hypothesis has been supported by the discovery of the transforming potential of DNA isolated from smooth muscle cells from plaques (Penn et al., 1986). In addition, it has been demonstrated that the DNA from atherosclerotic plaques, but not from normal arteries, could transform normal murine cells to malignant cells (Penn et al., 1986). If this hypothesis is proven to be true, the basic mechanism of the initiation of atherogenesis and tumourigenesis may be similar. To date, the injuring agent(s) or the genetic transforming elements remain elusive. Viruses, in particular, CMV, have been implicated as possible candidates in humans.

The above research observations have provided a substantial amount of knowledge concerning the mechanism that may link inflammation, immunity and infections to the molecular and cellular events in the arterial wall leading to the formation of atherosclerotic lesions. Evidence implicating the possible role of viral infections in the development of atherosclerosis is discussed below.

1.6 THE ASSOCIATION OF VIRAL INFECTIONS AND ATHEROSCLEROSIS

1.6.1 Studies in the avian model

In 1950, Paterson and Cottral reported the development of coronary atherosclerosis in chickens ill with Marek's lymphomatosis, whose etiologic agent was discovered to be a herpesvirus known as Marek's disease virus. In 1978, Fabricant et al.

observed that the inoculation of Marek's disease virus into pathogen-free chickens caused atherosclerosis as well as lymphomatosis. In their experiments, specific pathogen-free chickens susceptible to Marek's disease virus were divided into four groups. All of them were fed a normal low-cholesterol diet, but two of the groups were infected with a low-virulence strain of Marek's disease virus at 2 days of age. After 15 weeks, one infected and one uninfected group were fed a diet supplemented with 2% cholesterol; the other infected and uninfected groups remained on the normal diet. After an additional 15 weeks, aortas were examined for atherosclerotic lesions. It was found that only Marek's disease virus-infected chickens developed atherosclerosis, regardless of whether they were normocholesterolemic or hypercholesterolemic. Cholesterol acted as a risk factor by accelerating the disease process, in that cholesterol feeding increased the rate of visible and microscopic disease, and enhanced the fatty nature of the atherosclerotic plaques. Furthermore, specific viral antigens of Marek's disease virus were found in the smooth muscle cells of arteries of infected birds, as detected by immunofluorescence. Similar observations have been reported by Minick et al. (1979). Benditt et al. (1973) added to these observations by demonstrating nucleic acid sequences of Marek's disease virus in the arteries of infected chickens. In contrast, atherosclerotic plaques did not develop in uninfected chickens. The Marek's disease virus-induced plaques were strikingly similar to those seen in atherosclerosis in humans. These serendipitous observations led Benditt et al. (1973) to hypothesise a pathogenic role for herpesviruses in the pathogenesis of atherosclerosis. Immunisation with the related herpes virus of turkeys has been a common commercial practice to prevent Marek's disease in chickens. It was found that the development of atherosclerosis in Marek's disease virus-infected chickens was markedly reduced when they were first immunised with the herpes virus of turkeys (Fabricant, 1985).

The infection of cultured chicken arterial smooth muscle cells with Marek's disease virus has been demonstrated to lead to the accumulation of lipids, particularly cholesterol and cholesterol esters, in these cells (Fabricant et al., 1981). An increased lipid accumulation may be important in the pathogenesis of the Marek's disease virus-induced atherosclerosis. In addition, Hajjar et al. (1987) reported on a series of experiments focussing on the interaction between viruses and lipid metabolism and vessel wall injury. It was noted that the cells of chickens infected with Marek's disease virus had significant increases in aortic cholesterol, cholesterol ester, triglycerides and phospholipid accumulation, and altered cholesterol ester metabolism compared to mock-infected

chickens. These observations provided further evidence that Marek's disease virus infection may be a direct cause of atherosclerosis.

A second avian model of herpesvirus-related atherosclerosis was reported using strains of Japanese quail (Shih et al., 1983). This animal is widely used for nutritional and pharmaceutical studies of atherosclerosis, because of its susceptibility to spontaneously develop cholesterol-induced atherosclerosis. Shih and associates inbred strains of Japanese quail, and selected them for either susceptibility or resistance to atherosclerosis induced by dietary cholesterol. Dot-blot hybridisation experiments showed that DNA extracted from arteries or from embryos of susceptible quail contained sequences related to the avian (quail) herpesvirus, whereas DNA from tissues of resistant quail did not.

On the basis of these findings in viral infected chickens, it was postulated that herpesviruses may play a causative role in the development of atherosclerosis in humans.

1.6.2 The association of virus infections with human atherosclerosis in immunocompetent individuals

Following the discovery that a herpesvirus, Marek's disease virus, was capable of inducing the development of arterial vascular lesions in chickens similar to those seen in human atherosclerosis, the search for a viral etiological agent in the human arterial disease was initiated. Attention focused on five known herpesviruses that commonly infect humans: HSV types 1 and 2, Epstein-Barr virus, CMV, and varicella-zoster virus. Infection with all five viruses is widespread in the human population. The herpesviruses, HSV and varicella-zoster, are known to produce chronic and latent infections in the nervous tissue, while Epstein-Barr virus establishes a latent state in lymphocytes. The site of latency for CMV is unknown, although CMV has been isolated from a variety of tissues and cell types.

The response-to-injury hypothesis suggests that an injurious agent, such as a herpesvirus, might lead to viral-mediated endothelial cell injury, exposing the underlying smooth muscle cells, and triggering the development of atherosclerosis (Ross, 1993; Ross, 1999). Studies investigating the possibility of a viral-mediated endothelial injury have largely focussed on the role of CMV infection. In one of the initial epidemiological studies focussing on the association of CMV, HSV-1 and HSV-2 with atherosclerosis, Adam et al. (1987) studied 157 Caucasian male patients who underwent surgery for atherosclerotic vessel disease, and a matched control group of patients with high

cholesterol levels, but with no evidence of atherosclerotic disease on entry to the study. Blood samples were taken from both the atherosclerotic patients and the control subjects before surgery, and at the end of the 5-year study period. Two age-matched case control groups were studied. Group A included 134 pairs of patients who underwent vascular surgery, and a control group that did not have any signs of atherosclerosis. Group B had 46 pairs of patients, in which the surgical cases were matched with controls who, during the 5-year study period, had developed atherosclerosis. This study found that there were no significant differences in the detection of antibodies to HSV-1 and HSV-2, but differences in the detection of CMV antibodies between some of the groups. In group A, 70% of patients undergoing surgery for vascular disease had high levels of CMV antibodies compared with only 43% of control patients ($P < 0.001$). In group B, however, the surgical patients did not differ in the levels of CMV antibodies from the controls. These findings suggested that elevated levels of CMV antibodies may be associated with atherosclerotic plaque formation.

To further define a relationship between CMV and atherosclerosis, Sorlie et al. (1994) gathered data from the Atherosclerosis Risk in Communities study. Ultrasound examination of carotid arteries to determine the incidence of preclinical atherosclerosis, and serologic sampling for antibodies to CMV, were performed on 340 matched case-control pairs with no history of coronary disease. Cases were defined as patients with evidence of thickened carotid arteries, and controls had normal vessel walls. The case-control odds ratio for CMV antibodies was 1.55 ($P = 0.03$). However, the association was recalculated to be less impressive ($P = 0.20$) when possible confounding variables such as age, sex, race etc. were taken into account. This data was interpreted as suggesting a modest association between CMV and asymptomatic carotid wall thickening consistent with early atherosclerosis.

Using immunocytochemical techniques, Melnick et al. (1983) screened arterial tissues for evidence of infection with HSV-1, HSV-2 and CMV. This approach was based on the fact that cells that are abortively or persistently infected with a virus may express one or more proteins coded by the viral genome, even if no infectious virus is produced. Atherosclerotic plaque tissues were obtained from carotid endarterectomy patients, thus representing an advanced stage of the disease. A few atherosclerotic plaque tissues from abdominal aortas, iliac arteries and femoral arteries were also obtained. In addition, punch biopsies of the ascending aortas were secured from patients undergoing coronary artery bypass surgery. The latter samples were of uninvolved tissues, and showed minimal atherosclerotic changes on histological examination. None of the above mentioned tissues gave a positive reaction with any of the viral antisera tested. The arterial plaque tissue and

the uninvolved aortic tissues were subsequently analysed for the presence of viral proteins after culture of the tissue. The production of viral proteins is often higher in actively growing cells than in quiescent cells, such as those in differentiated tissue. Therefore, explant cultures of smooth muscle like cells were successfully isolated, and cultivated from involved and uninvolved aortic atherosclerotic tissues of the patients described above. The cells were tested during their first or second passage in culture, for the presence of antigens of both CMV and HSV. One quarter of the cell cultures obtained from the tissue samples contained antigens specific for CMV, whereas none had evidence of HSV antigens. CMV was not found to be replicating in any of the antigen positive cells, thereby suggesting that the arterial wall may be a site of latency for the virus.

Yamashiroya et al. (1988) demonstrated the presence of CMV, HSV type 1 and 2 DNA in the arterial tissue of young trauma patients without clinical heart disease. Coronary artery and thoracic aortic samples from 20 patients were removed at autopsy, and studied by *in situ* DNA hybridisation and immunoperoxidase methods. Both HSV and CMV DNA were detected in the coronary arteries of 8 subjects and in the aorta of 7 subjects. Positive HSV or CMV DNA findings were present in various layers of the vessel wall showing early atheromatous changes, including endothelial cells and smooth muscle cells. No infectious virus was detected in the arterial samples. These observations confirmed that the genomes of the herpes virus family are present in the arteries of young patients without clinically apparent atherosclerotic alterations.

In an effort to isolate CMV DNA from atherosclerotic tissue, Hendrix et al. (1989) obtained femoral arterial or abdominal aortic samples from 44 CMV seropositive patients undergoing vascular surgery. These samples were compared with a control group of 23 CMV seropositive patients who died from non-atherosclerosis-associated diseases, and who had no evidence of significant vascular disease. Using *in situ* and dot-blot hybridisation, they failed to demonstrate any significant difference between the two groups of patients in terms of CMV DNA probe reactivity. This study demonstrated the presence of CMV nucleic acid sequences in arterial samples from patients both with and without atherosclerosis. In a follow up study, the same group used the more sensitive PCR technology to demonstrate CMV DNA sequences in 90% of arterial tissue samples obtained from atherosclerotic patients compared to only 53% of patients with non-significant arterial disease ($P = 0.001$). This finding suggested that CMV remains latent in the human vascular wall, and that it may be a factor in the formation of atheromata (Hendrix et al., 1991). Recently, it has been demonstrated using *in situ* and dot-blot

hybridisation and nested PCR, that CMV DNA can be detected in a variety of organs from apparently healthy individuals, including aorta, spleen, pancreas, kidney and liver, suggesting that the arterial wall and peripheral blood leukocytes are not the only reservoir for latent CMV (Hendrix et al., 1997; Melnick et al., 1983, Taylor-Wiedman et al., 1993)

Tanaka et al. (1992) searched for possible evidence of a relationship between CMV and aortic disease by examining aortic lesions with atherosclerosis and/or inflammation for CMV DNA by PCR. They obtained aortic lesions from 41 patients. Patient conditions consisted of 33 abdominal aortic aneurysms, 6 thoracic aortic aneurysms and 2 junxternal high aortic occlusions. 39 (95%) of the 41 patients were seropositive for CMV. Control samples were obtained from the aortas of 16 patients at autopsy who were all seropositive for CMV. Using an IE gene primer, they demonstrated the presence of CMV DNA in 20 (61%) of 33 tissue samples from atherosclerotic aneurysms, in 5 of 6 (83%) samples from inflammatory aneurysms, and in all of two samples from aortic occlusions with inflammation. CMV DNA was also detected in 7 of 8 (88%) samples with inflammatory aortic diseases with periaortic fibrosis, whereas only 5 of 16 (31%) autopsy tissues that showed neither inflammation nor atherosclerosis contained CMV DNA. The peripheral blood leukocytes of all patients with aortic disease were negative for CMV DNA, indicating that the positive results obtained on aortic tissue were not due to leukocytes present in the aortic walls. The authors of this study concluded that CMV DNA was detected more frequently in inflammatory aneurysms and inflammatory aortic occlusions with retroperitoneal fibrosis than in atherosclerotic aneurysms.

The above studies suggested a possible association between CMV infection and the development of atherosclerosis. On the contrary, a few other studies suggest that CMV is not a major risk factor and is not associated with the development of atherosclerosis (Hendrix et al., 1991; Melnick et al., 1994; Gulizia et al., 1995; Adler et al., 1998). In one of the recent studies, Adler et al. (1998) studied the association between the rate of CMV positivity, the titre of antibodies to whole CMV antigens, CMV gB, and the presence and absence of coronary artery disease in approximately 900 patients undergoing coronary angiography. Briefly, using logistic regression, it was concluded that CMV positivity (0.462), the level of antibodies to CMV whole cell antigen ($P= 0.98$), or the level of antibodies to CMV gB ($P= 0.67$) were not significantly associated with coronary artery disease. However, other established cardiovascular risk factors such as age ($P< 0.001$), white race ($P< 0.001$), gender ($P< 0.010$ and hypercholesterolemia were identified to contribute to the development of atherosclerosis.

1.6.3 CMV, restenosis and the smooth muscle cell

There are several surgical treatments to relieve stenosis or narrowing of the coronary artery. Coronary angioplasty is one of the common intervention procedures, and involves the passing of a catheter with a balloon at its tip into an occluded artery, and then expanding the balloon. This enlarges the lumen by stretching the vessel wall and breaking up abnormal tissue deposits. Following the angioplasty procedure, patients develop restenosis, a vessel wall renarrowing process characterised by excessive proliferation of smooth muscle cells in the intimal layer of the vessel wall (intimal hyperplasia). In 1994, it was hypothesised that CMV was associated with the vessel wall injury sustained following angioplasty, and that the restenosis process might, in part, be caused by aberrant cellular growth and proliferation induced by CMV. Speir et al. (1994) examined 60 patients who had previously undergone coronary angioplasty and 20 patients undergoing the procedure as an initial intervention. They noted that 38% of the restenosis lesions and none of the initial lesions expressed p53. p53 is a tumour suppressor protein that is involved in the regulation of cell proliferation by stimulating the transcription of other specific cell cycle control genes (Levine et al., 1991). Normally, cells with wild type p53 are able to delay the progression from the G1 to the S phase of the cell cycle while abnormal DNA is repaired. Cells with inactivated or mutant p53 protein cannot do this, and thus the replication of abnormal DNA is not prevented. Consequently, inactivation of the wild type p53 gene product represents the most common genetic alteration in human carcinogenesis (Neil et al., 1997). By using PCR technology on extracted cellular DNA, CMV DNA was found in 85% of the p53-positive restenotic lesions examined, whereas it was present in only 27% of the p53-negative samples examined ($P<0.01$). Furthermore, smooth muscle cells grown from p53-positive lesions were shown to express the CMV-specific p86 protein and high amounts of p53. p86 was demonstrated to bind to p53, inhibiting its activity. It was concluded that the angioplasty procedure leads to the release or reactivation of latent CMV, which, by interfering with normal cellular regulatory processes such as cell cycle control, led to aberrant smooth muscle cell proliferation and contributed to the process of restenosis (Speir et al., 1994).

1.6.4 Transplant-associated atherosclerosis in recipients of solid-organ transplants

CMV has been reported to be associated with the development of a phenomenon termed transplant-associated atherosclerosis, accelerated transplant atherosclerosis, cardiac

allograft vasculopathy or chronic rejection in heart transplant patients. Transplant-associated atherosclerosis is the most common reason for retransplantation, and the single largest cause of death in patients surviving more than 6 months after heart transplantation (Conraads et al., 1998). Transplant-associated atherosclerosis is characterized by the occurrence of a diffuse concentric intimal proliferation in all arteries or arterioles of the transplanted organ (Billingham, 1994; Hayry et al., 1993; Ventura et al., 1995). The pathogenesis of the diffuse, concentric intimal thickening seen in heart allograft vasculopathy is believed to involve immunologic mechanisms operating in a milieu of non-immunologic risk factors (Hayry et al., 1995). These immunological events initiate stimuli which could cause endothelial injury, and, consequently, intimal hyperplasia and extracellular matrix synthesis (Dong et al., 1996). It is also postulated by some researchers, that allogeneic cytolytic T cells damage endothelial cells in the coronary arteries of human heart allografts through cytotoxic pathways. An allo-immune response, involving endothelial cells, monocytes and T lymphocytes may facilitate the synthesis and release of cytokines and growth factors into the vascular wall milieu. This, in turn, may modulate smooth muscle cell migration, proliferation and extracellular matrix synthesis, which ultimately would lead to lipid accumulation (Hayry et al., 1993; Ventura et al., 1995).

The influence of CMV on the development or progression of transplant-associated atherosclerosis was first studied by Grattan et al. (1989). The study involved 301 cardiac transplant recipients who were given immunosuppressive therapy and monitored from 1980 through to 1989. CMV infection was defined as a 4-fold rise in immunoglobulin G antibody levels to CMV, the isolation of CMV from clinical specimens in human fibroblast cultures, or the presence of CMV inclusion bodies in the tissues after transplantation. During this period, CMV infection was detected in 91 patients (CMV group), compared with 210 transplant recipients in whom CMV infection was not detected (non-CMV group). Routine coronary angiograph was performed annually to detect the presence of graft atherosclerosis. Coronary artery atherosclerosis was found to occur more frequently and earlier in the CMV group. By the first year after transplantation, it was noted that only 32% of the CMV group had survived in contrast to 68% of the non-CMV group. Of the patients who died by 5-years after transplantation, 8% of the CMV group patients had greater than 50% luminal obstructions, compared with only 0.8% of the non-CMV group patients. The rate of graft loss (which leads to death or retransplantation) was significantly greater in the CMV group (69%) compared with the non-CMV group (37%)

($P < 0.005$). In a subsequent update study, the death rate from atherosclerosis was 30% in the CMV group and only 10% in the non-CMV group (Grattan et al., 1991).

McDonald et al. (1989) reported on 102 immunosuppressed patients who had received a cardiac transplant between 1983 and 1989 and survived for at least 1 year after transplantation. Coronary artery disease was reported to develop in the graft of 31% of the post-transplant CMV-positive patients in contrast to 9% of the 70 CMV-negative patients.

These two initial studies were followed by several other studies in which a positive correlation between CMV infection and transplant-associated atherosclerosis in heart transplant recipients was demonstrated (Merigan et al., 1992; Loebe et al., 1990; Hruban et al., 1990, Decoene et al., 1996; Fernando et al., 1994; Koskinen et al., 1993a; Koskinen et al., 1993b; Koskinen et al., 1996b). Similar associations between CMV infection and transplant-associated atherosclerosis have been reported in recipients of lung (Sharples et al., 1996; Scott et al., 1997), liver (Jeng et al., 1994; de et al., 1998; de Otero et al., 1998) and renal allografts (Matas, 1994 ; Nadasdy et al., 1994; Andersen., 1997).

1.6.5 The rat model of transplant-associated atherosclerosis

The hypothesis that CMV infection plays a contributory role in the development of transplant-associated atherosclerosis in human transplant recipients has largely been supported by studies in the rat model, using rat CMV. The effect of rat CMV infection on the development of transplant-associated atherosclerosis has been extensively studied in rat allografts recipients. The first experimental model of rat CMV-infected aortic allografts using donor and recipient combinations of inbred Darkagouti (AG-B4, $RT1^a$) and Wistar-Furth (AG-B2, $RT1^v$) rats, respectively, was described by Lemstorm et al. (1993a, b). This served to investigate the interaction between rat CMV and the vascular wall of the transplant. The rat strain combinations Darkagouti to Darkagouti and Darkagouti to Wistar-Furth were used for syngeneic and allogeneic transplantation, respectively. The recipient rats were inoculated intraperitoneally either on day 1 (early infection) or on day 60 (late infection) after transplantation with 10^5 plaque forming units of rat CMV. The control rats were left uninfected. The grafts were removed at day 7 and day 14, and 1, 3 and 6 months after transplantation in the early infection group, and at day 7 and day 14, and 1 and 4 months after infection in the late infection group. The control rats were killed at 7 and 14 days and at 1, 2, 3, 6 and 12 months after transplantation. All

grafts were subsequently processed for histology and immunohistochemistry. The presence of viral infection was demonstrated by plaque assays from biopsies of the salivary glands, liver and spleen at sacrifice. The results of these experiments revealed that rat CMV infection significantly enhanced the development of transplant-associated atherosclerosis in the allograft in the rats infected early. The initial signs of transplant-associated atherosclerosis were an inflammatory influx of leukocytes, especially of monocytes/macrophages and T lymphocytes, starting at day 7 post-infection. In the syngeneic grafts, no effect of rat CMV on the development of transplant-associated atherosclerosis was noted. In the group infected late, there was no effect of rat CMV infection on transplant-associated atherosclerosis. These results suggest that early infection is more important in the generation of transplant-associated atherosclerosis than late infection, and that an acute alloimmune response must be associated with virus infection to induce transplant-associated atherosclerosis. In a follow-up to the above study, Lemstorm et al. (1994a) successfully demonstrated that triple drug immunosuppressive therapy significantly reduced the development of transplant-associated atherosclerosis in rat CMV-infected aortic allografts. This treatment reduced inflammation in the perivascular area, reduced the proliferation of smooth muscle cells, and reduced neointima formation by almost 50%. These data supported the concept that transplant-associated atherosclerosis is due to an immune reaction, and that rat CMV has a stimulatory effect in its development. Similar results have been described using allogeneic Brown Norway and Lewis rat strain combinations (Li et al., 1995; Li et al., 1996). In the latter system, an enhanced influx of monocytes/macrophages and T lymphocytes were observed in the rat CMV-infected rats receiving an aortic allograft. A neointima developed in the allografts. There was more neointima formation in the rat CMV-infected animals than in the controls. The rat CMV-induced effect on inflammation and on neointima formation was prevented when the recipients were treated with effective antiviral drugs (Lemstrom et al., 1994b).

The effect of rat CMV infection on the development or enhancement of transplant-associated atherosclerosis has been demonstrated in other transplanted organs in the rat. This includes transplants of the liver, kidney, and lungs (Martelius et al., 1998; Yilmaz et al., 1996; Lautenschlager et al., 1997a; Steinhoff et al., 1996). Hence, in the rat model, several studies implicate a contributory role of rat CMV infection in the development of transplant-associated atherosclerosis or chronic rejection in organ transplant allografts.

1.6.6 The proposed mechanism of CMV-enhanced transplant-associated atherosclerosis

The pathogenesis of transplant-associated atherosclerosis is a complex network of immunological, metabolic and haemodynamic events leading to a cascade of cellular and molecular events that enhance the development of this disease in the blood vessels of the transplanted organ (Hayry et al., 1997). The immunologic and molecular mechanisms involved in the pathogenesis of CMV-associated transplant-associated atherosclerosis are virtually unknown. However, a number of *in vitro* studies and *in vivo* studies in animals have provided interesting data of mechanisms that play a role in the development of this disease. From these studies, it has been postulated that the main cellular and molecular events in transplant-associated atherosclerosis proceeds with the infiltration of leukocytes, particularly T cells, monocytes, and platelets into the graft through the microvascular endothelium. This is facilitated by the expression of adhesion molecules on the surface of the endothelium and their corresponding ligands on leukocytes. Data derived from the biopsies of CMV-infected transplant-recipients, as well as from experimental models of transplantation, indicate that CMV infection can result in an up-regulation of such adhesion molecules, thereby facilitating the disease process (Sedmak et al., 1994; Yilmaz et al., 1996). Infection with CMV is also associated with an increased expression of MHC class II on multiple cell types (Ustinov et al., 1993; Waldman et al., 1993; You et al., 1996). Since the recognition of non-self MHC antigens is the major determinant of allograft rejection, an up-regulation of these molecules could contribute to graft failure. As a consequence of this interaction between the endothelium and the infiltrating leukocytes, cytokines like interleukin-2 and gamma- interferon are released from the leukocytes, which facilitate the maturation of B cells, cytotoxic T cells, and T helper cells *in situ* (Hayry et al., 1996; Hayry et al., 1997). The cytokines released by the leukocytes in the allograft adventitia and in the neointima, induce a continuous low-grade damage to the graft vascular endothelium. This is turn induces the endothelial cells to regenerate themselves via the secretion of peptide growth factors such as insulin growth factor-1, platelet-derived growth factor-BB, factors which are also released by smooth muscle cells and by the inflammatory cells. These growth factors, together with cytokines such as interleukin-1, induce smooth muscle cells of medial origin to undergo phenotypic transformation, replication, and migration into the neointima. As a consequence, the peripheral branches of arteries affected by the proliferative arteriopathy begin to occlude, leading to anoxic and fibrotic changes in the transplant.

A pro-inflammatory role for CMV infection has been demonstrated in a number of studies. The CMV IE gene products have autoregulatory features, and in addition are strong transactivators, known to stimulate the transcription of various viral and host cell genes (Thomsen et al., 1984). It is assumed that CMV may alter the expression of genes coding for proteins with pro-inflammatory activity, probably without the requirement for virus replication (Craigen et al., 1996; Scholz et al., 1996). *In vitro* experiments have shown that CMV induced the up-regulation of several adhesion molecules such as ICAM-1 and LFA-3 (Grundy et al., 1993; Craigen et al., 1996). There is evidence that CMV infection leads to an increase in the production of cytokines such as interleukin-1 and interleukin-6 in monocytes and endothelial cells, which could potentially enhance the inflammatory process (Iwamaoto et al., 1990; Kapasi & Rice, 1988; Woodroffe et al., 1993). Recently, it has been shown that CMV induced enhanced production of both C-X-C (interleukin-8 and Gro-α), and CC (RANTES and MIP-α) chemokines in different cell types (Michelson et al., 1997; Murayama et al., 1997; Craigen et al., 1997; Grundy et al., 1998). The rat model of transplant-associated atherosclerosis described above has been used to provide immunologic and molecular evidence on how rat CMV infection could enhance the generation of transplant-associated atherosclerosis (Lemstrom et al. 1997). The authors compared rat CMV-infected and non-infected Wistar-Furth rat recipients of Darkagouti rat heart allografts by monitoring structural evidence of adventitial inflammation and smooth muscle cell growth factor production in arterial walls. It was demonstrated that rat CMV infection led to an early, significant influx of T helper, T cytotoxic, and natural killer cells into the adventitia, and that these cells displayed enhanced levels of immune activation. In addition, rat CMV infection was demonstrated to be linked with an early, prominent platelet-derived growth factor-BB and tumour growth factor-β_1 mRNA expression in the allograft vascular wall (Lemstrom et al., 1997). This led to an enhanced neointima formation.

In conclusion, several studies have demonstrated that a variety of cells and molecules may regulate smooth muscle cell replication in the vascular wall, the migration of smooth muscle cells from the media to the intima, and the development of atherosclerotic lesions throughout the entire length of the vessel wall. These molecules include peptide growth factors, cytokines that are secreted *in situ* by inflammatory, endothelial and smooth muscle cells in the vascular wall. CMV infection of the vessel wall could contribute to the development of transplant-associated atherosclerosis by facilitating one or more of these processes, leading to neointima formation.

1.7 THE AIMS OF THE THESIS

Whilst there are numerous studies investigating the permissiveness of endothelial cells for CMV infection, relatively few studies exist on the permissiveness of smooth muscle cells. It is not known whether CMV infection of smooth muscle cells is abortive or fully permissive, and the few *in vitro* reports on CMV infection of smooth muscle cells have only used the high passage strain of CMV, AD169, which is now known to have large deletions of the viral genome compared to low passage clinical CMV strains (Cha et al., 1996). In addition, CMV strain AD169 replicates poorly in some cell types, such as endothelial cells and epithelial cells, (Friedman et al., 1981; Smiley et al., 1988), whilst these cells can be relatively permissive to infection with certain clinical isolates of CMV. An *in vitro* model of smooth muscle cells in culture was therefore set up to study their permissiveness for CMV infection, using the high passage CMV strain, AD169, and a low passage CMV strain, C1F. This model was then used to investigate the effect of CMV infection of smooth muscle cells on the cell surface expression of certain adhesion molecules and class I and class II MHC antigens. Finally, an organ culture system of the saphenous vein was developed to study CMV infection of an intact blood vessel wall. This system had the advantage over isolated monolayer cultures of single cells that the integrity and architecture of the vein wall was maintained, and this allowed the interaction between the endothelium and smooth muscle cells of the vein wall. This model was used to study which parts of the vessel wall were permissive for CMV infection. In addition, the overall effects of CMV infection on the structure of the vessel wall were assessed.

CHAPTER 2:

Materials and methods

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2.1 CELL CULTURE

2.1.1 Human aortic smooth muscle cells.

Human aortic smooth muscle cells at passage 20 were obtained from the European Collection of Cell Culture (Wiltshire, UK), and were grown in medium containing Ham's F12 (Sigma, Poole, UK) supplemented with 2mM glutamine, 1% non-essential amino acids, 50μg/ml ascorbic acid, 10μg/ml insulin, 30μg/ml endothelial cell growth supplement (Sigma) and 10% foetal calf serum. At confluency, cells were trypsinized with trypsin-ethylenediaminetetra-acetic acid (EDTA) (500μg/ml) for 5 minutes, split in a 1:3 ratio, as recommended by the supplier, and subsequently cultured in the above growth medium. They were used between passage 24 and 28. All cell culture reagents were obtained from GibcoBRL, Paisley, UK, unless otherwise stated.

2.1.2 Enzyme-dispersed smooth muscle cells.

Smooth muscle cells were isolated from the veins of human umbilical cords after prolonged enzymatic digestion of their luminal surfaces (Gimbrone et al., 1975). Briefly, veins in sterile segments of untraumatized umbilical cords were cannulated at both ends, rinsed with sterile phosphate buffered saline, and filled with 200IU/ml collagenase (Boehringer Mannheim, UK) in Hank's buffered saline solution. After incubation at 37°C for 10 minutes, gentle flushing released a suspension of cells, which grew to form a confluent monolayer in culture after 7 days. The cobblestone appearance suggested the vast majority of these cells were of endothelial origin. Smooth muscle cells were obtained by further incubation of the umbilical cord with 200IU/ml collagenase for 10 minutes, followed by gentle massaging of the umbilical cord. The resultant cell suspension after vigorous perfusion was centrifuged at 500g for 5 minutes. The cells pelleted from the supernatant were grown in Dulbecco minimal essential medium, supplemented with 10% foetal calf serum, penicillin (100IU/ml) and streptomycin (100g/ml). The latter cells obtained from the vessels of the umbilical cord were designated enzyme-dispersed smooth muscle cells. Enzyme-dispersed smooth muscle cells began to attach to the substratum within 2 days, with a confluent culture usually obtained in 2-3 weeks. At confluency, enzyme-dispersed smooth muscle cells were trypsinized with trypsin-EDTA (500μg/ml) for 5 minutes, split in a 1:2 ratio, and subsequently cultured in the above growth medium. All cell reagents were obtained from GibcoBRL, unless otherwise stated.

2.1.3 Explant-migrated smooth muscle cells.

Smooth muscle cells were also obtained from saphenous veins by explantation. Saphenous veins were obtained from patients undergoing coronary artery or lower limb bypass grafting, and were dissected with minimal handling. These non-diseased vein segments were collected under sterile conditions in Hank's buffered salt solution, or in the heparinized blood of the patient, and were processed within 6 hours of arrival at the laboratory. Following the removal of the external adventitial layer, the veins were cut longitudinally, and the endothelium scraped off with a scalpel blade. Figure 2.1 illustrates how the mid-portion of the saphenous vein media was used for explant culture (McMurray et al., 1991). The resulting saphenous vein media segment was then cut into 1-2mm vein pieces, which were then placed on the surface of a tissue culture flask (Falcon, UK). The explants were moistened with smooth muscle cell growth medium, of which the constituents were Dulbecco minimal essential medium, supplemented with 10% foetal calf serum, penicillin (100IU/ml) and streptomycin (100g/ml), and then incubated at 37°C in a humidified chamber containing 5% CO_2. The cells migrating from the vein wall were visible after 2-3 weeks, and reached confluency by 5-6 weeks. At confluency, the cells were trypsinized with trypsin-EDTA (500μg/ml) for 5 minutes, split in a 1:2 ratio, and subsequently cultured in the above growth medium. The smooth muscle cells obtained from saphenous veins by explantation were referred to as explant-migrated smooth muscle cells. Explant-migrated smooth muscle cells were used in experiments between passages 3 and 5. All cell reagents were obtained from GibcoBRL, unless otherwise stated.

All sources of smooth muscle cells were observed using an Olympus microscope equipped with photographic equipment. Photographs were taken using Olympus SPlan4PL or A20PL objectives using T-Max Kodak black and white film (Sigma).

2.1.4 Fibroblasts.

Human embryonic lung fibroblasts were isolated from foetal lung tissue (MRC Tissue Bank, London, UK). The tissue was cut into small pieces using a scalpel blade, and digested with trypsin-EDTA (500μg/ml) for 15 minutes at 37°C. The digested cells were then agitated using a Pasteur pipette, washed in Hanks buffered salt solution by centrifugation at 500g for 5 minutes, and finally resuspended and transferred to a tissue culture flask. Human embryonic lung fibroblasts were used between passages 7 and 19. The MRC-5 fibroblast cell line was obtained from the American Type Culture Collection (Rockville, MD, USA), and was used between

passages 23 and 27. HS68 foreskin fibroblasts were obtained from the European Collection of Cell Culture and were used between passages 20 and 35. All fibroblast cell lines were passaged using trypsin-EDTA (500μg/ml) in a 1:3 ratio, and were grown in minimal essential medium containing 10% foetal calf serum, 50 IU/ml penicillin, 50μg/ml streptomycin and 2mM L-glutamine and incubated at 37°C in 5% CO_2. All cell reagents were obtained from GibcoBRL, unless otherwise stated.

2.1.5 Epstein-Barr virus transformed B cells.

Epstein-Barr virus transformed B cells were obtained from Eira Rawlings (Royal Free and University College Medical School, London, UK) and used between passages 9 and 12. These suspension cells were passaged in a 1:3 ratio following centrifugation at 500g for 5 minutes. They were grown in RPMI1640 containing 10% foetal calf serum, 50 IU/ml penicillin and 50μg/ml streptomycin and incubated at 37°C in 5% CO_2. All cell reagents were obtained from GibcoBRL.

2.2 VIRUS STOCKS

2.2.1 The high passage CMV strain AD169.

The high passage CMV strain AD169 was obtained from the American Type Culture Collection. CMV strain AD169 was inoculated onto monolayer cultures of fibroblasts for 1 hour, and subsequently harvested from the supernatant of infected fibroblasts after clarification at 500g for 10 minutes. Following clarification, aliquots of viral inoculum were stored at –70°C until use. The CMV strain AD169 has been highly passaged through fibroblasts for a number of years. It was received at passage number 91 and was passaged a further 5-9 times after purchase before use.

2.2.2 The low passage CMV strain C1F.

The low passage strain of CMV, designated C1F, was a gift from Luci MacCormac (Royal Free and University College Medical School, London, UK). This virus strain had been isolated from the leukocytes of an HIV positive patient with CMV disease (MacCormac & Grundy, 1996). Briefly, the buffy coat from the blood of an HIV-positive patient was

plated onto fibroblast monolayers. These monolayers demonstrated evidence of CMV infection after one week post-culture. The CMV strain C1F was serially passaged by mixing infected cells with uninfected fibroblasts until 70-100% cytopathic effect was obtained in the fibroblast monolayer. CMV strain C1F was harvested from the supernatant of infected fibroblasts as described for the AD169 virus strain. It was received after five passages in fibroblasts, and was passaged a further three times before use.

All CMV virus stocks were handled in Class II (vertical laminar flow) biological safety cabinets. Laboratory coats and gloves were worn in the laboratory and removed when leaving the laboratory work areas. Hands were thoroughly washed in disinfectant before and after removal of gloves, as minute holes may permit the entry of the virus.

2.3 MYCOPLASMA TESTING OF CELLS AND VIRUS STOCKS

All cell cultures and virus stocks were tested for Mycoplasma contamination using the Mycoplasma Tissue Culture Rapid Detection System kit (Gen-Probe, San Diego, CA). This detection kit uses a [^{3}H]-labelled DNA probe homologous to Mycoplasma and Achoeplasma ribosomal RNA to detect the presence of Mycoplasma and related species. The instructions supplied with the kit were followed. Briefly, cells to be tested were passaged twice in antibiotic-free medium, and a 2ml aliquot of supernatant was removed at least 3 days after the second passage. Cell debris was removed by centrifugation at 500g for 5 minutes. The cell supernatant and thawed virus stocks of CMV strain AD169 and strain C1F was then incubated with a radiolabelled probe specific for Mycoplasma RNA for 18 hours at 42°C. The samples were then incubated at 42°C with the separation suspension provided, followed by three washes in the wash solution provided. The resulting pellet was resuspended in scintillation fluid, and the amount of radioactivity in each sample was determined by scintillation counting. The percentage of bound probe was calculated in relation to the amount of probe added, with Mycoplasma positive samples having a value greater than 2.4%. All cells and virus stocks were negative for Mycoplasma or Achoeplasma contamination using this detection system.

2.4 THE TITRE OF VIRUS STOCKS

The titre of virus stocks was determined using a plaque assay. Human embryonic lung fibroblasts were seeded into 48-well plates at 5.0 x 10^4 cells/well, and allowed to reach confluency. Virus stocks were diluted from 10^{-1} to 10^{-6}, in ten-fold serial dilutions, in minimal essential media containing 2% foetal calf serum and 10mM HEPES. 100µl of each virus dilution was inoculated onto fibroblast monolayers in triplicate wells and incubated for 1 hour at 37°C in 5% CO_2. After the incubation period, the monolayer was washed and overlaid with 0.5ml of minimal essential medium containing 1% methylcellulose (Sigma), 30% Leibovitz L-15 medium, 5% foetal calf serum, 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin and 0.2% sodium bicarbonate. All cell reagents were obtained from GibcoBRL, unless otherwise stated. The cells were then incubated at 37°C for 14 days. The monolayers were then fixed with 4% formyl saline (4g sodium chloride in 50ml formalin (BDH, Poole, UK) and 450ml distilled water) for 30 minutes at room temperature, and then washed thoroughly in distilled water. Fibroblast monolayers were then stained with 0.03% methylene blue (BDH) for 1 hour at room temperature. This procedure gave the plaques that had formed on the fibroblast monolayers a prominent blue colour. The plates were then washed thoroughly in water and allowed to dry. The number of plaques per well was counted using an inverted light microscope. The virus titre was expressed as the number of plaque forming units per ml (pfu/ml), which was calculated using the equation:

pfu/ml = mean number of plaques from triplicate wells x virus dilution factor x 10.

The multiplicity of infection is defined as the number of plaque forming units of virus particles per infected cell.

2.5 MONOCLONAL ANTIBODIES AND RELATED PROTEINS

Table 2.1 shows a summary of antibodies, their isotypes, and respective optimal concentrations used in experiments described in this thesis.

2.5.1 Antibodies specific for cytoplasmic filaments.

The monoclonal antibody, anti-α-smooth muscle-1, specific for smooth muscle α-actin was obtained from Sigma and was used at a dilution of 1:800. Another monoclonal

antibody, specific for smooth muscle α-actin purified from chicken gizzard actin was obtained from Chemicon, UK, and was used at a working dilution of 1:100. Both monoclonal antibodies were used to demonstrate the presence of smooth muscle α-actin filaments in cultured cells. Anti-vimentin (Amersham, Buckinghamshire, UK) was used to demonstrate the presence of vimentin intermediate filaments in cultured cells, and was used at a dilution of 1:100. Working dilutions for the above mentioned antibodies was determined by indirect immunofluorescence staining of smooth muscle cells.

2.5.2 A fibroblast-specific monoclonal antibody.

A fibroblast-specific monoclonal antibody reactive with the cell surface, designated FibAS02, was obtained from Dianova, Hamburg, Germany. An antibody titre of 5μg/ml was determined to be optimal on human embryonic lung fibroblasts by flow cytometric analysis (see section 2.8.4).

2.5.3 Antibodies specific for adhesion molecules.

The anti-human ICAM-1 antibody (Hybridoma clone, BB1G-11) and the anti-human VCAM-1 antibody (Hybridoma clone, BB1G-V1) were obtained from R &D Systems, Abingdon, UK. An optimum working dilution of 1:100 of a 1mg/ml stock solution for both antibodies was determined by flow cytometry on activated and non-activated human umbilical vein endothelial cells. The anti-human LFA-3 antibody, (hybridoma clone, AICD58.9) was obtained from Boehringer Mannheim, Germany. Anti-human LFA-3 was used at a dilution of 1:400 from a 1.5mg/ml stock. This optimal working dilution was determined on human embryonic lung fibroblasts by flow cytometry. The anti-human CD51/CD61 monoclonal antibody (clone, 23C6), which recognizes the intact complex formed between CD51 and CD61 antigens ($\alpha_v\beta_3$) was purchased from Chemicon. An optimal working dilution of 1:50 of a 1mg/ml stock solution was determined by flow cytometry on Epstein-Barr virus transformed B cells.

2.5.4 MHC-specific antibodies.

The class I MHC-specific hybridoma (Clone PA.26), which recognizes a conformational determinant of the class I heterodimer was harvested by ammonium sulphate precipitation from the supernatant fluids of a hybridoma obtained from the American Type Culture Collection. The class II MHC-specific antibody, DR2, recognizing non-polymorphic class

II epitopes was similarly harvested from the supernatant fluids of a hybridoma, obtained from the Royal Free and University College Medical School, London, UK (Janossy et al., 1986). Antibody dilutions of 1:200 and 1:5 were determined to give optimal staining on Epstein-Barr virus transformed B cells by flow cytometry for class I and class II MHC molecules, respectively.

2.5.5 CMV-specific antibodies.

The monoclonal antibody E13, which is specific for the CMV p72 and p86 antigens, was obtained from Biosoft, Tissue Culture Services, UK and used at a dilution of 1:50. These antigens are found from very early times in CMV-infected cell cultures and persist throughout the later stages of infection (Marezon et al., 1992). The ascites preparation of anti-CMV pp65 (Clone 14) was a gift from the late Dr. Jim Booth (St. George's Medical School, London, UK) and was used at a 1:500 dilution. The ascites monoclonal antibody preparation, designated 63.27, which is specific for the CMV P72 immediate-early antigen and the murine tissue culture supernatant antibody, 7-17, which is specific for the late glycoprotein B antigen were kind gifts from Dr. William Britt, University of Alabama at Birmingham, Alabama, USA. These were used at a dilution of 1:1600 and 1:16, respectively. The optimal working dilution of these antibodies was determined by indirect immunofluorescence detection of the respective antigens on CMV-infected fibroblasts (see section 2.6 below).

2.5.6 Immunoglobulin G molecules.

Mouse myelomas IgG1, IgG2a, IgG3 proteins (Sigma) were reconstituted to a concentration of 1mg/ml. IgG molecules were centrifuged at 13,000g for 20 minutes in a microcentrifuge before use to remove any aggregated protein. They were used in experiments as irrelevant isotype-matched control antibodies at a concentration similar to that of the test primary antibody.

2.5.7 Secondary conjugated antibodies.

Affinity purified $F(ab')_2$ fractions of fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse secondary antibodies were obtained from Sigma, and were used at a dilution of 1:100. The optimal working dilution was determined by indirect immunofluorescence staining of CMV-infected fibroblasts (see section 2.6 below).

2.6 CYTOPLASMIC IMMUNOFLUORESCENCE STAINING FOR THE DETECTION OF CMV ANTIGENS

Smooth muscle cells and fibroblasts were seeded in 8-well chamber slides at a density of 2 x 10^4 cells per well, and incubated for 24 hours in their respective culture medium containing 10% foetal calf serum (see sections 2.1.1 and 2.1.2). They were either left uninfected, or infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 5 pfu/cell. At the specified intervals post-infection, cells were removed and washed with phosphate buffered saline for 5 minutes, and fixed with a fixation method appropriate for the antigen being detected as described below in Table 2.2. The cells were then washed twice in phosphate buffered saline, and stained with the appropriate primary antibody for 1 hour at 37°C in a humidified chamber. Irrelevant isotype-matched control antibodies at the same concentration as the primary antibody were used in parallel. The cells were then washed and stained with a FITC-conjugated $F(ab')_2$ goat-anti-mouse IgG secondary antibody for 1 hour at 37°C. After washing, slides were mounted with citifluor (UKC Chemical laboratory, Canterbury, UK), and the staining observed using an Olympus microscope equipped for fluorescence and photography. Photographs were taken using a SPlan 40PL/0.70 objective (Olympus, UK) on T-Max Kodak black and white film.

2.7 FLOW CYTOMETRY

2.7.1 The detection of the expression of CMV-specific IE antigen by flow cytometry.

Smooth muscle cells and fibroblasts were seeded at a density of 1.0 x 10^5 cells/well in 12-well plates and incubated for 24 hours. The cells were infected with CMV strain AD169 or strain C1F at a multiplicity of infection of from 0.3-10 pfu/cell as indicated in the text. At 24 hours post-infection, infected cells were removed by trypsinization using trypsin-EDTA (500µg/ml). The cells were washed twice with phosphate buffered saline by centrifugation at 500g for 5 minutes, and fixed with phosphate buffered saline: acetone (66:34) for 20 minutes at 4°C. The cells were then washed and incubated with a monoclonal antibody specific for CMV IE antigen, designated E13, for one hour at 37°C in a humidified chamber. Irrelevant isotype-matched antibodies at the same concentration as the primary antibody were used in parallel. The cells were then washed and incubated with a FITC-conjugated $F(ab')_2$ fraction of a goat anti-mouse IgG antibody at 37°C for 30 minutes. Following washing, the cells were fixed in 2% paraformaldehyde, and analysed

by flow cytometry using a FACScan (Benton Dickinson, Mountain view, CA) as described in section 2.7.4.

2.7.2 The detection of a fibroblast-specific cell surface marker by flow cytometry.

Smooth muscle cells and fibroblasts were seeded into 6-well plates at a density of 2.5 x 10^5 cells per well, and cultured for 24 hours. The cells were trypsinized, washed twice in phosphate buffered saline, and incubated with a fibroblast-specific monoclonal antibody, designated FibAS02, or with an irrelevant isotype-matched control antibody for 1 hour at 37°C. The cells were again washed in phosphate buffered saline, and stained with a FITC-conjugated $F(ab')_2$ fraction of a goat-anti-mouse IgG antibody for 1 hour at 37°C. Following incubation, the cells were washed twice with phosphate buffered saline, fixed in 2% paraformaldehyde, and analysed by flow cytometry using a FACScan as described in section 2.7.4.

2.7.3 The detection of the cell surface expression of adhesion molecules and class I and class II MHC by flow cytometry.

Smooth muscle and fibroblasts were seeded into 6-well plates at 3 x 10^5 cells/well, and 24 hours after the initial seeding were either left uninfected, or infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10 pfu/cell. In some experiments, the CMV-infected smooth muscle cells were either stimulated with 10 ug/ml IFN-gamma (Sigma), or left unstimulated. The cells were detached by trypsinization, and washed in phosphate buffered saline containing 0.1% sodium azide (BDH) and 0.1% bovine serum albumin (Sigma). The cells were then stained with monoclonal antibodies specific for certain adhesion molecules, class I or class II MHC-determinants, or their appropriate isotype-matched control antibody, at their saturating concentrations (see section 2.5) for 30 minutes. This was followed by staining with FITC-conjugated $F(ab')_2$ fraction of a goat anti-mouse IgG antibody for a further 30 minutes. After washing, the cells were fixed in 2% paraformaldehyde (BDH), and subsequently analysed by flow cytometry using a FACscan as described in section 2.7.4.

2.7.4 The acquisition and analysis of flow cytometric data

For all flow cytometric analyses using the FACScan IV flow cytometer, 5000-10000 events were collected using a logarithmic amplifier, and the data obtained was processed using the consort 30 and lysis II software programmes (Becton Dickinson) or the Win MDI version 1.3.3 programme (Verity Software House, Maine, USA). After exclusion of the dead cells from the original cell

population using the forward and side scatter dot plot profiles, the data from the viable cells was obtained using a logarithmic scale, and presented either as histogram profiles, the percentage of cells positive for the appropriate antigen, or the median fluorescent intensity in arbitrary units. When stated in the text, flow cytometric data were converted from logarithmic values (median fluorescence intensity), to median linear channel values, using the lysis II software programme, and subsequently to fluorescence intensity units (FIU). The background fluorescence of parallel samples stained with an irrelevant isotype-matched antibody was subtracted from the values obtained for the test samples. The median linear channel values were then converted to FIU using the relationship $FIU=10^{(x/235)}$, where x is the difference in median linear channel value of the sample from that of the control cells (Watson, 1992). The value 235, represents the shift in channel values that generated a 10-fold increase in signal brightness as determined using electronic test pulses on the FACScan. Briefly, this was achieved by recording the median fluorescence intensity of a given test signal, amplifying the signal by a factor of 10 using the manually controlled amplifier on the machine, and recording the median fluorescence intensity of the amplified signal. This provided a measure of the increase in median fluorescence intensity, which represented a 10-fold increase in brightness of the signal, which was then converted to channel values. For the machine used in this study, this value as determined using the FL1 detector at 1024 channels resolution was found to be 235 channels. In this study, control cells were uninfected cells stained for the molecule under investigation, and FIU values thus represented fold increases or decreases from this control cell value. Statistical analysis was performed on these values using the Mann-Whitney t-test.

2.8 ORGAN CULTURE OF SAPHENOUS VEINS

2.8.1 Vein collection procedure.

Saphenous vein samples were obtained from patients undergoing coronary artery or lower limb bypass grafting at the London Chest Hospital (London, UK). These non-diseased vein segments were dissected with minimal handling, collected under sterile conditions, and placed immediately in cold (4°C) calcium-free Krebs solution, or in the heparinized blood of the patient. They were transported to the laboratory at ambient temperature, and processed within 6 hours for organ culture. Only veins that were macroscopically normal were included in the study.

2.8.2 Organ culture procedure.

Vein segments were placed in sterile RPMI 1640 tissue culture medium containing 20mmol/L HEPES buffer in the bottom of a circular petri dish (Corning, UK). The vein culture system used was based on that developed by Soyombo et al. (1990). Briefly, excess fat and adventitial tissue was dissected from the vessels using a scalpel blade. The vessels were then cut transversely along their length into 0.5cm ring segments. A dissected vein segment was then assessed for endothelial cell coverage using the trypan-blue exclusion technique (Pederson & Bowyer, 1985). This involved the incubation of the vein segment for 1-2 minutes at room temperature in phosphate buffer saline, pH 7.6 containing 0.01% trypan Blue. To visually assess endothelial integrity, veins denuded of endothelium stained blue, while those with an intact endothelial cell lining did not pick up the stain. Veins with denuded endothelial cell lining were excluded from the study. In order to confirm the presence of an intact endothelial cell lining, another vein segment was immediately fixed in formalin and processed for histology and immunohistochemistry. The rest of the vein segments were then immobilized on a layer of preformed sylgard resin (Dow Corning, Seneffe, Belgium) in the bottom of a glass petri dish (BDH), with minuten pins (size A1, Watkins and Doncaster, Kent, UK). They were cultured in RPMI1640 containing L-glutamine (2mmol/ml), 30% (v/v) foetal calf serum, penicillin (100IU/ml) and streptomycin (100g/ml), and incubated at 37°C in a humidified atmosphere of 5% CO_2 for up to 21 days, with a change of medium every 2-3 days. At the end of the culture period, the segments were fixed overnight with 10% formalin, followed by processing and paraffin embedding, which was performed by the Department of Histopathology, Royal

Free Hospital, UK. Figure 2.2 shows a schematic illustration of the allocation and processing of vein segments for organ culture.

2.8.3 The infection of saphenous vein segments with CMV.

Saphenous vein segments obtained as described above were infected either by using a standard virus adsorption technique or by centrifugal inoculation. The standard virus adsorption technique involved the submersion of vein segments in 1ml of virus stock at 1 x 10^6 pfu/ml for one hour at 37°C. Mock-infected vein segments were placed in 1ml of culture medium. For centrifugal inoculation, vein segments were individually placed in 15ml conical tubes (GibcoBRL), to which 1ml of either CMV strain AD169 or strain C1F at a titre of 1x 10^6 pfu/ml was added. Vein segments were then centrifuged at 1500g for 1 hour. Centrifugal inoculation has been demonstrated to significantly enhance the infectivity of CMV strains in endothelial (MacCormac & Grundy, 1999) and monocyte/macrophage cell cultures (Ho et al., 1993). Mock-infected vein segments were centrifuged in 1ml of culture medium. Following centrifugal inoculation, vein segments were then placed for a further hour of virus absorption at 37°C in a humidified atmosphere containing 5% CO_2. The vein segments were then removed from the virus supernatant, and transferred to glass petri dishes, where they were cultured and processed as described above. Figure 2.3 shows a schematic illustration of the allocation, infection and processing of vein segments.

2.8.4 Primary antibodies used in immunohistochemical staining.

Table 2.3 summaries the specificity, dilution and appropriate antigen retrieval procedure (see below) for monoclonal antibodies used for immunohistochemical analysis.

For the detection of CMV-infected cells in vein segments, the monoclonal antibodies CCH2 and E13, which specifically react with proteins from different phases of viral replication, were used. The antibody CCH2 (Dako, UK) reacts with a 43kD DNA-binding protein, p52, of CMV (Plachter et al., 1992), and was used at a dilution of 1:25. The initiation of the synthesis of this protein requires the presence of viral IE proteins. It is expressed early, and more abundantly late, after infection with CMV. The specificity and concentration of the monoclonal antibody, E13 has been described in section 2.5.5. In paraffin-embedded tissue sections, the antibodies CCH2 and E13 stain the nuclei of morphologically unaltered as well as cytomegalic cells. The working dilution of these

antibodies was determined by immunohistochemical staining of a section of ulcerated colon from a patient who died from disseminated CMV disease. This tissue served as a positive control for CCH2 and E13 staining, and was processed in parallel to the vein segments in all experiments.

For the identification of smooth muscle cells in vein segments, a 1:20,000 dilution of anti-α-smooth muscle-1 (clone 1A4, Sigma) was used. Endothelial cells were detected using a panel of endothelial cell specific antibodies: anti-von-Willebrand factor (Dako), anti-CD34 (Dako), anti-PECAM-1 (Dako). Endothelial cells were also detected with the glycoprotein, Ulex Europeus lectin (Dako) which is known to interact with α-L fucosyl residues in oligosaccharides present on the membranes of human endothelial cells. The working dilutions of the smooth muscle cell and endothelial cell specific antibodies were determined by immunohistochemical staining of sections of an umbilical cord. For the identification of lymphocytes and monocytes/macrophages in vein segments, monoclonal antibodies specific for CD45 and CD68 antigens were used, respectively. The working dilutions of these monoclonal antibodies were determined by immunohistochemical staining of sections of human tonsils.

2.8.5 Immunohistochemistry.

For the application of immunohistochemistry, 4μm sections of paraffin-embedded tissues were mounted on slides coated with poly-L-lysine (Sigma), or 3-aminopropyl trietoxy silane (Sigma), to increase their adherence, and dried overnight at 56°C in an incubator (Griffin & George Ltd, UK). They were deparaffinized through three washes in xylene (BDH) for 5 minutes, rehydrated in absolute alcohol (BDH) and then rinsed in distilled water. Sections were then treated with 3% hydrogen peroxide (Sigma) for 10 minutes at room temperature, to block any endogenous peroxidase activity present in the tissues. An antigen retrieval procedure involving proteolytic digestion with trypsin or pepsin at 37°C was used to improve the accessibility of monoclonal antibodies to their target antigens. This procedure is crucial for the successful immunohistochemical staining of formaldehyde-fixed, paraffin-embedded tissues. Trypsin retrieval solution was prepared by dissolving 1mg of trypsin with buffer salt tablets (Sigma) in 1 ml of distilled water at room temperature, pH 7.8. Pepsin retrieval solution was prepared by dissolving 2g of powder pepsin (Dako, UK) in 500ml of 0.2M hydrochloric acid, pH 7.8. The proteolytic agents were applied to tissue sections for 30 minutes at 37°C. Following the antigen

retrieval procedure, slides were rinsed in distilled water, and then allowed to equilibrate in Tris buffered saline (BDH) for 5 minutes at room temperature. They were then incubated with normal goat serum (Dako) (1:10, diluted in Tris buffered saline) for 15 minutes in a humidified chamber at room temperature, to block non-specific binding sites. Excess normal goat serum was removed, and the sections were either then incubated with the appropriate dilution of primary antibody, or with an irrelevant isotype-matched antibody at the same concentration for 1 hour at room temperature.

Following incubation, the sections were washed in Tris buffered saline and sequentially incubated at room temperature for 30 minutes in 1:100 dilutions of biotin-conjugated goat-anti-mouse/rabbit immunoglobulin and streptavidin/horseradish peroxidase, with Tris buffered saline washes after each of the steps. Both reagents were obtained from the StrepABComplex Horseradish Peroxidase Mouse/Rabbit Duet Kit, (Dako). The working dilutions of these antibodies were obtained from the manufacturer's instructions. The slides were then washed three times in Tris buffered saline, and incubated with a 0.05% solution of diaminobenzidine (Sigma) containing 0.03% hydrogen peroxide in Tris buffered saline for colour development. Sections were washed and counterstained with Mayers haematoxylin (Sigma) for 5 minutes at room temperature, then dehydrated through a graded alcohol series from 70% to absolute alcohol, then in citroclear (BDH), and finally mounted in DPX (BDH).

2.8.6 Double-staining immunohistochemistry.

An indirect immunostaining method combining the streptavidin-biotin-peroxidase and streptavidin-biotin-alkaline phosphastase techniques was employed to detect CMV antigens and various cell marker antigens sequentially. In the first step, CMV early antigens were stained using the monoclonal antibody CCH2 as described above. After prior washing, the binding of the primary antibody was followed by incubation for 30 minutes with a biotinylated goat anti-mouse antibody (1:500, Vector Laboratories, UK). The optimal working dilution of the secondary antibody was determined by immunohistochemical staining of a section of CMV-positive colon tissue. Following the biotinylated antibody reaction, sections were incubated with streptavidin/horseradish peroxidase (1:100, Dako) for a further 30 minutes at room temperature. After visualization of CMV antigens with a 0.05% solution of diaminobenzidine, the respective cell marker proteins were labelled in the same way with the following modifications: Following the application of, and incubation with, the second primary antibody, slides were washed in Tris buffered saline, and then incubated for 30 minutes in biotinylated

goat anti-rabbit antibody. They were then washed again in Tris buffered saline, and incubated with streptavidin-biotin-alkaline phosphatase for a further 30 minutes. Both reagents were obtained from the Vector ABC-alkaline phosphatase kit (Vector Laboratories) and were used according to the manufacturer's instructions. Visualization was performed using the Vector red substrate kit (Vector Laboratories), which was also used according to the manufacturer's instructions. After the second staining, slides were dehydrated as above and mounted in VectaMount (Vector Laboratories). Cells positive for CMV antigens stained brown (peroxidase positive) while those positive for cell marker antigens stained red (alkaline phosphatase positive).

A specimen of ulcerated colon taken from a patient who died from disseminated CMV infection served as a positive control for the simultaneous detection of CMV antigens and endothelial cell markers in the same tissue.

2.8.7 The demonstration of elastin fibres in sections: Millers elastin/ van Gieson staining

Sections of paraffin-embedded tissues were mounted on slides coated with poly-L-lysine or 3-aminopropyl trietoxy silane, and dried overnight at 56°C in an incubator. Slides were de-paraffinized through three washes in xylene for 5 minutes, rehydrated in absolute alcohol and then rinsed in distilled water. The sections were then treated with 0.5% potassium permanganate (BDH) for 5 minutes at room temperature, washed in distilled water, and subsequently treated with 1% oxalic acid (BDH) for 1 minute at room temperature. The sections were then washed in distilled water, followed by absolute alcohol, and then stained with Millers elastin biological stain (BDH) for 3 hours at room temperature. After the staining period, sections were washed in distilled water, rinsed in absolute alcohol, and then stained with van Gieson stain (BDH) for 5 minutes. Slides were then blotted dry with an absorbant tissue, rehydrated by washing through two changes of absolute alcohol and then in citroclear for 1min, before being mounted in DPX.

2.8.8 The detection of neointima formation and the measurement of neointimal thickness

Measurements of the neointimal thickness of cultured vein segments were made using a computerized image analysis system (Leica Q500MC, Leica, UK). The neointima was defined as the new cellular layer developing in culture beneath the endothelium on the

luminal side of the internal elastic lamina. The internal elastic lamina was localized using Miller's elastin/van Gieson stain as described above. This stains the internal elastic lamina a prominent or distinct black colour. A total of 25 measurements were taken along the length of each vein section, from which the average of these readings was calculated and denoted as the neointimal thickness of that section or vein segment.

2.8.9 The quantification of CMV positive cells in the vein wall.

The number of cells staining positively for CMV antigens in the vessel wall of saphenous veins was determined using an image analysis system (Seescan, Cambridge, UK) at x40 magnification. The area of each field was determined with the image analyser by drawing a frame around it, from which the computer measured an area. Areas of neointima and adventitia were outlined, and the number of positive cells within each measured framed area was counted. Ten fields each were counted around the neointima or adventitia, and the number of CMV positive cells counted divided by the measured area. The number of CMV positive cells in the vessel wall was expressed as the number of positive cells/unit area (μm^2).

2.9 STATISTICAL ANALYSIS

In order to determine the significance of the flow cytometric data, the median channel values obtained from triplicate mock-infected or infected sample of cells were compared using the Mann-Whitney t-test. The Wilcoxon non-parametric test was used to analyse the significance of virus titres released into the supernatant fluid of paired infected cell cultures with a 95% confidence interval. The differences in the neointimal thickness that developed in cultured vein segments were analysed by the Mann-Whitney t-test, with a 95% confidence interval.

Table 2.1. Primary murine monoclonal antibodies used in this study to characterize and analyse cultured vascular smooth muscle cells[1]

Antigen Specificity	Clone	Isotype	Optimal working concentration/ Dilution
Adhesion molecules			
ICAM-1	BB1G-1	IgG1	5μg/ml
LFA-3	AICD58.9	IgG3a	5μg/ml
VCAM-1	BBIG-VI	IgG1	5μg/ml
$\alpha_v\beta_3$	23C6	IgG1	1:50
Human HLA molecules			
Class I HLA	PA 2.6	IgG1	1:200
Class II HLA	RFDR2	IgG2a	10μg/10^5cells
Cytoskeletal proteins			
Smooth muscle α-actin	1A4	IgG2a	1:800
Chicken gizzard actin	-	IgG1	1:100
Fibroblast specific marker	AS02	IgG1	5μg/ml
Vimentin	-	IgG1	1:2
CMV antigens			
CMV p72 and p86	E13	IgG1	1:50
CMV p72	63.27	IgG1	1:1600
CMV pp65	Clone 10/11	IgG1	1:500
CMV glycoprotein B	7-17	1gG3	1:16

[1]The source of antibodies used is given in the text.

Table 2.2. Fixation methods for cytoplasmic immunofluorescent staining

Antigens	Fixation
α-actin, vimentin	Ice-cold methanol-acetone (BDH) (1:1), at -20°C for 10 min
CMV p72 and p86 CMV glycoprotein B	Acetone at 4°C for 20 minutes
CMV pp65	5% paraformaldehyde with 2% sucrose (Sigma) in phosphate buffered saline for 10 minutes at room temperature. Then permeabilize with 0.5% NP-40 (BDH) with 10% sucrose and 1% foetal calf serum in phosphate buffered saline.

Table 2.3. The panel of monoclonal antibodies and reagents used to analyse CMV viral antigen expression and cell specific markers in cultured vein segments by immunohistochemistry

Cell specificity	Antigen specificity	Clone	Donor species	Source	Dilution	Antigen retrieval
Endothelial cells	[1]CD34 antigen	QB-END10	Mouse	Dako	1/20	Pepsin 30 minutes
Endothelial cells	PECAM	JC/70A	Mouse	Dako	1/25	Pepsin 30 minutes
Endothelial cells	von Willebrand factor	-	Mouse	Dako	1/10000	Pepsin 30 minutes
Endothelial cells	von Willebrand factor	M616	Rabbit	Dako	1:1000	Pepsin 30 minutes
Endothelial cells	Ulex Europeus	-	-	Vector Laboratories	1/4000	Trypsin 15 minutes
Lymphocytes	CD45 antigen	2B11 + PD7/26	Mouse	Dako	1:20	Trypsin 30 minutes
Monocytes Macrophages	CD68 antigen	PGM1	Mouse	Dako	1/200	Trypsin 10 minutes
Smooth muscle cells	α-actin	1A4	Mouse	Sigma	1/10000	No Treatment
-	CMV	CCH2	Mouse	Dako	1/25	Trypsin 30 minutes
-	CMV	E13	Mouse	Biosoft	1/100	Trypsin 30 minutes

[1]CD = Cluster of differentiation.

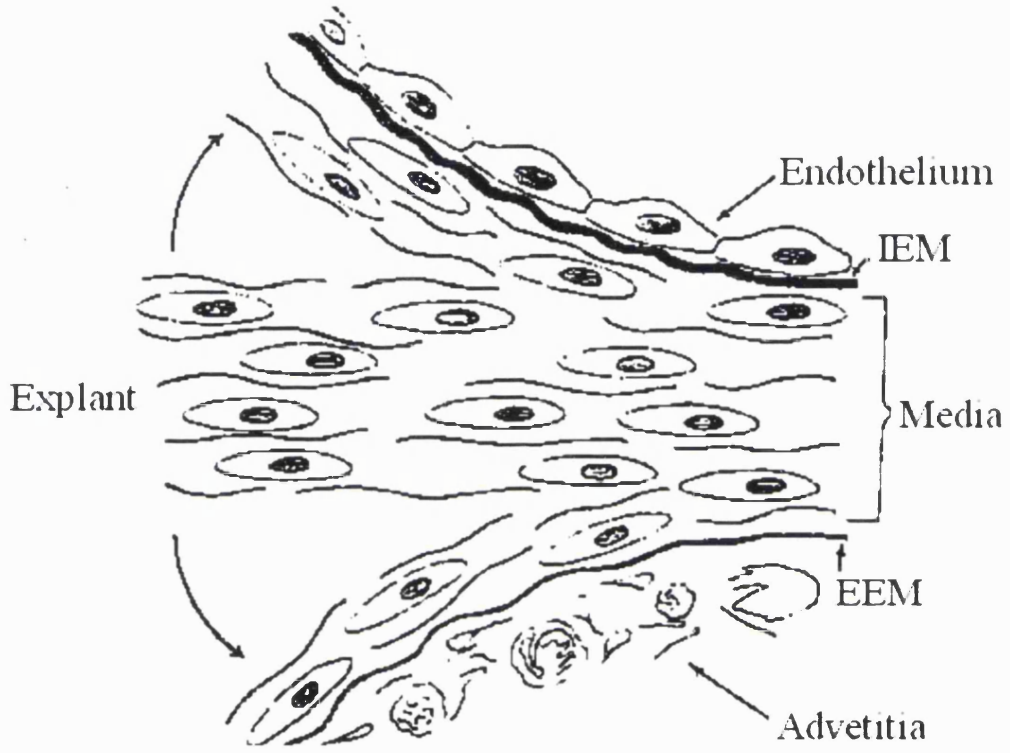


Figure 2.1. A diagram showing how the mid-portion of the media of the saphenous vein used for explant culture was obtained.

The inner portion of the media, including the intima, and the outer portion of the media, including the adventitia, were stripped off with a scalpel and discarded. IEM: Internal elastic membrane; EEM: External elastic membrane.

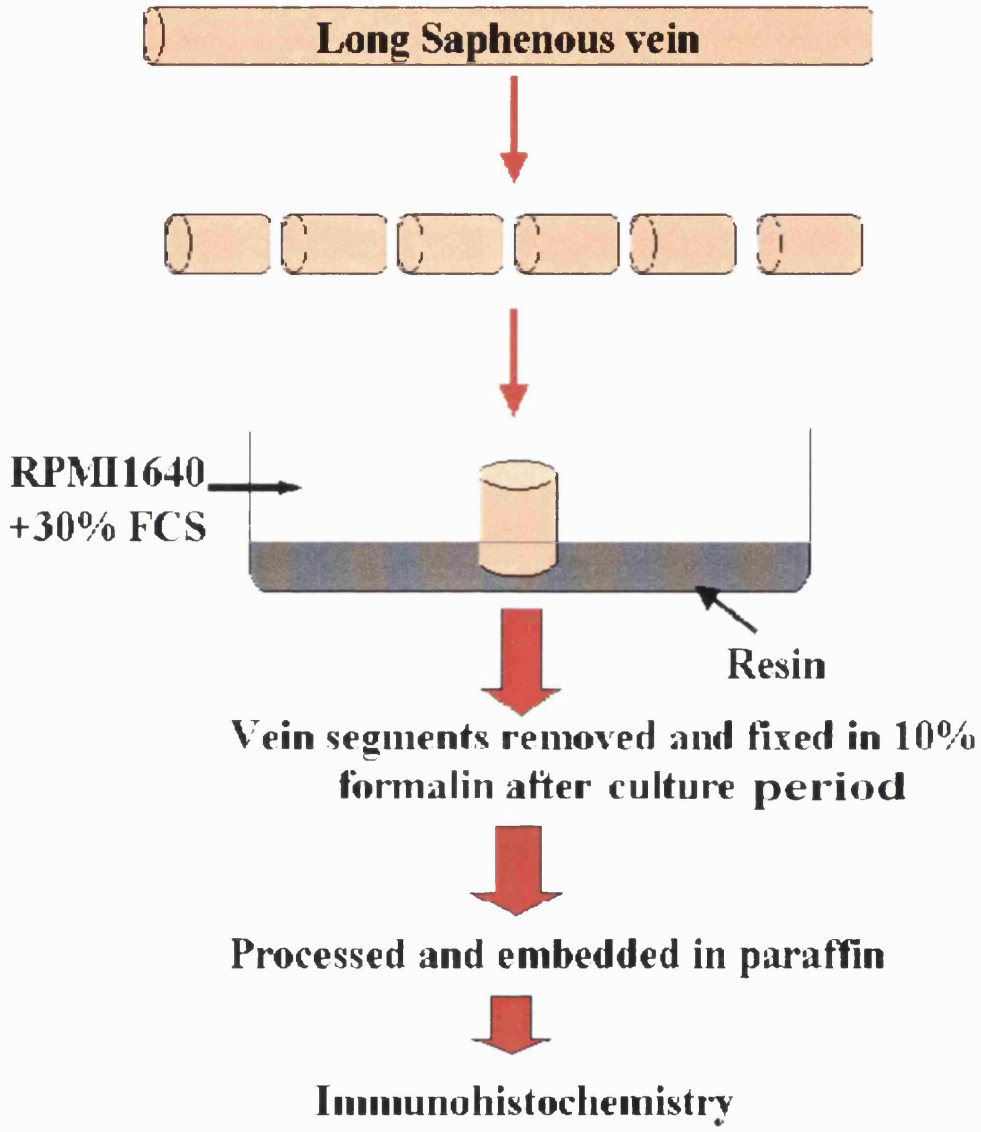


Figure 2.2. A schematic diagram of the processing of vein segments.

Saphenous vein samples were obtained from patients undergoing coronary artery or lower limb bypass grafting. The vessels were cut transversely along their length into 0.5cm ring segments, and then immobilized on a layer of preformed sylgard resin in the bottom of a glass petri dish with minuten pins. The vein segments were cultured in RPMI 1640 containing 30% (v/v) foetal calf serum, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Vein segments were completely submerged in culture medium. At the end of the culture period, the segments were fixed overnight with 10% formalin, followed by processing and paraffin embedding for immunohistochemical analysis.

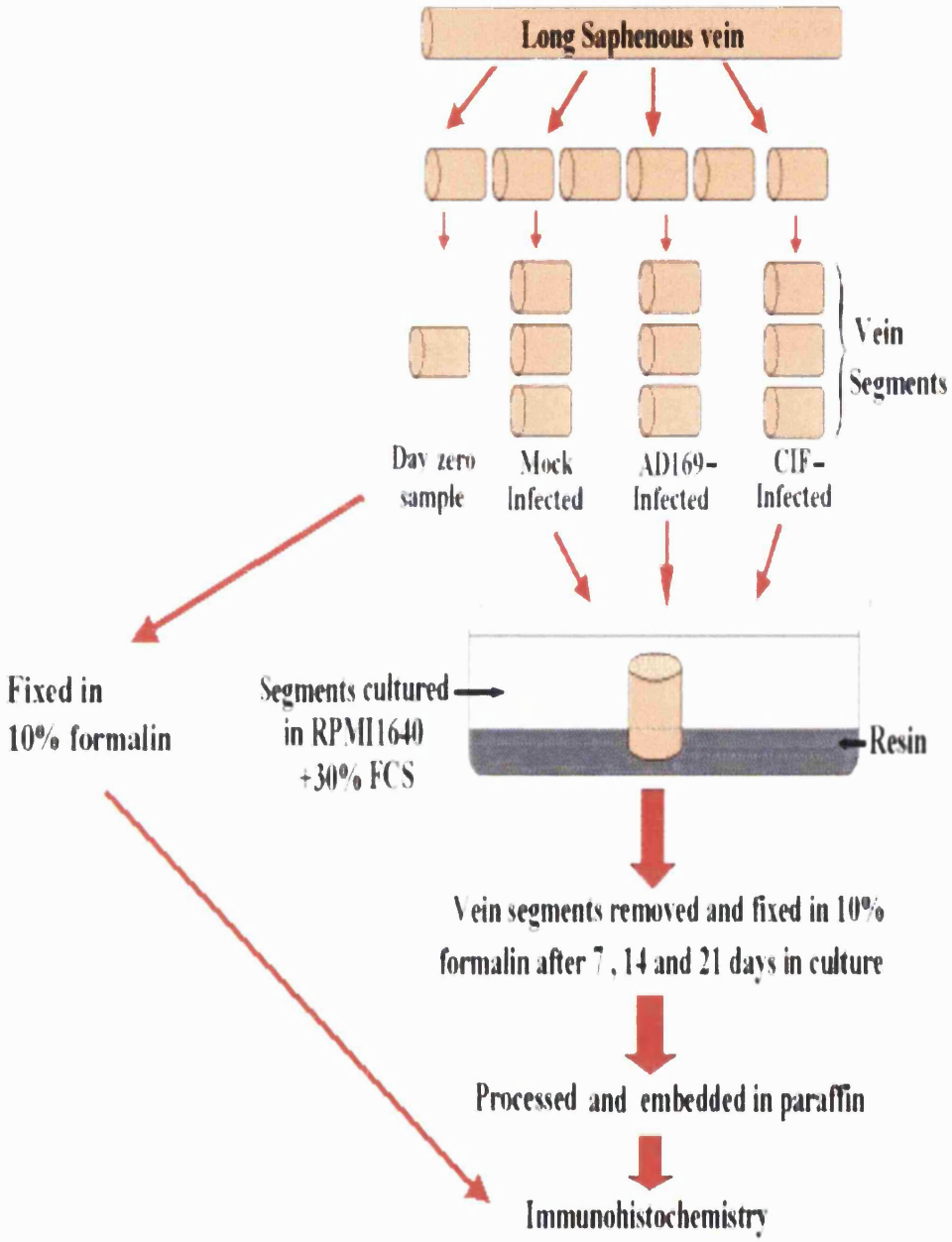


Figure 2.3. A schematic illustration of the allocation, infection and processing of vein segments.

Saphenous vein samples were obtained and processed for culture as described in Figure 2.2. The vein segments were infected with CMV strain AD169 or strain C1F either by a standard virus adsorption technique or by centrifugal inoculation. Mock-infected vein segments were exposed to culture medium. Following the infection of vein segments, they were cultured in RPMI 1640 containing 30% (v/v) foetal calf serum, and incubated at 37°C in a humidified atmosphere of 5% CO_2. At the end of the culture period, the vein segments were fixed overnight with 10% formalin, followed by processing and paraffin embedding for immunohistochemical analysis.

CHAPTER 3:

The isolation, culture and characterization of vascular smooth muscle cells

3.1 INTRODUCTION

Smooth muscle cells in the blood vessel wall are involved in the development of atherosclerotic plaques, and infection of these cells with CMV has been proposed to play a role in the progression of atherosclerosis. In any attempt to investigate the possible role of CMV infection of smooth muscle cells in the pathogenesis of atherosclerosis *in vitro*, it is imperative to obtain pure cultures of smooth muscle cells to use in the study. There are two general methods for the preparation of primary cultures of vascular smooth muscle cells. One involves the direct isolation of cells from enzymatically digested vessels (enzymatic isolation method), and the other involves isolating the cells that migrate out from small tissue samples (explant method). In the present study, smooth muscle cells were isolated from the vessels of umbilical cords by enzymatic digestion, and from tissue samples of the saphenous vein by the explant method. The rational basis of these methods are based on the fact that the media layer of the blood vessel wall consists entirely of smooth muscle, and by removing the adventitia and intimal layers of the vessel wall, cultures of vascular smooth muscle cells can be obtained (McMurray et al., 1991). In addition, aortic smooth muscle cells were obtained from the European Collection of Cell Culture. These cells had already been characterised as smooth muscle cells, and thus, served as a positive control for the smooth muscle cell phenotype. In addition, the aortic smooth muscle cells served as alternatives to smooth muscle cells isolated from the venous vasculature of the human blood vessels. The efficiencies of the isolation methods were compared in terms of the viability, yield and purity of the resultant smooth muscle cell populations.

Smooth muscle cells isolated by enzymatic digestion or explantation have the risk of fibroblast contamination due to incomplete removal of the adventitia, and as such, it is important to be able to distinguish smooth muscle cells from other cell types in culture. One strategy frequently applied to determine the origin of a cell of uncertain lineage is to examine its phenotype by using immunohistochemical and/or biological markers, such as cytoskeletal proteins. The contractile proteins and other proteins associated with the regulation of contraction represent logical candidates for use in studying the differentiation and characterization of vascular smooth muscle cells. They include smooth muscle α-actin (Owens, 1995; Gabbiani et al., 1981), smooth muscle myosin heavy chain (Rovner et al., 1986), smooth muscle myosin light chain (Hasegawa et al., 1992) and smooth muscle α-tropomyosin (Lees-Miller & Helfman, 1991). In the present study, the characterization and purity of smooth muscle cells isolated from the vessels of umbilical cords and from the explant tissue of the saphenous vein was determined by immunofluorescent staining

using monoclonal antibodies specific for smooth muscle α-actin. In primary cultures of vascular smooth muscle cells, smooth muscle α-actin synthesis is rapidly induced in contact inhibited, growth-arrested cultures, suggesting that the composition of the medium and the degree of confluency may influence the level of smooth muscle α-actin mRNA, synthesis, and content (Rovner et al., 1986; Owen et al., 1986). Furthermore, a fibroblast specific monoclonal antibody, designated, FibAS02 was used to determine the likelihood of contamination of the isolated smooth muscle cell populations with fibroblasts, by assessing the reactivity of this antibody with the smooth muscle cell populations.

A large proportion of human smooth muscle cells in arteries and veins express only vimentin as an intermediate filament protein (Giabbiani et al., 1981; Schmid et al., 1982). Vimentin intermediate filaments have been reported to exist in various cultured cells. It has been demonstrated that vascular smooth muscle cells differ from smooth muscle cells of the digestive, respiratory and urinogenital tract by the presence of large amounts of vimentin filaments (Gabbiani et al., 1981). Vimentin, in contrast to the other intermediate filament, desmin, and several other contractile and structural proteins of smooth muscle cells, is not down-regulated in primary cultures of smooth muscle cells. It was therefore of interest to investigate the relative composition of vimentin filaments in the smooth muscle cells isolated in this study. Hence, the demonstration of the expression of actin and vimentin filaments served as a diagnostic means of characterizing the isolated vascular smooth muscle cells used in this study.

3.2 RESULTS

3.2.1 The isolation and culture of smooth muscle cells from the saphenous vein.

The saphenous vein possesses an inner as well as an outer layer of medial smooth muscle cells, and as such, is an excellent source of smooth muscle cells. The isolation of these cells can be achieved by the procedure of explantation. Explantation involves the removal or stripping of the intima and adventitial layers, which theoretically gives rise to vein segments consisting solely of the media layer of the vein wall. The removal of these two blood vessel layers is followed by the attachment of the saphenous vein explants to the surface of a tissue culture flask, and the observation of the outgrowth of medial smooth muscle cells from the explant.

When saphenous vein explants were placed in culture and the bathing media replaced every 2-3 days, cells migrated out from the explant onto the surface of a tissue culture flask. Figure 3.1 shows a photomicrograph of cells migrating out from the explant after a few weeks in culture. Cell migration and subsequent growth from the explant was observed as early as 2 weeks post-culture, with the migrated cells reaching confluency and forming a continuous circular sheet of cells around the explant at approximately 4-6 weeks (Figure 3.2). At this point, the explant tissues were removed, and the confluent cultures of migrating cells were harvested using trypsin-EDTA. A suspension of migrating cells from the various explant cultures from the same vein tissue were collected in a 50ml centrifuge tube. This cell suspension was spun at 500g for 10 minutes, the resulting cell pellet was resuspended in growth medium, and the percentage of viable cells determined by estimating the number of cells excluding trypan blue, using a haemocytometer. Cell viability was always greater than 95% in the cells isolated using the explant method. The yield of cells was in the order of 0.5-1 x 10^5 cells/6cm vein tissue. These cells were plated into tissue culture flasks, and were observed to flatten within a few hours. The size and shape of the cells varied depending on the density of cell seeding. Generally, migrated cells had a ribbon or spindle shape morphology, with an oval or sausage shaped nucleus containing two or more nucleoli, as illustrated in figure 3.3. In the presence of standard 10% foetal calf serum, subcultured migrated cells grew in overlapping layers until they reached a certain density, forming a "hill and valley" growth pattern. The hills denote areas of cells that have piled upon one another to form as many as 10-15 layers, whereas the valleys may contain no cells or 1-3 layers of cells. These primary and subcultured cells were ready for use within 2-3 weeks after the isolation from the saphenous vein.

3.2.2 The isolation and culture of smooth muscle cells from the umbilical cord.

Enzymatic digestion of the vessels of umbilical cords served as another reliable method to obtain primary cultures of vascular smooth muscle cells. Briefly, the vessels of untraumatized umbilical cords were cannulated at both ends and filled with the digestive enzyme, collagenase. Following the initial digestion of endothelial cells lining the vessel lumen, enzyme-dispersed smooth muscle cells from the vessels of umbilical cords were obtained following a further digestion of the lumenal vessels with collagenase. The enzyme-cell suspension were then transferred to 50ml centrifuge tubes and spun at 500g for 10 minutes. The resulting cell pellet was gently resuspended in nutrient medium and an aliquot of the cell suspension was taken for the determination of cell viability. Cell viability was always greater than 95% in these cells, similar to that of cells isolated using the explant method. The cell concentration was then adjusted to the required level, and the cells plated out into tissue culture flasks. The yield of cells varied considerably due to the variability in the length of umbilical cords digested, and averaged approximately 1-2.5 x 10^4 cells/umbilical cord after the centrifugation of the enzyme-cell suspension. The enzyme-dispersed smooth muscle cells attached to the plastic substrate of a tissue culture flask within 2 days. The cells appeared to have an irregular, elongated ribbon or spindle shape, as illustrated in Figure 3.4. After about one week, the cells began to proliferate, reaching confluency and exhibiting a "hill and valley" growth pattern. Confluent cultures were subcultured with trypsin-EDTA, and again approached confluency after a further 2-3 weeks. These primary and subcultured cells were ready for use within 4-5 weeks after their isolation from the umbilical cord.

3.2.3 Human aortic smooth muscle cells.

Growing cultures of aortic smooth muscle cells were received at passage 24 in 25cm^2 tissue culture flasks from a commercial source, and took approximately 4 weeks to reach confluency. At confluency, they were observed to grow in a "hill and valley" morphology, characteristic of smooth muscle cells in culture. Confluent cultures, at approximately 0.5 x 10^5 cells/cm^2 were subcultured in a 1:3 ratio, as recommended by the suppliers, and were observed to exhibit a relatively slow growth rate with confluency achieved after approximately 4-5 weeks in culture. This limited the use of these cells in future studies. Figure 3.5 shows a phase contrast photomicrograph of aortic smooth muscle cells at passage 24.

3.2.4 The demonstration of smooth muscle α-actin in cultured smooth muscle cells using the monoclonal antibody, anti-α-smooth muscle-1 by immunofluorescence staining.

Smooth muscle cells isolated from umbilical cords and from the explant tissue of saphenous veins could easily be contaminated with fibroblasts, due to the incomplete removal of the adventitial connective tissue layer of the vein wall. Endothelial cells would not grown in these cultures because of their requirements for specific growth factor supplements. In most vascular biology studies, monoclonal antibodies specific for smooth muscle α-actin are used to characterize and identify the phenotype of smooth muscle cells. In the present study, a monoclonal antibody specific for smooth muscle α-actin, anti α-smooth muscle-1, was used to characterize the cells isolated from the saphenous vein and from the vessels of the umbilical cord as smooth muscle cells, and to verify that there was no contamination of the smooth muscle cell population with fibroblasts. Primary cultures of smooth muscle cells tend to lose α-actin filaments *in situ* and as a result, its synthesis must be induced for the successful demonstration of α-actin in cultured smooth muscle cells. The induction of smooth muscle α-actin was achieved by culturing confluent smooth muscle cells overnight in medium containing 1% foetal calf serum.

Explant-migrated and enzyme-dispersed smooth muscle cells were both harvested at passage 3, and aortic smooth muscle cells at passage 25. They were seeded into chamber slides and cultured in their appropriate growth media containing 10% foetal calf serum for 24 hours after the initial seeding. The cells were then cultured for a further 24 hours in 1% foetal calf serum, and then removed and stained for smooth muscle α-actin. Human embryonic lung fibroblasts, MRC-5 fibroblasts and human foreskin fibroblasts served as negative controls. Immunofluorescent staining using anti-α-smooth muscle-1 on explant-migrated, enzyme-dispersed and aortic smooth muscle cells showed that smooth muscle α-actin was always expressed in these cells. The α-actin staining pattern in these cells was exclusively in long, straight, non-interrupted fibrils, scattered densely throughout the cytoplasm. There was no nuclear staining, and no staining of the cytoplasm between the fibrils. Figure 3.6 illustrates the α-actin staining pattern in the smooth muscle cell cultures examined. The relative proportion of the expression of smooth muscle α-actin in these cells were approximately 95-100%, suggesting that the vast majority of the cells in these smooth muscle cell populations were indeed of the smooth muscle cell phenotype. However, in contrast to published reports, significant smooth muscle α-actin staining was also seen in cultured human embryonic lung fibroblasts, MRC-5 fibroblasts and human

foreskin fibroblasts stained in parallel. Figure 3.7 shows the demonstration of smooth muscle α-actin filaments in the various fibroblast cultures examined. Smooth muscle α-actin staining was seen in approximately 30-40% of fibroblasts. These observations suggested that, contrary to previous publications, the anti-α-smooth muscle-1 monoclonal antibody was not strictly specific for smooth muscle cells, but that the antigen it detects is also expressed in subpopulations of fibroblasts from various sources. It was therefore impossible to obtain a clear distinction of the phenotype between smooth muscle cells and fibroblasts using the monoclonal antibody, anti α-smooth muscle-1.

3.2.5 The reactivity of smooth muscle cells with a fibroblast specific marker as assessed by flow cytometry.

Based on the premise that the anti α-smooth muscle-1 monoclonal antibody was incapable of distinguishing smooth muscle cells from fibroblasts, a monoclonal antibody (FibAS02) that had been described to specifically react with a cell surface marker on fibroblasts was used in order to determine whether the isolated cultures of smooth muscle cells described above were contaminated with fibroblasts. Explant outgrowths of smooth muscle cells at passage 3, enzyme-dispersed smooth muscle cells at passage 3, and aortic smooth muscle cells at passage 25 were seeded into 6-well plates. 24 hours after the initial plating, the cells were trypsinized, stained with FibAS02, and then assessed by flow cytometry for the presence of the cell surface fibroblast marker. The reactivities of FibAS02 with human embryonic lung fibroblasts and Epstein-Barr virus transformed B cells were used as positive and negative controls, respectively. The flow cytometric profiles for the reactivity of FibAS02 with the various smooth muscle cells, human embryonic lung fibroblasts and Epstein-Barr virus transformed B cells are shown in Figure 3.8. The data showed that FibAS02 was reactive on cells from all smooth muscle cell cultures and human embryonic lung fibroblasts with almost 100% of cells reactive with the antibody. Epstein-Barr virus transformed B cells were unreactive with this antibody. It was unlikely that the reactivity of the freshly isolated smooth muscle cells with the fibroblast-specific antibody was due to contamination with fibroblasts, since almost 100% of the cells were positive for the marker. In addition this was also the case with aortic smooth muscle cell cultures, which had been supplied as cultures of pure smooth muscle cells. Thus it was probable that the marker was not fibroblast specific, but was also

present on cultured smooth muscle cells. Hence, this supposedly fibroblast-specific marker failed to distinguish the two cell types.

3.2.6 The demonstration of smooth muscle α-actin by immunofluorescence in cultured smooth muscle cells using a monoclonal antibody raised against actin derived from chicken gizzard smooth muscle.

The failure of the monoclonal antibody anti-α-smooth muscle-1 to specifically differentiate smooth muscle cells from fibroblasts and the reactivity of the FibAS02 with both cell types, created the need to search for an antibody that could distinguish between the two cell types. As anti α-smooth muscle-1 was supposed to be specific for smooth muscle cells, an alternative monoclonal antibody specific for smooth muscle α-actin was a possible candidate. A monoclonal antibody raised against actin derived from chicken gizzard smooth muscle had been described in the literature to specifically distinguish smooth muscle cells from fibroblasts. Hence, the reactivity of an anti-chicken gizzard α-actin monoclonal antibody with cultured smooth muscle cells and fibroblasts was studied. Explant-migrated, enzyme-dispersed, and aortic smooth muscle cells were seeded into chamber slides, and 24 hours after the initial seeding the medium was replaced with medium containing 1% foetal calf serum. The cells were cultured for a further 24 hours, and then stained for smooth muscle α-actin, using the anti-chicken gizzard α-actin monoclonal antibody. The fibroblast cultures of human embryonic lung fibroblasts, MRC-5 fibroblasts and human foreskin fibroblasts were stained in parallel, and served as negative controls in these experiments. It was found that this monoclonal antibody specifically stained explant-migrated, enzyme-dispersed and aortic smooth muscle cells for smooth muscle α-actin as illustrated in Figure 3.9. The proportion of cells staining positively for smooth muscle α-actin was approximately 95-100%. The staining pattern was similar to that obtained with the monoclonal antibody, anti-α-smooth muscle-1, which consisted exclusively of long, straight fibrils scattered throughout the cytoplasm. On the other hand, this antibody failed to react with fibroblasts. The α-actin staining pattern on all fibroblasts examined was weak, diffuse, and indistinguishable from that obtained with an irrelevant isotype-matched control antibody. Figure 3.10 shows the staining pattern obtained on the various cultured fibroblast populations by immunofluorescence using the anti-chicken gizzard smooth muscle α-actin antibody.

Thus, the anti-chicken gizzard α-actin monoclonal antibody enabled a clear distinction to be made between smooth muscle cells and fibroblasts, and conclusively demonstrated that the vast majority of the freshly isolated smooth muscle cell populations, and indeed, the commercially purchased aortic smooth muscle cells were of a smooth muscle cell lineage.

3.2.7 The demonstration of vimentin by immunofluorescence staining

The expression of vimentin filaments has been described to be abundant in vascular smooth muscle cells. In this study, the expression of vimentin filaments was determined in explant-migrated and enzyme-dispersed smooth muscle cells, both at passage 3, and aortic smooth muscle cells at passage 25. The fibroblast cultures as above were included in the study to serve as positive controls, since this cell type is known to express vimentin. Smooth muscle cells and fibroblasts were seeded into chamber slides and stained for vimentin filaments using an anti-vimentin monoclonal antibody. Vimentin immunostaining was prominent on all smooth muscle cell cultures, suggesting the expression and synthesis of this protein in the cells. The relative proportion of vimentin positive cells was approximately 90-100% in all smooth muscle cells cultures. Similarly, fibroblasts from various sources also stained positively for vimentin filaments, with similar proportions of vimentin positive cells observed to those of the smooth muscle cell cultures. Figures 3.11 and 3.12 show the staining pattern of the various smooth muscle cell cultures and fibroblast cultures, respectively, with the anti-vimentin monoclonal antibody. The cells reacted in immunofluorescence with a wavy cytoplasmic filamentous system distinct from that of microtubules and actin filaments. Thus, it was demonstrated that the isolated cultured vascular smooth muscle cells expressed vimentin intermediate filaments in a large number of cells.

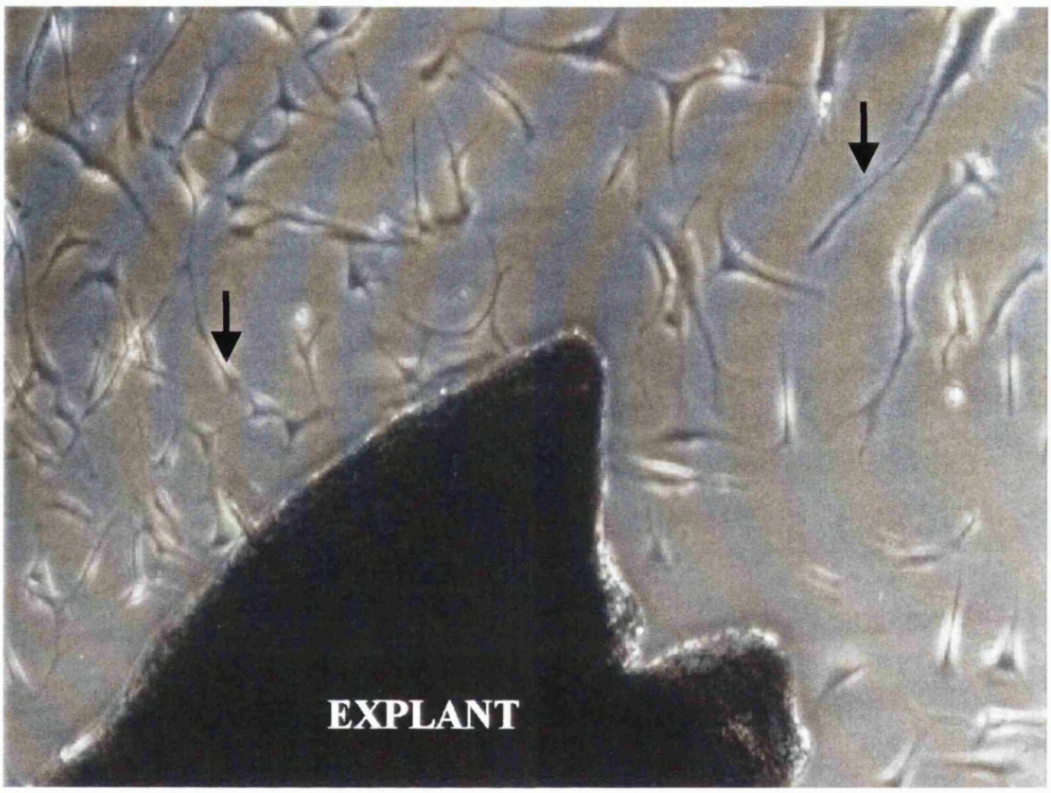


Figure 3.1. The initial outgrowth of smooth muscle cells from an explant of a saphenous vein.

A phase contrast photomicrograph of cells migrating out from an explant of saphenous vein after 3 weeks in culture. The migrated cells are sparsely distributed with active cytoplasmic projections (arrowed). Magnification x400.

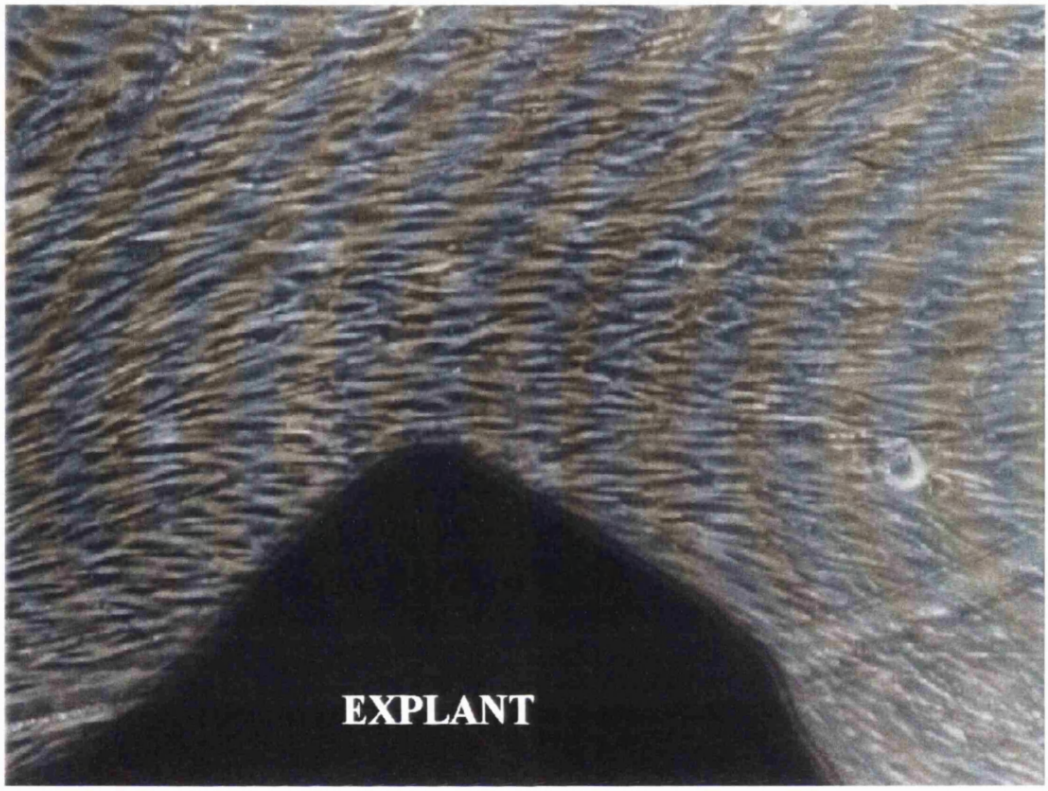


Figure 3.2. The outgrowth of smooth muscle cells from an explant of saphenous vein at confluency.

A phase contrast photomicrograph of cells migrating out from an explant of saphenous vein after 6 weeks in culture. The migrating cells are shown to have formed a continuous sheet of cells around the explant. Magnification x200.

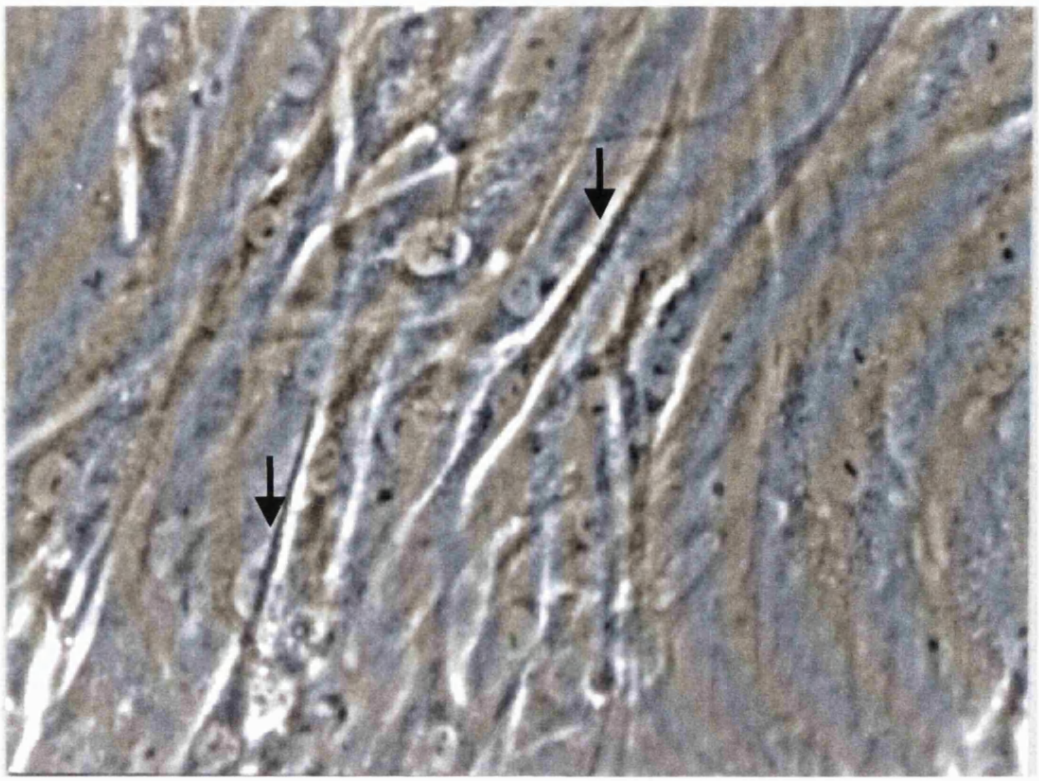


Figure 3.3. Explant-migrated smooth muscle cells in culture.

A phase contrast photomicrograph of smooth muscle cells obtained by explantation from the saphenous vein at passage 3. The smooth muscle cells (arrowed) appear ribbon or spindle shaped, with an oval or sausage shaped nucleus containing two or more nucleoli. Magnification x800.

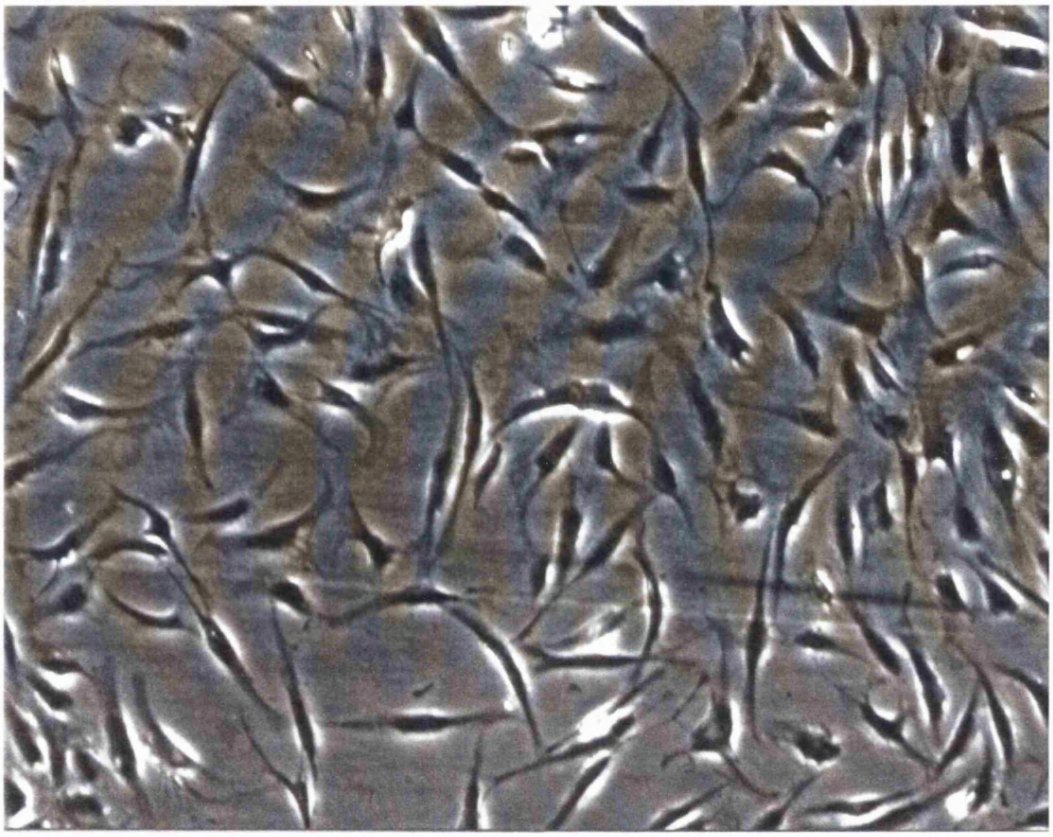


Figure 3.4. Enzyme-dispersed smooth muscle cells in culture.

A phase contrast photomicrograph of smooth muscle cells isolated from the vessels of an umbilical cord by collagenase digestion, at day 7 post-culture. Magnification x400.

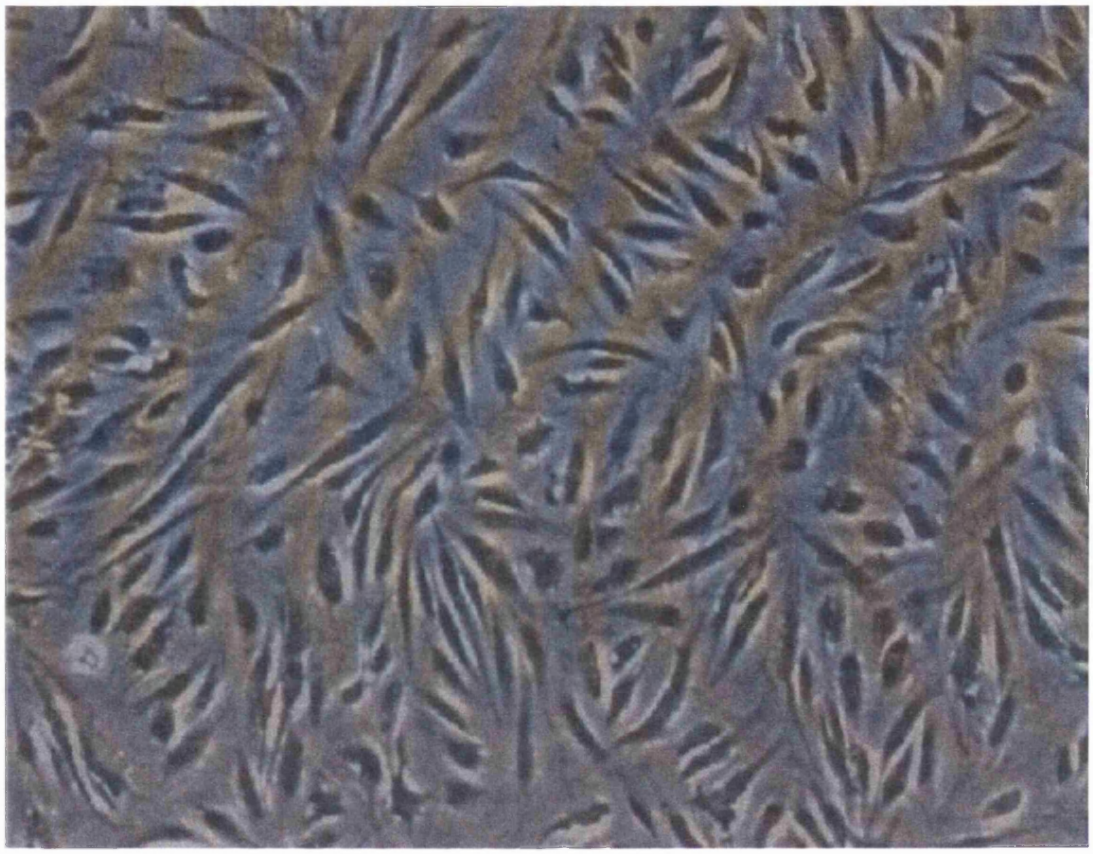


Figure 3.5. Aortic smooth muscle cells in culture.

A phase contrast photomicrograph of aortic smooth muscle cells at passage 24. Magnification x400.

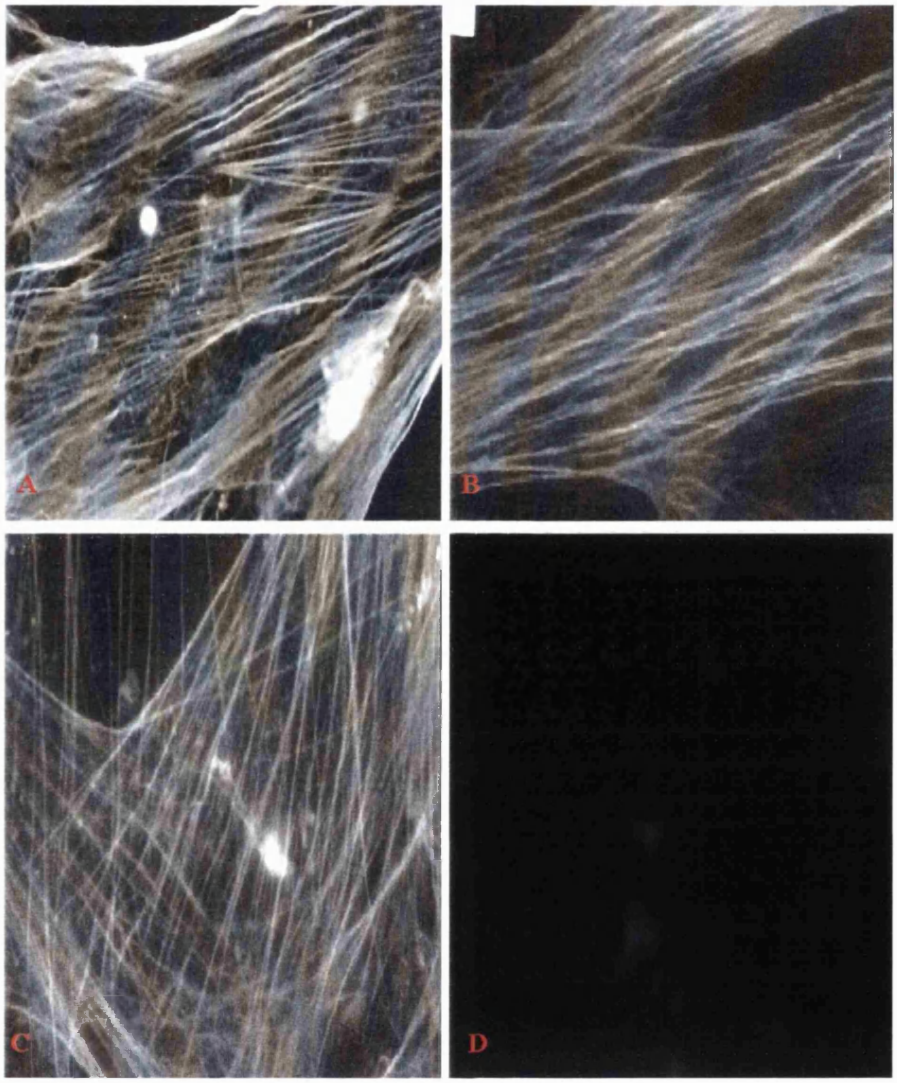


Figure 3.6. The detection of smooth muscle α-actin in cultured smooth muscle cells by immunofluorescence using the monoclonal antibody, anti-α-smooth muscle–1.

Aortic smooth muscle cells at passage 25 (A), explant-migrated smooth muscle cells at passage 3 (B), and enzyme-dispersed smooth muscle cells at passage 3 (C), were seeded into 8-well chamber slides at 2.5 x 10^4 cells/well. 24 hours after the initial seeding, their respective growth media containing 10% foetal calf serum was substituted for growth media containing 1% foetal calf serum, and the cells cultured for a further 24 hours. The cells were then removed, and the presence of smooth muscle α-actin was detected by immunofluorescence staining. As illustrated, all cell types stained positively for α-actin. α-actin staining is seen as long, straight, non-interrupted fibrils, scattered densely throughout the cytoplasm. A representative photomicrograph of the staining pattern obtained with an irrelevant isotype-matched control antibody for explant-migrated smooth muscle cells is shown in D. Magnification x900.

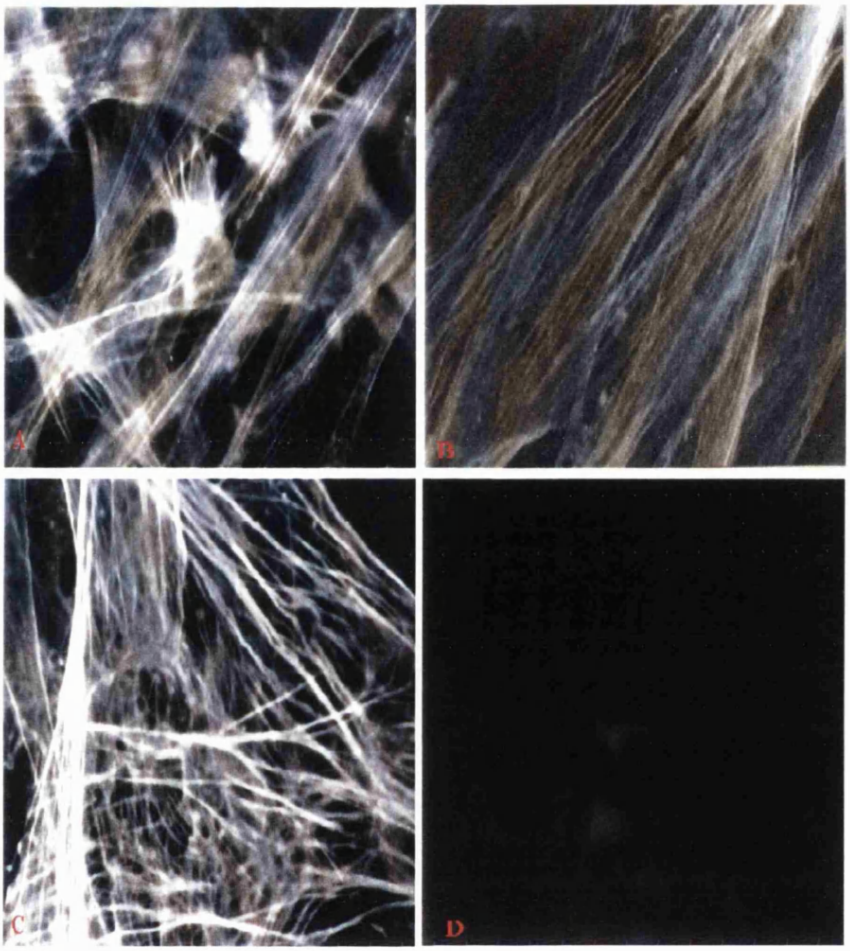


Figure 3.7. The detection of smooth muscle α-actin in cultured fibroblasts by immunofluorescence using the monoclonal antibody, anti-α-smooth muscle–1.

Human embryonic lung fibroblasts at passage 12 (A), MRC-5 fibroblasts at passage 24 (B), and human foreskin fibroblasts at passage 26 (C), were treated in parallel with the smooth muscle cells shown in figure 3.6 for the detection of smooth muscle α-actin by immunofluorescence staining. As shown, a proportion of cells stained positively for α-actin. The α-actin staining pattern shown is similar to that observed on smooth muscle cells in figure 3.6. A representative photomicrograph of the staining pattern obtained with an irrelevant isotype-matched control antibody for human embryonic lung fibroblasts is shown in D. Magnification x900.

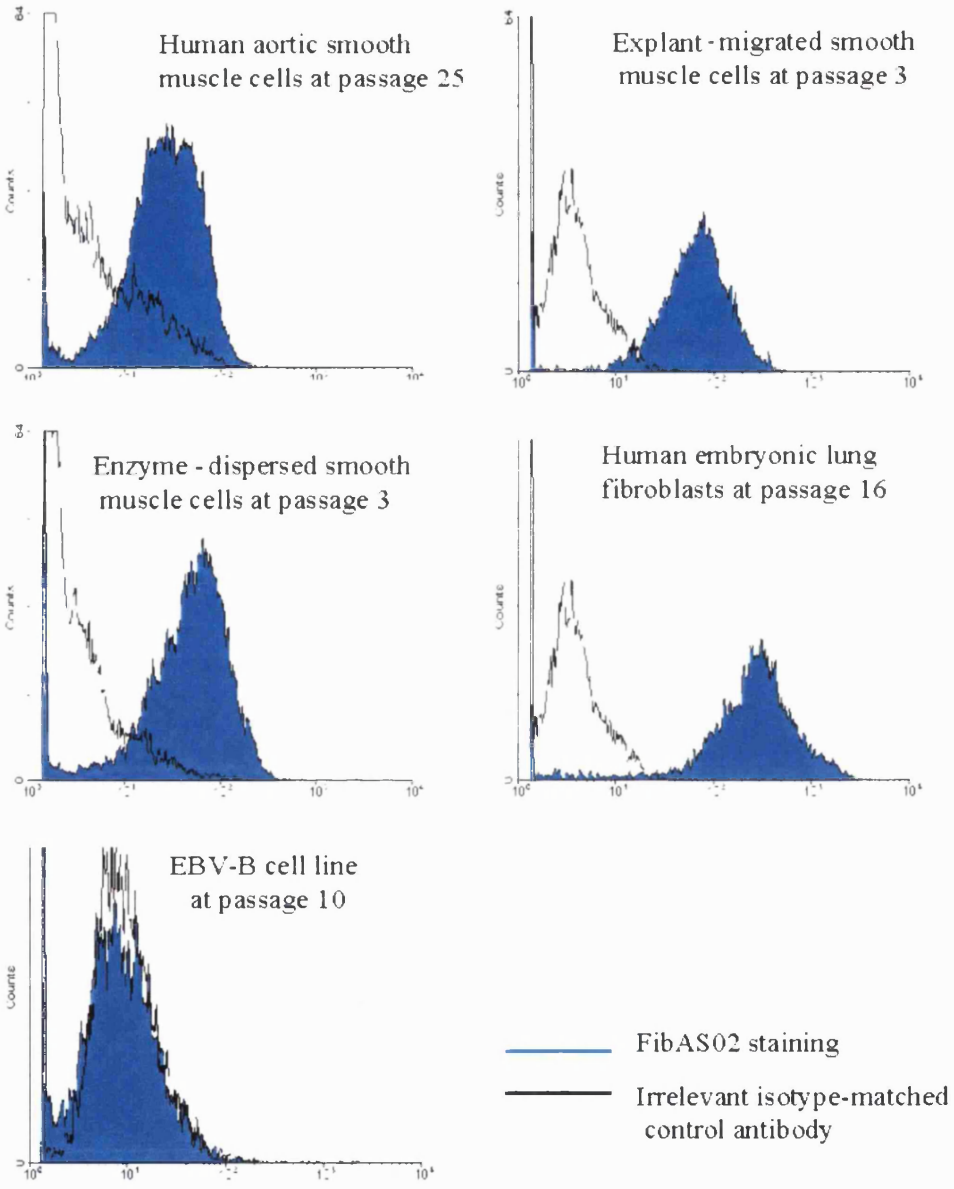


Figure 3.8. Flow cytometric analysis of the binding capacity of a fibroblast-specific monoclonal antibody, FibAS02 to various cell types.

Cultured cells as shown above were seeded into 6–well plates at 3 x 10^5 cells/well. 24 hours after the initial seeding the cells were trypsinized, and analysed by flow cytometry for the presence of a fibroblast specific cell surface marker, using the antibody FibAS02. As shown, nearly 100% of smooth muscle cell cultures and fibroblasts were reactive with FibAS02. The binding capacity of FibAS02 is shown compared to the binding of an irrelevant isotype-matched control antibody. An EBV-transformed B cell line was used as a negative control.

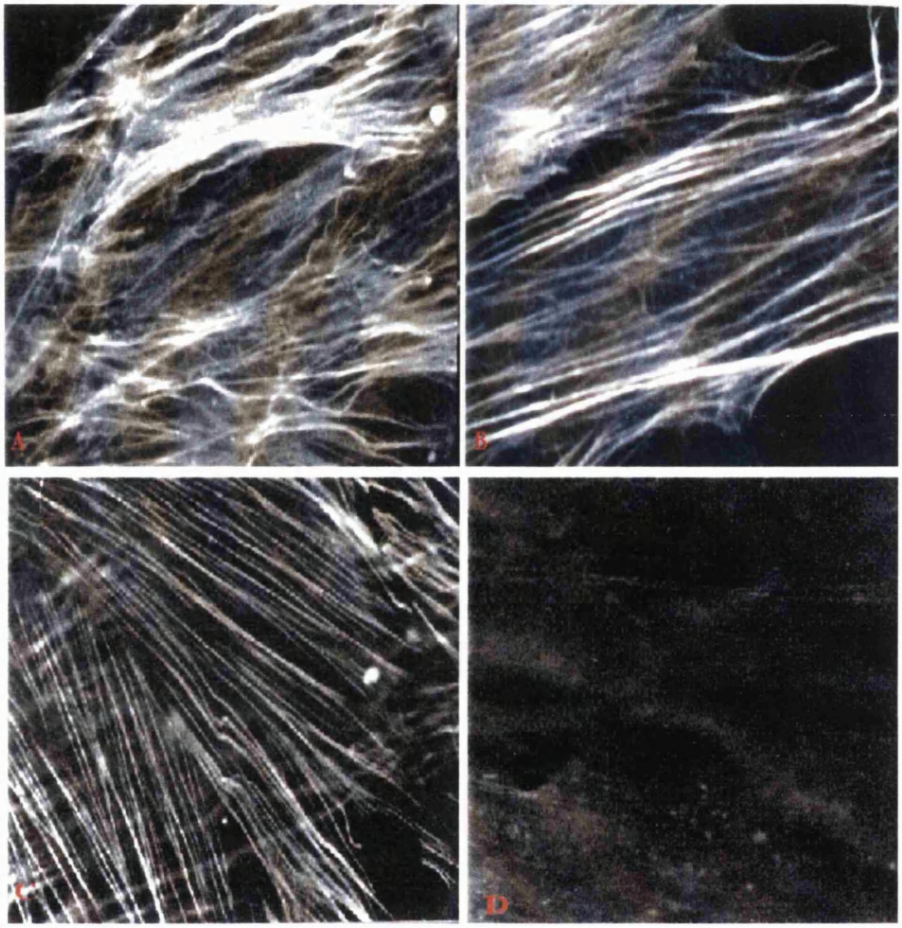


Figure 3.9. The detection of smooth muscle α-actin in cultured smooth muscle cells by immunofluorescence using a monoclonal antibody raised against actin derived from chicken gizzard smooth muscle.

Aortic smooth muscle cells at passage 25 (A), explant-migrated smooth muscle cells at passage 3 (B), and enzyme-dispersed smooth muscle cells at passage 3 (C), were processed as described in figure 3.6, except that they were stained with a monoclonal antibody raised against actin derived from chicken gizzard smooth muscle. As illustrated, all cell types stained positively for α-actin. α-actin staining is depicted by long, straight, non-interrupted fibrils, scattered densely throughout the cytoplasm. A representative photomicrograph of the staining pattern obtained with an irrelevant isotype-matched control antibody for explant-migrated smooth muscle cells is shown in D. Magnification x900.

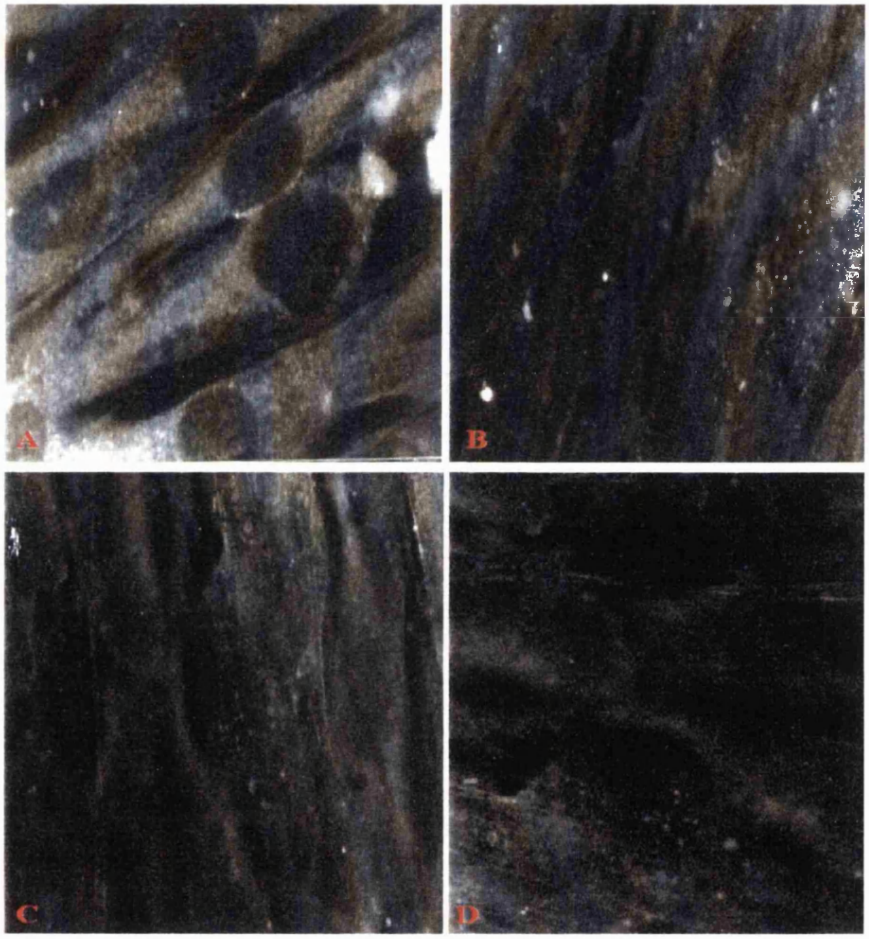


Figure 3.10. The detection of smooth muscle α-actin in fibroblasts by immunofluorescence using a monoclonal antibody raised against native actin derived from chicken gizzard smooth muscle.

Human embryonic lung fibroblasts at passage 12 (A), MRC-5 fibroblasts at passage 24 (B) and human foreskin fibroblasts at passage 26 (C) were processed as described in figure 3.6, except that they were stained with a monoclonal antibody raised against actin derived from chicken gizzard smooth muscle. As illustrated, smooth muscle α-actin staining for all fibroblast cultures was weak and indistinguishable from that obtained with an irrelevant isotype-matched control antibody on human embryonic lung fibroblasts (D). Magnification x900.

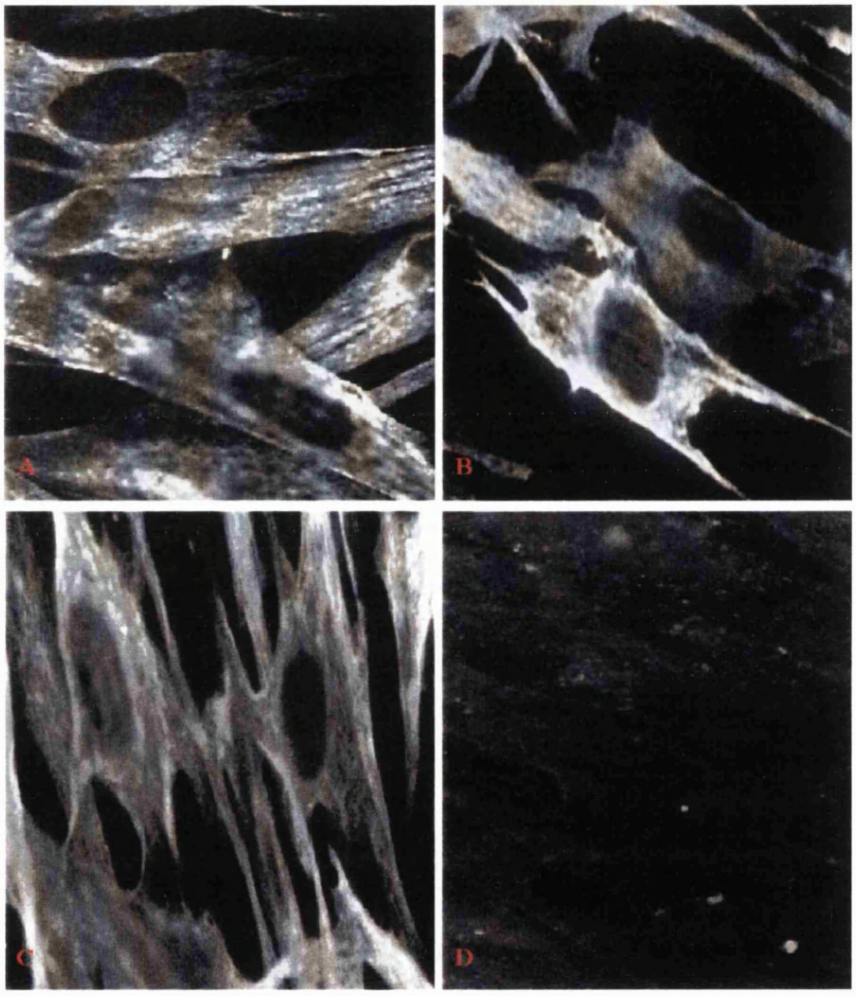


Figure 3.11. The detection of vimentin filaments in cultured smooth muscle cells by immunofluorescence using an anti-vimentin antibody.

Aortic smooth muscle cells at passage 26 (A), explant-migrated smooth muscle cells at passage 3 (B), and enzyme-dispersed smooth muscle cells at passage 3 (C), were seeded into 8-well chamber slides at 2.5 x 10^4 cells/well. 24 hours after the initial seeding, they were removed and the presence of vimentin was detected by immunofluorescence staining. As illustrated, all cell types stained positively for vimentin, revealing a wavy cytoplasmic filamentous system distinct from the appearance of actin fibres. A representative photomicrograph of the staining pattern obtained with an irrelevant isotype-matched control antibody in explant-migrated smooth muscle cells is shown in D. Magnification x900.

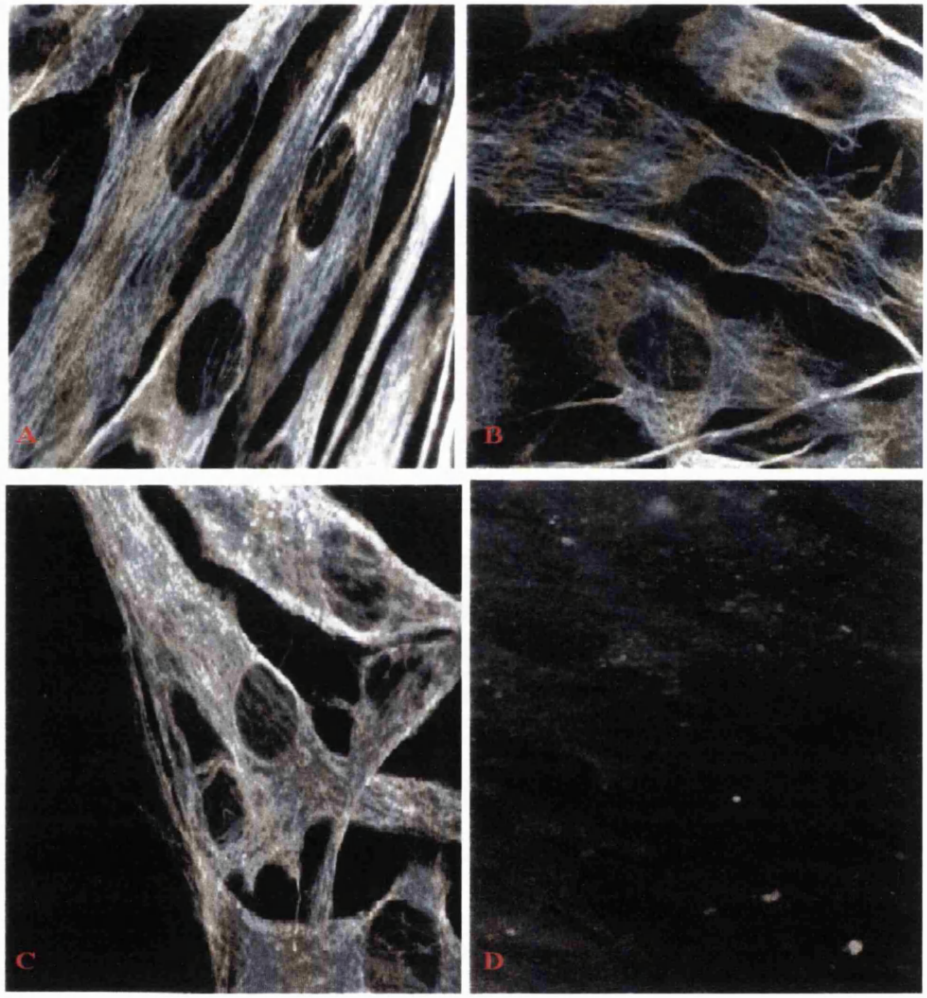


Figure 3.12. The detection of vimentin filaments in cultured fibroblasts by immunofluorescence using an anti-vimentin antibody.

Human embryonic lung fibroblasts at passage 14 (A), MRC-5 fibroblasts at passage 25 (B) and human foreskin fibroblasts at a passage 26 (C), were processed as described in figure 3.11. As illustrated, all cell types stained positively for vimentin showing a wavy cytoplasmic filamentous system distinct from that seen for actin fibres. A representative photomicrograph of the staining pattern obtained with an irrelevant isotype-matched control antibody in human embryonic lung fibroblasts is shown in D. Magnification x900).

3.3 DISCUSSION

In the present study, smooth muscle cells were obtained from primary explants of the saphenous vein and from the vessels of umbilical cords. These two main isolation techniques are generally used to obtain a morphological homogenous population of vascular smooth muscle cells from the blood vessel wall. Both methods of cell isolation were found to be a relatively simple way of obtaining single smooth muscle cells from human tissue sources. Viability, as determined by exclusion of trypan blue, was always greater than 95% in the cells isolated using the two methods. Whereas, 4-6 weeks were required for cells to grow out of explant tissue, enzyme-dispersed smooth muscle cells were ready for use within 3-4 weeks. However, the yield of smooth muscle cells from explant tissue was far greater than that obtained with enzymatic digestion. This is probably due to the fact that the arteries of umbilical cords were not perfused with heparin during the isolation procedure. Perfusion of the arteries of umbilical cords with heparin has been suggested to decrease the arterial resistance, possibly by mediating vasorelaxation. The resulting increase in the luminal surface may facilitate the access of the digestive enzymes to the vessel wall, thereby further increasing the number of smooth muscle cells isolated. Smooth muscle cells isolated by enzyme digestion of the blood vessel wall provide a population of cells which are initially in the contractile phenotype (Campbell et al., 1981). They spontaneously and gradually undergo a change in phenotype from a "contractile" to a "synthetic" phenotype with time in culture. Problems associated with this isolation technique include variability in the yield of viable cells, in part due to the variability in the enzymatic digestion regimen used due to variation in the specific activity of the digestive enzyme. Furthermore, enzyme-dispersed cells are no longer in their normal environment, and may be removed from important influences, such as contact with adjacent cells and with the extracellular matrix. The protein composition of the plasma membrane in these cells may be modified by enzymatic digestion, thus causing abnormal behaviour (McMurray et al., 1991). Smooth muscle cells obtained by explantation, on the other hand, provide a system in which at the initiation of culture, the cells are in the contractile phenotype and the majority of cells are in their normal spatial relationship with one another, and with the surrounding extracellular matrix. As a result, explanted cells provide a potentially good system for the study of differentiation of smooth muscle cells and the complex events associated with the growth of the smooth muscle cells in atherosclerosis.

The characterization of explant-migrated, enzyme-dispersed and aortic smooth muscle cells was initially performed using a monoclonal antibody specific for smooth muscle α-actin and by their characteristic morphology at confluency. The monoclonal antibody, anti-α-smooth muscle -1, clone 1A4, which was raised against the acetylated NH_2-terminal synthetic decapeptide of smooth muscle α-actin, and had been reported to react specifically with smooth muscle α-actin, was used to demonstrate the expression of α-actin stress fibres in the above mentioned smooth muscle cell populations. The results obtained showed that smooth muscle α-actin, as detected using anti-α-smooth muscle-1, was expressed in explant-migrated, enzyme-dispersed and aortic smooth muscle cells, but also in a range of various fibroblast cell cultures originally used in this study as negative controls. This finding is in conflict to several previous reports in which the smooth muscle cell phenotype has been determined by immunofluorescence staining, using anti-α-smooth muscle–1 (Skalli et al., 1986; Leavitt et al., 1985; McMurray et al., 1991). However, the lack of smooth muscle cell specificity using anti-α-smooth muscle–1 is consistent with the findings of Desmouliere et al. (1992), who demonstrated that anti-α-smooth muscle-1 was not specific for smooth muscle cells, but was reactive with various fibroblast cell cultures, clones and subclones. The latter authors concluded that smooth muscle α-actin expression in fibroblast cultures was not due to contaminant cells, but was a feature of fibroblasts themselves. It has been suggested that the immunization of mice with the NH_2-terminal synthetic decapeptide conjugate of smooth muscle α- actin resulted in at least two antibody populations: one specific for smooth muscle α-actin, and the other cross-reacting with the other actin isoforms (Skalli et al., 1986). This suggests that the NH_2-terminal synthetic decapeptide of smooth muscle α-actin bears at least two epitopes; one shared by different actin isoforms, and the other specific for smooth muscle α-actin. Thus, it is possible that the anti-α-smooth muscle-1 antibody reacts with the former and not with the latter. Hence, the monoclonal antibody, anti-α-smooth muscle-1 is not a specific marker for smooth muscle cells in culture, and its reactivity with it fails to offer a reliable indication that a particular cell population is of a smooth muscle cell lineage.

To further characterize the isolated cultures of smooth muscle cells, which had already been demonstrated to express smooth muscle α-actin, but were indistinguishable from fibroblasts in terms of the presence of smooth muscle-specific α-actin fibres, a fibroblast specific monoclonal antibody was employed to detect any contamination with fibroblasts in the smooth muscle cell cultures. It was hoped that this monoclonal antibody

would be non-reactive on the various smooth muscle cell populations, indicating the absence of fibroblasts in these cultures. Using this antibody, it was observed that all smooth muscle cell populations, and indeed human embryonic lung fibroblasts, were reactive with this antibody, but it was not reactive with a B-cell line used in this study to serve as a negative control. Using flow cytometry and immunohistochemistry, Saalbach et al. (1996) demonstrated that the antibody, FibAS02, reacted exclusively with fibroblasts of human origin, and was non-reactive on smooth muscle cells in tissue sections. Unfortunately, FibAS02 was not previously tested on smooth muscle cells in culture. It therefore seems, that the development of phenotypic modulation in cultured smooth muscle cells, probably enabling them to exhibit fibroblast-like features, is a possible reason for the reactivity of FibAS02 on vascular smooth muscle cells in culture.

Subsequently, the expression of α-actin stress fibres was assessed in the explant-migrated, enzyme-dispersed and aortic smooth muscle cells, using a monoclonal antibody raised against actin derived from chicken gizzard smooth muscle (Groschet-Stewart et al., 1976). Chamley et al. (1977) described the use of this monoclonal antibody to stain cultures of guinea-pig vas deferens and taenia coli, rabbit thoracic aorta, rat ventricle and chicken skeletal muscle. They showed that this monoclonal antibody reacted with cardiac cells, skeletal muscle myotubes and smooth muscle cells. Most importantly, smooth muscle cells, which had undergone morphological "differentiation", still stain intensely with this anti-α-actin antibody. In the present study, the results indicated that the determinant recognized by this monoclonal antibody was detected in explant-migrated, enzyme-dispersed and human vascular smooth muscle cells, but not in various fibroblast cell cultures. This observation is consistent with Chamley et al. (1977), and hence this monoclonal antibody served to distinguish smooth muscle cells from fibroblasts. This observation suggests that the anti-α-actin monoclonal antibody raised against actin derived from chicken gizzard smooth muscle is directed against a particular sequence of amino acids in the muscle actin molecule, which is not homologous with the non-muscle actin. Using this antibody, it was possible to conclude that the smooth muscle cells isolated by enzymatically digesting the veins of the umbilical cord, and from the explant tissues of the saphenous vein were of a smooth muscle cell lineage. This conclusion was supported by the fact that the cells exhibited the typical "hill and valley" growth pattern and the spindle-shape appearance characteristic of primary and subcultured smooth muscle cells. In addition, the data obtained from the human aortic smooth muscle cell culture was consistent with that obtained from the isolated smooth muscle cell cultures.

However it is also known that α-actin can be transiently expressed by a variety of mesodermally derived cells during development, tissue repair and neoplastic growth (Sappino et al., 1990). α-actin expression can be induced in a number of non-smooth muscle cell types in culture, including microvascular endothelial cells and myofibroblasts, by treatment with transforming growth factor- β (Kocher et al., 1989; Verbeek et al., 1994; Roelof et al., 1998). Thus, smooth muscle-α-actin expression alone does not provide definite evidence for smooth muscle cell lineage. The use of smooth muscle specific myosin monoclonal antibodies has been suggested to serve as specific markers for smooth muscle cells (Rovner et al., 1986; Babij et al., 1992). It has been demonstrated in sub-confluent and confluent cultured smooth muscle cells that the mRNA of two smooth muscle specific myosin heavy chain isoforms, SM1 (204kDa) and SM2 (200kDa), are decreased by 30-45% and 80%, respectively. In addition, there is little or no effect on SM1 or SM2 myosin heavy chain mRNA levels upon serum reduction. However, a nonmuscle myosin heavy chain is synthesized (Babij et al., 1992). The decrease in smooth muscle specific myosin heavy chain and the synthesis of non-muscle myosin in vascular smooth muscle cells in culture limits the use of monoclonal antibodies against myosin as cell specific markers for cultured vascular smooth muscle cells. Additionally, vascular smooth muscle cells also express a number of specific proteins that are part of the cytoskeleton, and are hypothesized to be involved in the regulation of contraction. These proteins include calponin (Winder et al., 1991), SM-22α (Winder et al., 1991), h-caldesmon (Sobue et al., 1988), γ-vinculin (Geiger et al., 1980), α and β-metavincullin (Geiger et al., 1980). The vast majority of these proteins are found predominantly in smooth muscle cells of the contractile phenotype, but the synthesis of their mRNAs is progressively decreased in culture, and hence are undetectable by standard immunocytochemical and biochemical techniques. They are thus of little use for the characterization and identification of smooth muscle cells in culture (Ueki et al., 1987; Glukhova et al., 1988; Birukov et al., 1991). In an attempt to identify other possible markers for smooth muscle cells in culture, Shanahan et al. (1993) isolated specific markers of both differentiated and proliferating vascular smooth muscle cells by differential screening, using RNA from cultured rat aortic vascular smooth muscle cells. They reported the isolation of a distinct cDNA of a putative membrane channel protein, CHIP28, which was not highly expressed in other smooth muscle tissue and may therefore be a new vascular smooth muscle cell marker. At present, no monoclonal antibody specific for this protein exists. This protein may be a specific marker for cultured vascular smooth muscle cells in the future. In addition, Kramer et al. (1999) recently described a

novel, large isoform of smoothelin, a structural protein of differentiated, contractile smooth muscle cells. The protein, which is highly conserved in mouse and in humans, shows homology with other cytoskeleton-associated smooth muscle cell proteins, and contains an actinin-type actin-binding domain. Northern blot analysis from various mouse organs identified short and long smoothelin mRNA forms, which exhibit tissue-specific expression patterns. The short form is expressed in visceral muscle tissues, while the long mRNA is expressed in all vascularised organs. These results may provide new tools and approaches to study both smooth muscle cell differentiation and proliferation in vascular diseases.

Electron microscopy has shown that vascular smooth muscle cells contain abundant intermediate-sized filaments (Campbell et al., 1971; Campbell et al., 1974). In the present study, vimentin was demonstrated to be present by immunofluorescent staining in explant-migrated, enzyme-dispersed and aortic smooth muscle cells. The abundance of vimentin in vascular smooth muscle cells suggests a close relationship between smooth muscle cells and other mesenchymal cells, such as fibroblasts, that are also characterized by having vimentin as the predominant, if not exclusive, intermediate filament protein (Lazarides, 1980).

. In conclusion, homogenous populations of smooth muscle cells were isolated from the vessels of umbilical cords by enzymatic digestion and from the medial layer of the saphenous vein by explantation. The isolated smooth muscle cell cultures were demonstrated to be of smooth muscle cell lineage by their expression of smooth muscle-specific α-actin and by their characteristic pattern of growth in culture. In the experiments described in chapters 4 and 5, explant-migrated smooth muscle cells were initially used because of the readiness in obtaining vast amount of these cells in culture, and the results obtained were compared with enzyme-dispersed and human aortic vascular smooth muscle cell cultures. This ensured that any possible alterations in smooth muscle cell phenotype and behaviour, due to differences in their anatomical location could be assessed. In addition, since it has been reported that phenotypically modulated smooth muscle cells in culture become senescent after undergoing five cumulative population doublings, explant-migrated and enzyme-dispersed smooth muscle cells were used in experiments at passages 3 and 4. On the other hand, since the aortic smooth muscle cells used have been reported to undergo a total of 30 to 35 passages before the onset of senescence (European Collection of Cell Culture Ref No: 94102702), these cells were used in experiments between passage 24 and 28.

CHAPTER 4:

CMV infection of vascular smooth muscle cells

4.1 INTRODUCTION

There have been very few studies on the replication of CMV in smooth muscle cells *in vitro*. In one study, Tumilowicz et al. (1985) reported that the infection of cultured smooth muscle cells with the high passage CMV strain AD169 was delayed and prolonged, a finding that led to the suggestion that the virus induced latency in these cells. Several observations consistent with this view came from studies in which attempts to isolate CMV from smooth muscle cell biopsies from patients with advanced atherosclerotic complications were unsuccessful (Bruggeman and Van Dam-Mierras, 1991). Hosenpud et al. (1991), on the other hand, suggested that smooth muscle cells were not sites for CMV latency, since they found that smooth muscle cells were permissive for CMV infection with the high passage CMV strain AD169. Hence, the level at which CMV strain AD169 is able to replicate in smooth muscle cells remains unclear. Furthermore, it is known that the high passage CMV strain AD169 replicates poorly in some cell types, such as endothelial cells and epithelial cells (Friedman et al., 1981; Smiley et al., 1988) whilst these cells can be relatively permissive to infection with certain low passage strains of CMV. Thus, the question arises as to whether smooth muscle cells are permissive for CMV replication, and if so, whether they are more permissive to the low passage strains of CMV than the high passage CMV strain AD169.

In order to investigate the potential pathogenic role of CMV infection of smooth muscle cells in the development of atherosclerosis, the replication of CMV in cultured smooth muscle cells was studied. Generally, when CMV infects a permissive cell, three broad classes of CMV genes are progressively transcribed. These three phases of CMV gene expression have been operationally defined in human fibroblasts, and have been termed α (IE), β (early) and γ (late) genes (Honess & Roizman, 1974). The progression of CMV infection and replication in a permissive cell is normally determined by the detection of the expression of the corresponding IE, early or late antigens in the infected cell. The present study was divided into three parts. In the first part of the study, the initiation of CMV infection of smooth muscle cells was investigated by monitoring the expression of IE antigen by flow cytometry, using a monoclonal antibody specific for IE antigen at 24 hours post-infection. Flow cytometric analysis of CMV-infected smooth muscle cells allowed for a rapid and efficient way of quantitating the percentage of cells expressing the CMV-specific IE antigen. The synthesis and subsequent detection of IE antigen in infected smooth muscle cells would indicate the initiation of the sequence of events leading to a properly programmed cycle of CMV replication. In the second part of this study, the investigation proceeded to determine whether a full cycle of CMV

replication could be achieved in smooth muscle cells, or was limited to the expression of IE antigen and thus an abortive infection in these cells. Immunofluorescence staining was carried out on smooth muscle cells infected with CMV to detect and monitor the expression of IE, early and late antigens. Flow cytometry was not used to detect the expression of the early and late CMV antigen because of the lack of suitable antibodies that could detect these antigens by this technique. The demonstration that CMV infection of smooth muscle cells leads to the synthesis of IE, early and late antigens would be suggestive of the fact that a full cycle of replication could be achieved in this cell type. Furthermore, as CMV infection of fibroblasts is known to induce characteristic cytopathic effects with alterations to the cell morphology of the infected cell (Sinzger et al., 1993b; Sinzger et al., 1995), observations were made using a light microscope to investigate the possible development of these cytopathic effects in CMV-infected smooth muscle cells.

In the final part of this study, the production of infectious virus progeny in the supernatant of CMV-infected smooth muscle cells was investigated using a plaque assay. This served to determine whether CMV infection of smooth muscle cells led to the release of the virus from the cell into the surrounding medium.

In the previous chapter, it was discussed that vascular smooth muscle cells *in vivo* exhibit a range of phenotypic differences in their magnitude of expression of smooth muscle specific proteins and thus, reflect differences in their interactions to certain stimuli such as viral infection (Halayko et al., 1997). Hence, the above studies were initially carried out on explant-migrated smooth muscle cells, and then performed on smooth muscle cells isolated by enzymatic digestion from the vessels of the umbilical cord, and on aortic smooth muscle cells. This allowed for the identification of possible differences in infection by CMV between the various smooth muscle cell cultures. For comparative purposes, human embryonic lung fibroblasts, which are fully permissive to CMV infection, were studied in parallel. All studies were performed using both the AD169 and C1F strains of CMV. This would allow possible differences in the susceptibility of smooth muscle cells to CMV infection between the two virus strains to be identified.

4.2 RESULTS

4.2.1 The determination of the percentage of CMV infection of cells by flow cytometry

Previous work in this laboratory had suggested that the percentage of cells expressing IE antigen, and hence susceptible to CMV infection, could be quantitated by flow cytometry. This is because the percentage of cells positive for IE antigen correlated with varying dilutions of the viral inoculum used to infect the cells, whilst the virus dose used had no effect on the mean fluorescence intensity of antigen expression obtained. To illustrate this point in the present study, human embryonic lung fibroblasts were seeded into 6-well plates and infected with varying multiplicities of infection of the high passage CMV strain AD169. At 24 hours post-infection, the cells were stained with the monoclonal antibody, E13, and subsequently analysed by flow cytometry. The data obtained was processed using the consort 30 and the lysis II software programmes. After the exclusion of cell debris and dead cells by the application of viable cell gates, two distinct peaks, representing cells positive and negative for IE antigen expression, were seen (Figure 4.1). The percentage of cells was determined by placing markers around the IE antigen positive peak using the lysis II software programme, from which the percentage of cells expressing IE antigen was deduced. Figure 4.1 shows the flow cytometric profile obtained for fibroblasts infected with varying multiplicities of infection of CMV strain AD169. It could be seen that, regardless of the viral multiplicity of infection, the mean fluorescence intensity of IE antigen expression did not change. On the other hand, the percentage of cells positive for IE antigen expression increased with increasing multiplicity of infection of the virus used to infect the cells. Thus, this method of assessing the percentage of cells infected with CMV was used to study the susceptibility of smooth muscle cells to CMV.

4.2.2 A comparison of the initiation of CMV infection in explant-migrated smooth muscle cells and fibroblasts.

In order to compare the initiation of CMV infection between smooth muscle cells and fibroblasts, the percentage of cells expressing IE antigen was determined by flow cytometry, 24 hours after the infection of these cells with the high passage CMV strain AD169. A range of viral doses was used in order to allow for the possibility that different

amounts of virus might be required to initiate infection in the two cell types. Subconfluent monolayers of explant-migrated smooth muscle cells at passage 3 and human embryonic lung fibroblasts at passage 15 were infected with the high passage CMV strain AD169 over a range of multiplicities of infection from 0.3-10. In each experiment, the different cell types were infected under identical conditions, and analysed in parallel. The percentage of cells infected with CMV strain AD169 was determined by placing markers around the IE antigen positive peak as described above. The flow cytometric profiles of a representative experiment of the expression of IE antigen in explant-migrated smooth muscle cells and human embryonic lung fibroblasts at 24 hours post-infection with the CMV strain AD169, are shown in figures 4.2 and 4.3, respectively. The analysis of explant-migrated smooth muscle cells infected with the high passage CMV strain AD169 revealed that a significant proportion of smooth muscle cells expressed the IE antigen. The results showed that, in comparison to human embryonic lung fibroblasts, slightly fewer explant-migrated smooth muscle cells expressed IE antigens following infection with CMV strain AD169. For example, at a multiplicity of infection of 10, 82% of explant-migrated smooth muscle cells were positive for IE antigens as compared to 90% of fibroblasts infected with the same multiplicity of infection of CMV strain AD169. This profile of IE antigen expression in fibroblasts versus explant-migrated smooth muscle cells was maintained over the range of multiplicities of infection examined, with slightly more infected fibroblasts expressing IE antigen than explant-migrated smooth muscle cells. For both cell types, the higher the input virus, the greater the number of cells expressing IE antigens. The Mann-Whitney t-test was used to determine the significance of the differences in the percentage of cells expressing IE antigen at each multiplicity of infection between CMV-infected smooth muscle cells and fibroblasts. This statistical analysis revealed that there was no significant difference in the percentage of cells expressing IE antigen between the two cell types over the range of multiplicity of infection examined using the data from three separate experiments ($P > 0.05$). The mean fluorescence intensity of IE expression between the two cell types was always of equal intensity between the two cell types, regardless of the virus multiplicity of infection. These results indicated that CMV infection of explant-migrated smooth muscle cells with the high passage CMV strain AD169 led to the initiation of CMV replication in these cells as deduced by the expression of CMV-specific IE antigen. The percentage of cells expressing IE antigen was slightly less than those obtained in fibroblasts infected with an equivalent amount of virus, but this difference was not statistically significant.

A comparison of the initiation of CMV infection between the explant-migrated smooth muscle cells and fibroblasts was also investigated using the low passage CMV strain C1F. These cells were seeded into 12-well plates, and at 24 hours after the initial seeding were infected with CMV strain C1F over a range of multiplicities of infection from 0.3-10. At 24 hours post-infection, the cells were harvested, stained with the monoclonal antibody E13, and subsequently analysed by flow cytometry. The percentage of cells expressing IE antigen was determined as previously described. The flow cytometric profiles of a representative experiment of the expression of IE antigen in explant-migrated smooth muscle cells and human embryonic lung fibroblasts at 24 hours post-infection with the low passage CMV strain C1F, are shown in figures 4.4 and 4.5, respectively. The results obtained suggested that infection of explant-migrated smooth muscle cells with the low-passage CMV strain C1F induced the expression of IE antigens in these cells, initiating the cycle of CMV replication. As indicated in Table 4.1, the percentage of cells expressing IE antigen were of equivalent proportions for both cell types. For example, at a multiplicity of infection of 10, the mean percentage of cells expressing IE antigen in explant-migrated smooth muscle cells and fibroblasts were 84% and 83%, respectively. There was no significant difference in the percentage of cells expressing IE antigen at each multiplicity of infection between explant-migrated smooth muscle cells and fibroblasts over the range of multiplicity of infection examined in three separate experiments, as determined using the Mann-Whitney t-test ($P > 0.05$) for each multiplicity of infection. As seen previously with CMV strain AD169, the mean fluorescence intensity of IE expression between the two cell types was always of equivalent intensity, regardless of the virus multiplicity of infection. Figure 4.6 shows a comparison of the percentage of cells expressing IE antigen, as determined in three separate experiments, in explant-migrated smooth muscle cells and fibroblasts infected with CMV strain AD169 and strain C1F.

In conclusion, the data obtained revealed that CMV infection of smooth muscle cells led to the expression of IE antigen, initiating CMV replication in these cells. The percentage of cells expressing IE antigen was always slightly higher in fibroblasts than in explant-migrated smooth muscle cells infected with an equivalent amount of the high passage CMV strain AD169. On the other hand, infection of cells with the low passage CMV strain C1F, resulted in similar proportions of cells expressing IE antigen in both cell types over the range of multiplicities of infection examined. However, both virus strains were clearly able to initiate CMV infection in smooth muscle cells to the stage of IE antigen expression.

4.2.3 The study of the initiation of CMV infection in smooth muscle cells from different sources.

In order to see whether the ability of CMV to initiate infection in explant-migrated smooth muscle cells was representative of smooth muscle cells from other vascular sources, the infection of smooth muscle cells isolated from the vessels of the umbilical cords and the aorta was studied. Enzyme-dispersed and aortic smooth muscle cells, at passage 3 and 24 respectively, were seeded into 12-well plates, and 24 hours after the initial seeding were infected with CMV strains AD169 or C1F over a similar range of multiplicities of infection as described above for explant-migrated smooth muscle cells. They were then stained for the CMV IE antigen, and subsequently analysed by flow cytometry. Human embryonic lung fibroblasts were analysed in parallel for comparative purposes.

Figure 4.7 shows the effect of varying the multiplicity of infection of the CMV strain AD169 and strain C1F on the percentage of cells expressing IE antigen using data from three separate experiments in enzyme-dispersed smooth muscle cells and fibroblasts. The results obtained indicated that the high passage CMV strain AD169 could initiate CMV infection in enzyme-dispersed smooth muscle cells. In comparison to fibroblasts infected with the high passage CMV strain AD169, it was observed that the percentage of cells expressing IE antigen was slightly higher than those obtained in enzyme-dispersed smooth muscle cells, infected with an equal multiplicity of infection of the virus. When enzyme-dispersed smooth muscle cells were infected with the low passage CMV strain C1F, it was observed over the course of three experiments, that the percentage of cells expressing IE antigen were of equal proportions to fibroblasts infected in parallel. The Mann-Whitney t-test was used to determine the significance of the differences in the percentage of cells expressing IE antigen between enzyme-dispersed smooth muscle cells and fibroblasts infected with the CMV strain AD169 and strain C1F. At each multiplicity of infection examined, this statistical analysis revealed that there was no significance between the percentage of cells expressing IE antigen between enzyme-dispersed smooth muscle cells and fibroblasts infected with CMV strain AD169 ($P > 0.05$). Similarly, there was no significance difference in the percentage of cells expressing IE antigen between both cell types infected with CMV strain C1F ($P > 0.05$).

Figure 4.8 shows the effect of varying the multiplicity of infection of CMV strain AD169 and strain C1F on the percentage of cells expressing IE antigen using data in aortic

smooth muscle cells and fibroblasts from three separate experiments. The results obtained indicated that the high passage CMV strain AD169 could initiate CMV infection in aortic smooth muscle cells. In comparison to fibroblasts infected with the high passage CMV strain AD169, it was observed that the percentage of cells expressing IE antigen was slightly less than those obtained in aortic smooth muscle cells, infected with an equal multiplicity of infection of the virus. When aortic smooth muscle cells were infected with the low passage CMV strain C1F, it was observed in three separate experiments, that the percentage of cells expressing IE antigen was of an equal proportion to that of fibroblasts infected in parallel. The Mann-Whitney t-test was used to determine the significance of the differences in the percentage of cells expressing IE antigen between aortic smooth muscle cells and fibroblasts infected with the CMV strain AD169 and strain C1F. At each multiplicity of infection examined, this statistical analysis revealed that there was no significant difference in the percentage of cells expressing IE antigen between aortic smooth muscle cells and fibroblasts infected with CMV strain AD169 ($P > 0.05$). Similarly, statistical analysis revealed that there was no significance difference in the percentage of cells expressing IE antigen between both cell types infected with CMV strain C1F ($P > 0.05$).

When the percentage of cells expressing IE antigen in CMV-infected enzyme-dispersed and aortic smooth muscle cells at each multiplicity of infection were compared, it was observed that there was no significant difference in the percentage of cells expressing IE antigen between the two cell types, as determined using the Mann-Whitney t-test ($P > 0.05$), at each multiplicity of infection examined. Thus, the level of IE expression between the two cell types was equivalent, regardless of the virus multiplicity of infection and the strain of virus used to infect the cells.

These results suggested that CMV could initiate infection in smooth muscle cells isolated from the veins of humans. It appeared that CMV strain AD169 was slightly, but not significantly, more efficient in initiating infection in fibroblasts than in any of the three anatomical sources of smooth muscle cells, whereas equivalent proportions of cells expressed IE antigen in all smooth muscle cell cultures and fibroblasts infected with the low passage CMV strain C1F.

4.2.4 The analysis of IE, early and late antigen expression following CMV infection of explant-migrated smooth muscle cells.

The above results suggested that CMV could enter smooth muscle cells and initiate a cycle of replication in these cells with an efficiency comparable to that achieved in fibroblasts. Therefore, the next question to be addressed was whether the virus could continue with a full cycle of replication in smooth muscle cells, and if so, whether this occurred in a similar time span to that observed in fibroblasts. The sequential synthesis of IE, early and late antigens, which are indicative of a complete replication cycle of CMV, was investigated by immunofluorescence, at various times post-infection following the infection of explant-migrated smooth muscle cells with the high passage CMV strain AD169. Fibroblasts were studied in parallel for comparative purposes. Explant-migrated smooth muscle cells and human embryonic lung fibroblasts were seeded into 8-well chamber slides and infected with CMV strain AD169 at a multiplicity of infection of 5. At 24, 72 and 120 hours post-infection, cells were fixed and examined by immunofluorescence for the presence of the p72 antigen, the lower matrix phosphoprotein pp65, an early antigen, and the major envelope glycoprotein B, a late antigen. The CMV-specific monoclonal antibodies used were as follows: the antibody 63.27 for the p72 antigen, the C14 antibody for the pp65 early antigen and the antibody 7-17 for the late gB antigen.

Immunofluorescence staining using the above monoclonal antibodies to detect IE, early and late antigens of CMV, revealed that all three temporal classes of CMV antigen were expressed in infected explant-migrated smooth muscle cell monolayers. At 24 hours post-infection of explant-migrated smooth muscle cells and fibroblasts, both the IE and early antigens were clearly detectable in the nuclei of virtually all cells (Figures 4.9 and 4.10, respectively). The late antigen, glycoprotein B, was not detectable in either cell type at this time point (Figure 4.11). At 72 hours post-infection, IE antigen expression continued to be detected in the nuclei of both cell types (Figure 4.12). The staining for the early antigen, pp65, exhibited cytoplasmic fluorescence, in addition to nuclear fluorescence, at this time point. The expression of glycoprotein B was also observed at 72 hours post-infection, and the antigen was found to be located at the perinuclear region of the cell in both smooth muscle cells and fibroblasts (Figures 4.13 and 4.14, respectively). The distribution of the IE antigen was predominantly nuclear at all time points in both cell types, however the level of expression appeared to be reduced at 120 hours post-infection (Figure 4.15). At this time point, pp65 cytoplasmic fluorescence began to predominate over nuclei fluorescence (Figure 4.16), while the late glycoprotein B continued to be detectable at the perinuclear region of the infected cell in both cell types (Figure 4.17). There was

no immunofluorescence staining observed on uninfected cells using any of the above CMV-specific antibodies at all time points examined. Thus, the infection of explant-migrated smooth muscle cells and fibroblasts infected with the high passage CMV strain AD169 was observed to lead to the ordered expression of IE, early and late CMV antigens in these cells. There was no discernable difference in the kinetics and ordered expression of these antigens between the two cell types.

Similarly, the synthesis of IE, early and late antigens was investigated in explant-migrated smooth muscle cells infected with the low passage CMV strain C1F. As described above, explant-migrated smooth muscle cells were seeded into chamber slides, and then infected with strain C1F at a multiplicity of infection of 5. The cells were examined for the expression of IE, early and late CMV antigens by immunofluorescence at 24, 72 and 120 hours post-infection. As observed in explant-migrated smooth muscle cells infected with the high passage CMV strain AD169, immunofluorescence staining revealed that all three classes of CMV viral antigens were expressed in explant-migrated smooth muscle cells infected with the low passage CMV strain C1F. There was no difference in the observed pattern of expression of these antigens between cells infected with CMV strain AD169 and strain C1F (data not shown). To illustrate that a complete cycle of replication was achieved in explant-migrated smooth muscle cells infected with the low passage CMV strain C1F, Figure 4.18 shows a photomicrograph of the expression of the CMV late glycoprotein B antigen at 72 hours post-infection in these cells. The pattern of expression of IE, early and late antigens were similar to those observed in fibroblasts treated in parallel (data not shown).

In conclusion, immunofluorescence staining revealed that the infection of explant-migrated smooth muscle cells with high (AD169) and low (C1F) passage CMV strains led to the sequential expression of IE, early and late CMV viral antigens. There was no difference in the number of infected cells expressing these antigens between virus strains, or between explant-migrated smooth muscle cells and fibroblasts treated in parallel.

4.2.5 The analysis of the expression of IE, early and late antigens following CMV infection in smooth muscle cells from different sources.

The question of whether CMV could similarly induce the sequential expression of IE, early and late antigens in enzyme-dispersed and aortic smooth muscle cells was investigated. These cells were seeded into 8-well chamber slides and infected at a

multiplicity of infection of 5 with a high (AD169) or low (C1F) passage strain of CMV. The sequential expression of the respective CMV-encoded antigens was studied by immunofluorescence staining at 24, 72 and 120 hours post-infection.

Immunofluorescence staining using the monoclonal antibodies directed against the IE, early and late antigens of CMV, revealed that all three temporal classes of CMV antigen were expressed in infected enzyme-dispersed and aortic smooth muscle cells. The sequential expression of these antigens was similar to that observed in explant-migrated smooth muscle cells and fibroblasts described above (data not shown). To illustrate the expression of CMV IE antigen, Figure 4.19 shows a photomicrograph of the expression of IE antigen in enzyme-dispersed and aortic smooth muscle cells, following their infection with CMV strain AD169. These results are consistent with the data described above, where the synthesis of IE antigen was detected in these cells by flow cytometry, indicating CMV infection has been initiated in these cells. To illustrate the expression of the late antigen, Figure 4.20 shows the expression of glycoprotein B in enzyme-dispersed and aortic smooth muscle cells, following their infection with CMV strain AD169. Similar results were obtained in both cell types when infected with the low passage CMV strain C1F (data not shown).

These observations suggested that a complete replication cycle, including the synthesis of structural viral proteins of CMV, was achieved in smooth muscle cells isolated from the veins of humans in a similar manner to that seen in fibroblasts.

4.2.6 A comparison of the effect of CMV infection on the cell morphology of smooth muscle cells and fibroblasts

The development of the characteristic cytopathic effects of CMV infection has been carefully studied in cultured fibroblasts. The question of whether similar morphological alterations are observed following the infection of smooth muscle cells with CMV was studied by phase microscopy. Explant-migrated smooth muscle cells at passage 3 were seeded into 8-well chamber slides, and infected 24 hours after the initial seeding with CMV strain AD169 or strain C1F at a multiplicity of infection of 5. The cells were then examined daily over a 7-day period, to examine any alterations in cell morphology compared to mock-infected cell cultures. Figure 4.21 illustrates the ability of CMV strain AD169 to induce morphological alterations in explant-migrated smooth muscle cells. The cytopathic effects were observed as early as 24 hours post-infection in these cells. Cytopathic effects included the swelling and rounding of infected cells.

Morphologically altered cells appeared throughout the cell monolayer, although infected cells were also observed to form clusters at this time point. The observed cytopathic effects increased with time post-infection. Peak cytopathic effects were observed at approximately 96 and 120 hours post-infection. At 120 hours post-infection, the nucleus was observed to adopt a kidney-shaped morphology, and become indented (Figure 4.21). Nuclear and cytoplasmic inclusions were also seen at this time point. The infected cultures progressed to the complete destruction of the cell monolayer by 168 hours post-infection. There were no noticeable alterations in cell morphology in mock-infected cell cultures.

The features of cytopathic effect observed in CMV-infected smooth muscle cells were similar to those observed in fibroblasts, both in terms of morphological alterations, and also in the time taken for these features to develop following CMV infection. There was no difference in the occurrence and features of the observed cytopathic effects between explant-migrated smooth muscle cells infected with the low passage CMV strain C1F and CMV strain AD169. Furthermore, analysis of the CMV induced cytopathic effects for enzyme-dispersed and human aortic smooth muscle cells infected with CMV revealed that they were similar to those observed with explant-migrated smooth muscle cells and fibroblasts. The features of cytopathic effect in enzyme-dispersed and aortic smooth muscle cells were similar following infection with either the CMV strain AD169 or the low passage strain C1F.

Hence, the morphological alterations characteristic of CMV-infected fibroblasts are induced in smooth muscle cells isolated from various anatomical sources, and occur in a similar time span similar to that observed in fibroblasts.

4.2.7 A comparison of the production of infectious virus progeny following infection of smooth muscle cells and human embryonic lung fibroblasts with CMV strain AD169 and C1F.

In section 4.2.5, it was demonstrated that CMV infection of smooth muscle cells led to the expression of the late CMV structural protein, glycoprotein B. The expression of this late antigen, and the development of cytopathic effects described above, suggested that a full cycle of CMV replication had been initiated in smooth muscle cells, but did not prove that infectious virus progeny was released from the cells into the surrounding medium. Hence, in order to investigate the possibility of an incomplete cycle of CMV replication in smooth muscle cells, and to assess the ability of these cells to produce

extracellular virus, the production and release of infectious virus progeny into the supernatant of CMV-infected smooth muscle cells was measured. Monolayers of explant-migrated smooth muscle cells, and for comparison, human embryonic lung fibroblasts, were infected with CMV strain AD169 or strain C1F at an input multiplicity of infection of 5. After an hour of virus adsorption, the cells were washed, the media replaced with the appropriate maintenance medium containing 4% foetal calf serum, and incubated at 37°C. The supernatant fluid from the infected cell cultures was removed at various intervals post-infection, and the relative amounts of infectious virus particles in these supernatants fluids was determined by titration in a plaque assay using fibroblasts. Three separate experiments using explant-migrated smooth muscle cells from three different veins were performed. Prior to infection, the various cell monolayers were of equal density, and were inoculated at the same multiplicity of infection of virus. Figure 4.22 shows the development of infectious virus in the supernatant fluid of CMV-infected explant-migrated smooth muscle cells and human embryonic lung fibroblasts. The data showed that CMV-infected smooth muscle cells produced infectious virus progeny.

Following infection with equivalent amounts of the high passage CMV strain AD169, the production and release of infectious virus into supernatant fluids occurred slightly more rapidly from human embryonic lung fibroblasts than from explant-migrated smooth muscle cells. In both cell types, the synthesis of infectious virus began to increase at day 3 post-infection, and reached a titre of approximately 10^7pfu/ml at day 4 post-infection. These observations correlated with the results obtained from immunofluorescence studies, in which it was demonstrated that the late CMV structural protein was synthesized and expressed in infected smooth muscle cell cultures beyond day 3 post-infection. A rapid decline in virus titre was seen beyond day 4 post-infection. When the Wilcoxon non-parametric test was used to compare the data from the paired groups from three separate experiments at the different time points, it was computed that there was no significant difference in the titre of extracellular virus released into the supernatant fluids between the two cell types ($P>0.05$).

When the low passage strain of CMV C1F was used to infect explant-migrated smooth muscle cells and human embryonic lung fibroblast cultures, the production of extracellular virus was observed to be of equivalent amounts between the two cell types. There was no significant difference in the virus titre released into the supernatant fluids of these infected cultures in three separate experiments when infectious virus titres were examined with the Wilcoxon non-parametric test at each time point ($P> 0.05$). A

comparison of the titre of virus released into the supernatant fluids of AD169-infected explant-migrated smooth muscle cells and C1F-infected explant-migrated smooth muscle cells, revealed that there was no significant difference in the titre of virus released between the two strains of virus at any time point ($P > 0.05$). However, it was observed that AD169-infected human embryonic lung fibroblasts released significantly more virus into the supernatant fluids when compared to C1F-infected human embryonic lung fibroblasts at each time point ($P < 0.05$).

These studies indicated that infection of smooth muscle cells with the high passage CMV strain AD169 and the low passage CMV strain C1F could lead to the production and release of infectious virus progeny. The CMV replication cycle in smooth muscle cells was fairly similar in kinetics when compared to the time course of CMV replication in human embryonic lung fibroblasts, and these finding demonstrated that smooth muscle cells can support productive CMV replication in a manner very similar to that of fibroblasts.

Table 4.1. The initiation of CMV infection with high (AD169) and low (C1F) passage strains of CMV in explant-migrated smooth muscle cells and human embryonic lung fibroblasts[1].

	Percentage of cells positive for IE antigen			
Cell type	Explant-migrated smooth muscle cells		Human embryonic lung fibroblasts	
CMV Strain	AD169	CIF	AD169	CIF
MOI				
10.0	82	84	90	83
0.50	80	77	88	80
0.25	72	69	81	71
0.125	58	55	65	57
0.0625	35	35	39	37
0.03	22	18	25	21

[1]Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 12-well plates, and 24 hours after the initial seeding were infected with varying multiplicities of infection (MOI) of CMV strains AD169 or C1F. They were then analysed at 24 hours post-infection for IE antigen expression by flow cytometry using the monoclonal antibody, E-13. The data shown is the percentage of cells expressing IE antigen as determined using the Lysis II software programme, and represents the mean derived from three separate experiments.

Table 4.2. The initiation of CMV infection with high (AD169) and low (C1F) passage strains of CMV in enzyme-dispersed smooth muscle cells, human aortic vascular smooth muscle cells and human embryonic lung fibroblasts[1].

	Percentage of cells positive for IE antigen					
Cell type	Enzyme –dispersed smooth muscle cells		Human aortic vascular smooth muscle cells		Human embryonic lung fibroblasts	
CMV Strain	AD169	C1F	AD169	C1F	AD169	C1F
MOI						
10.0	79	67	79	64	82	66
0.50	73	63	76	60	75	61
0.25	51	53	73	53	53	52
0.125	40	39	61	40	48	40
0.0625	27	26	47	21	34	27
0.03	14	13	35	9	22	14

[1]Enzyme-dispersed smooth muscle cells at passage 3, and human aortic vascular smooth muscle cells at passage 24, were infected as previously described for explant-migrated smooth muscle cells in Table 4.1. They were then analysed at 24 hours post-infection for IE antigen expression by flow cytometry using the monoclonal antibody E-13. Human embryonic lung fibroblasts at passage 5 were treated in parallel for comparative purposes. The data shown is the percentage of cells expressing IE antigen as determined using the Lysis II software programme, and represents the mean derived from three separate experiments.

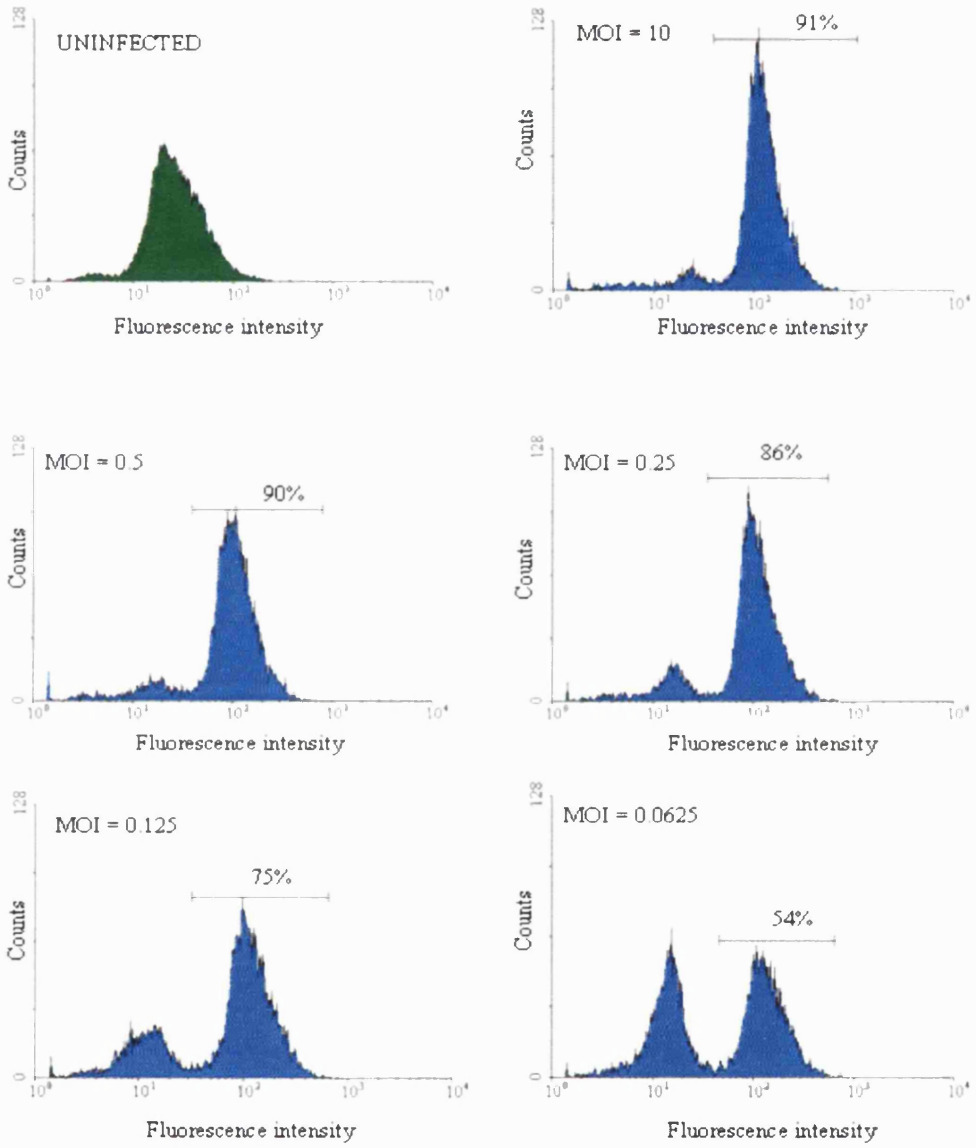


Figure 4.1. The effect of varying the multiplicity of infection on the expression of IE antigen in CMV-infected fibroblasts.

Human embryonic lung fibroblasts at passage 15 were seeded into 6-well plates, and 24 hours after the initial seeding were infected with varying multiplicities of infection of the high passage CMV strain AD169 (blue) or left uninfected (green). They were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. After the exclusion of cell debris and dead cells using viable cell gates, two distinct peaks representing cells positive (right) and negative (left) for IE antigen were obtained. The percentage of cells positive for IE antigen expression is indicated on each histogram.

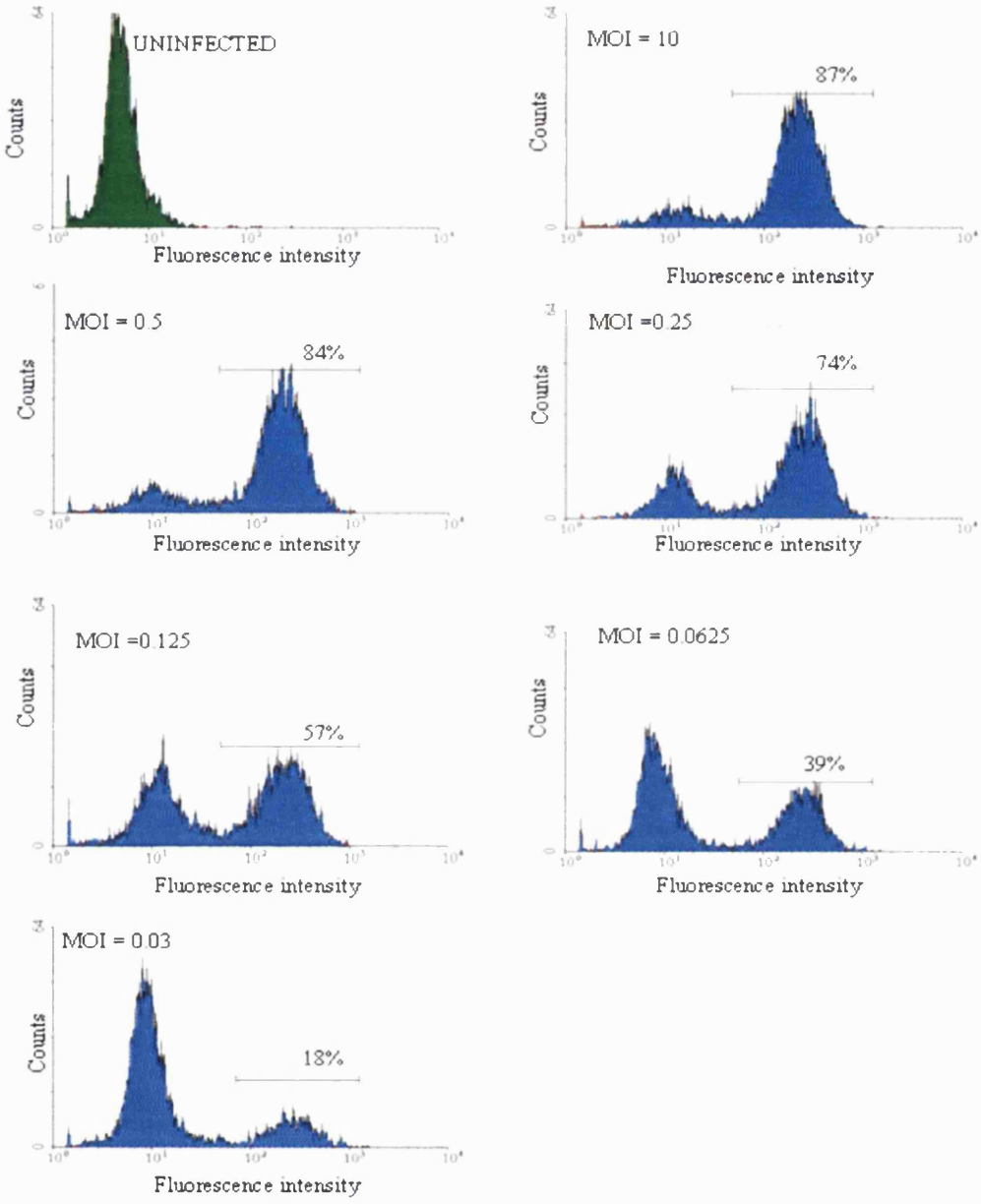


Figure 4.2. Flow cytometric profiles showing the reactivity of the CMV IE antigen-specific monoclonal antibody E13 with smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were seeded into 12 well plates, and infected with the high passage CMV strain AD169 (blue) at the multiplicity of infection indicated or left uninfected (green). The cells were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. The percentage of cells positive for IE antigen is indicated on each histogram, as determined by markers set using the lysis II software programme.

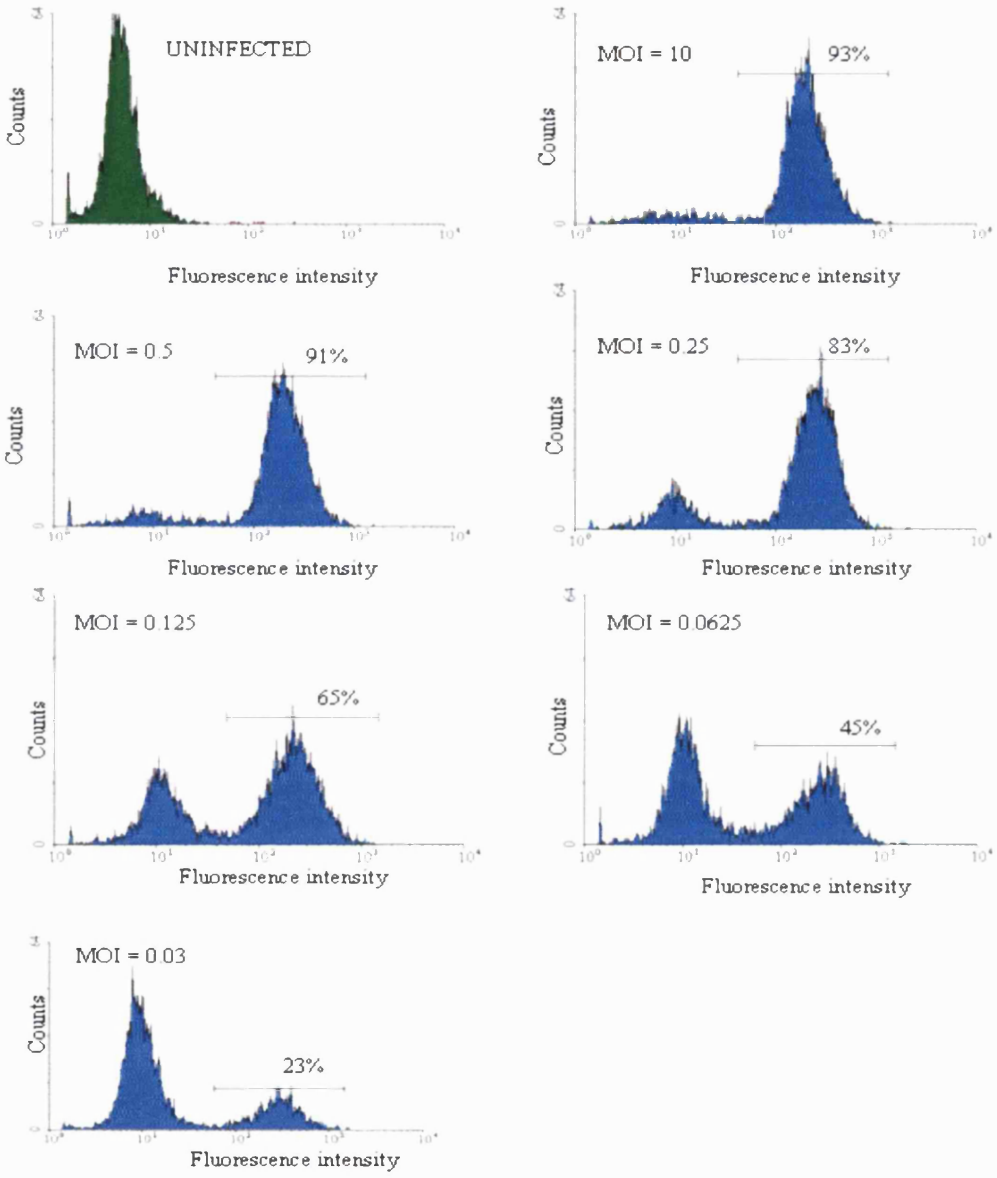


Figure 4.3. Flow cytometric profiles showing the reactivity of the CMV IE antigen-specific monoclonal antibody E13 with fibroblasts infected with CMV strain AD169.

Human embryonic lung fibroblasts at passage 15 were either left uninfected (green) or infected with the high passage CMV strain AD169 (blue), and subsequently analysed for the expression of IE antigen, as previously described for explanted-migrated smooth muscle cells in Figure 4.2. The percentage of cells positive for IE antigen is indicated on each histogram, as determined by markers set using the lysis II software programme.

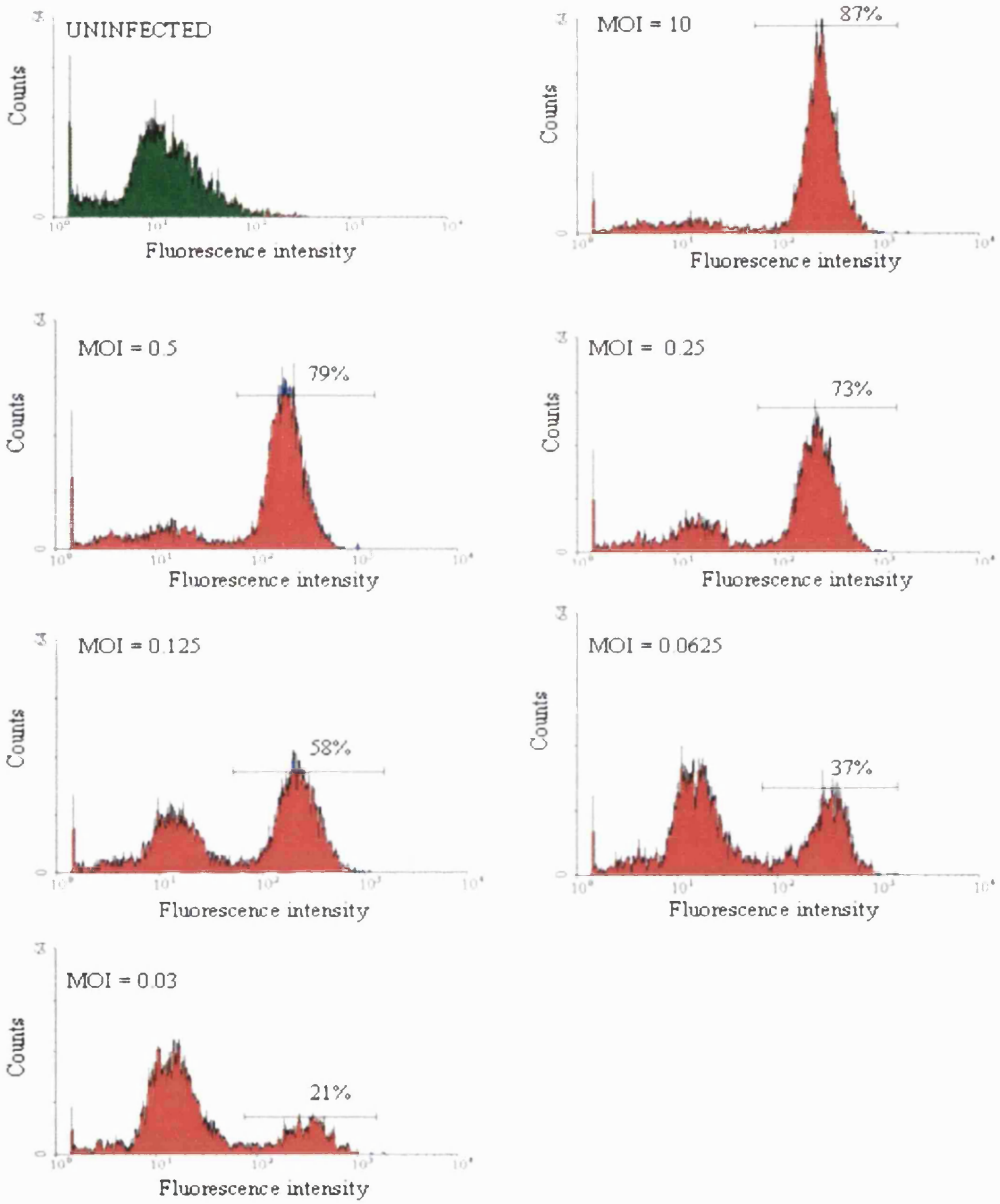


Figure 4.4. Flow cytometric profiles showing the reactivity of the CMV IE antigen-specific monoclonal antibody E13 with smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were seeded into 12-well plates, and 24 hours after the initial seeding were infected with the low passage CMV strain C1F (red) at the multiplicity of infection indicated or left uninfected (green). The cells were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. The percentage of cells positive for IE antigen is indicated on each histogram, as determined by markers set using the lysis II software programme.

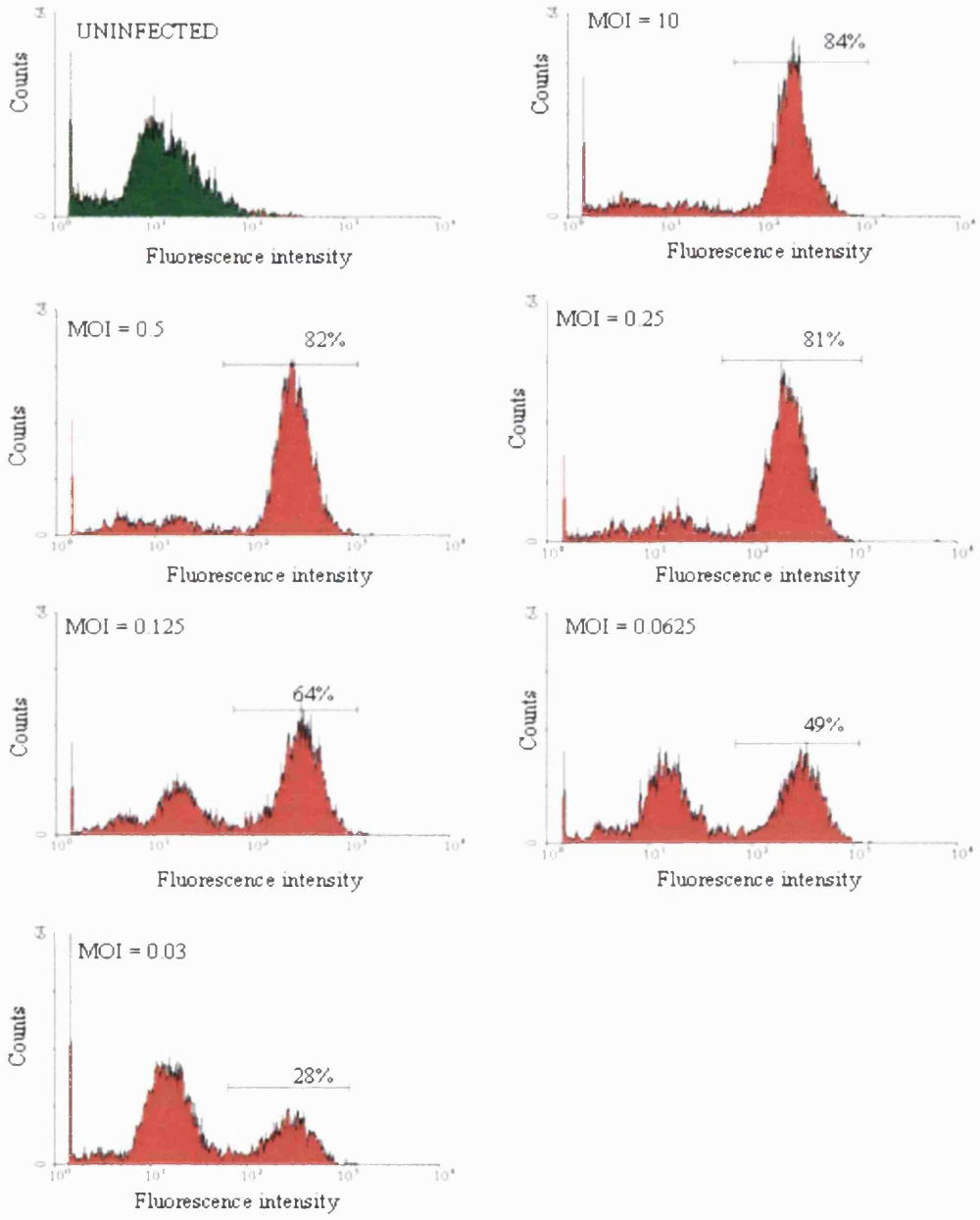
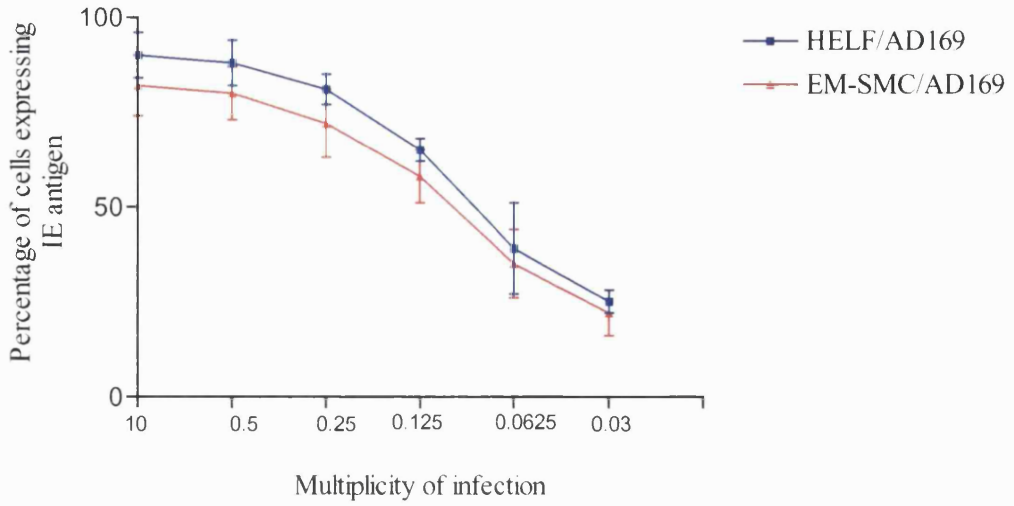


Figure 4.5. Flow cytometric profiles showing the reactivity of the CMV IE antigen-specific monoclonal antibody E13 with fibroblasts infected with CMV strain C1F.

Human embryonic lung fibroblasts at passage 15 were either left uninfected (green) or infected with the low passage CMV strain C1F (red), and subsequently analysed for the expression of IE antigen, as previously described for explanted-migrated smooth muscle cells in Figure 4.4. The percentage of cells positive for IE antigen is indicated on each histogram, as determined by markers set using the lysis II software programme.

A



B

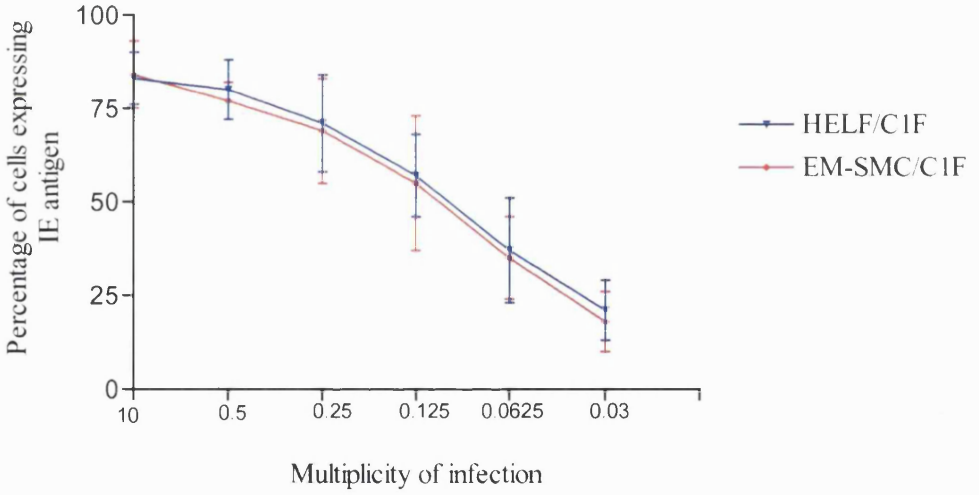


Figure 4.6. The effect of varying the multiplicity of infection of CMV strains AD169 and C1F on the percentage of cells expressing IE antigen, following infection of explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells (EM-SMC) at passage 3, and human embryonic lung fibroblasts (HELF) at passage 15, were seeded into 12-well plates, and 24 hours after the initial seeding were infected at various multiplicities of infection with CMV strains AD169 or C1F. They were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. The data shown is the percentage of cells expressing IE antigen in EM-SMC and HELF infected with CMV strain AD169 (A) or C1F(B), as determined using the Lysis II software programme, and represents the mean ± standard deviation of data derived from three separate experiments.

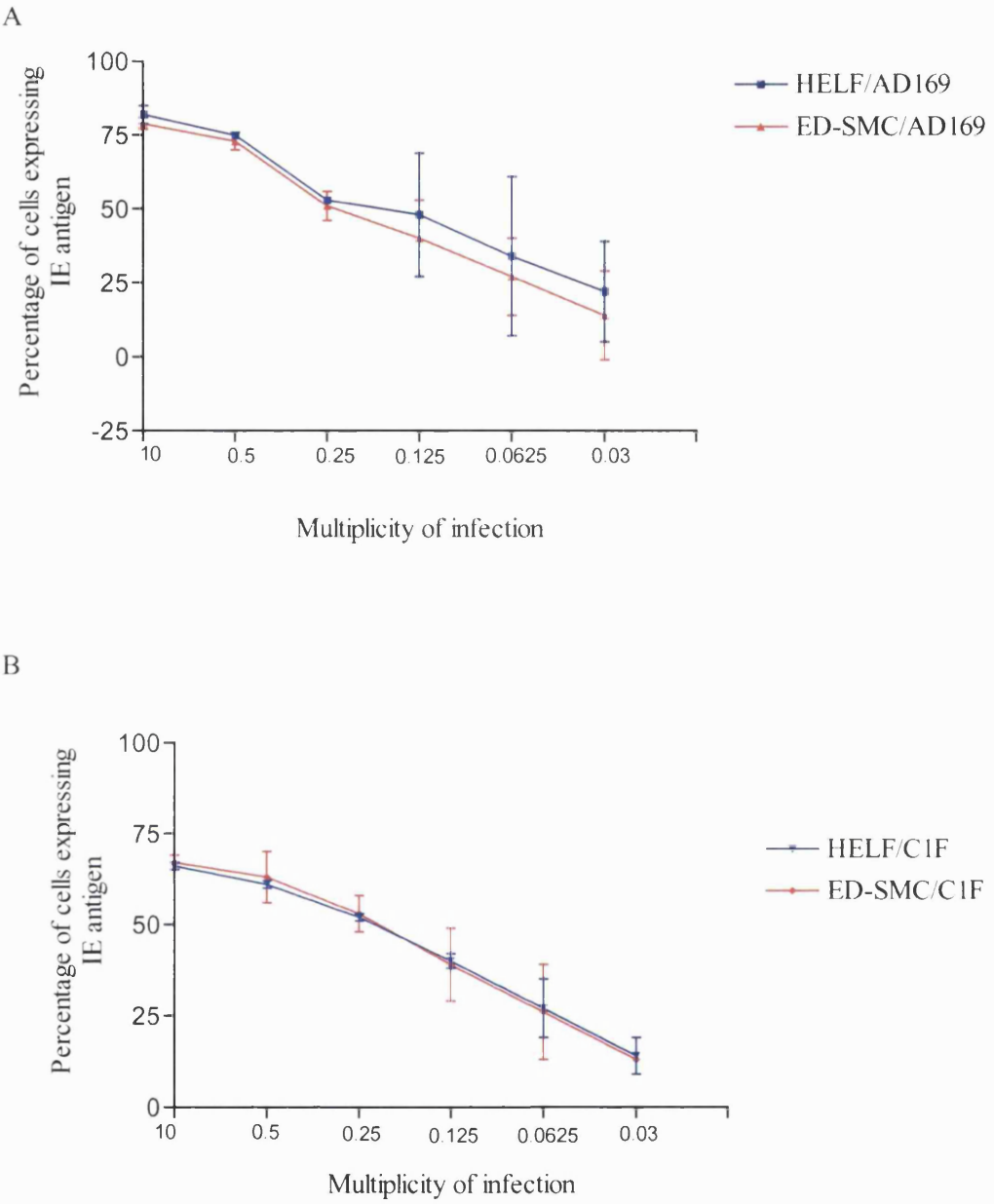
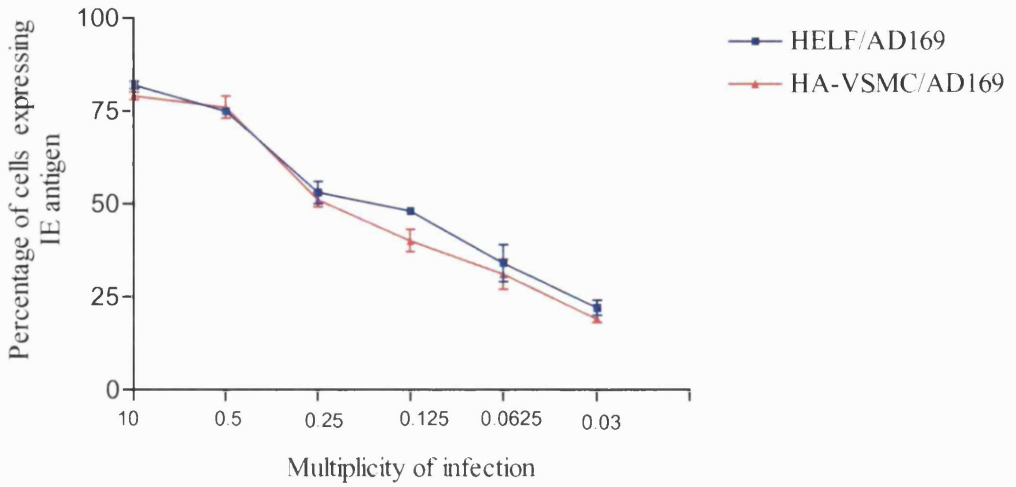


Figure 4.7. The effect of varying the multiplicity of infection of CMV strains AD169 and C1F on the percentage of cells expressing IE antigen following infection of enzyme-dispersed smooth muscle cells and fibroblasts.

Enzyme-dispersed smooth muscle cells (ED-SMC) at passage 3, and human embryonic lung fibroblasts (HELF) at passage 15, were seeded into 12-well plates, and 24 hours after the initial seeding were infected at various multiplicities of infection with CMV strains AD169 or C1F. They were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. The data shown is the percentage of cells expressing IE antigen in ED-SMC and HELF infected with CMV strain AD169 (A) or C1F (B), as determined using the Lysis II software programme, and represents the mean ± standard deviation of data derived from three separate experiments.

A



B

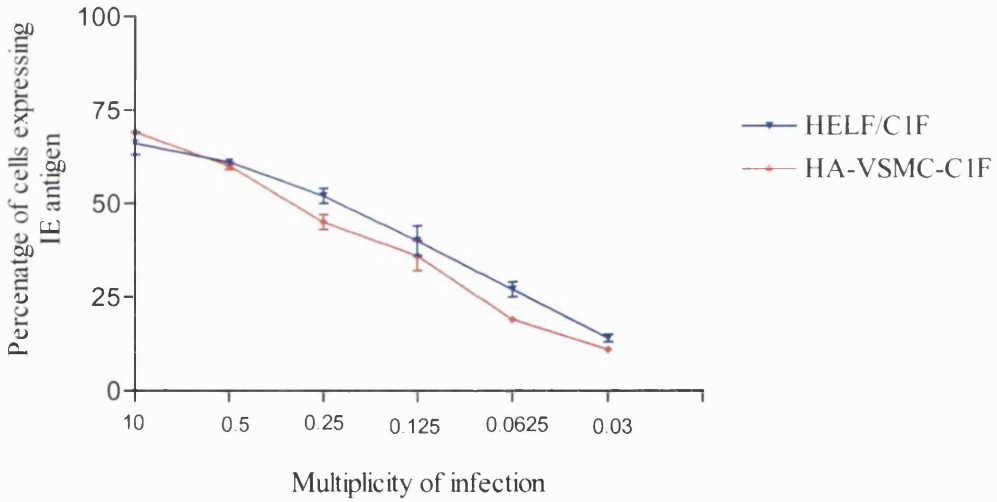


Figure 4.8. The effect of varying the multiplicity of infection of CMV strains AD169 and C1F on the percentage of cells expressing IE antigen following infection of aortic smooth muscle cells and fibroblasts.

Aortic smooth muscle cells (HA-VSMC) at passage 3, and human embryonic lung fibroblasts (HELF) at passage 15, were seeded into 12-well plates, and 24 hours after the initial seeding were infected at various multiplicities of infection with CMV strains AD169 or C1F. They were then analysed at 24 hours post-infection for the expression of IE antigen by flow cytometry using the monoclonal antibody E13. The data shown is the percentage of cells expressing IE antigen in HA-VSMC and HELF infected with CMV strain AD169 (A) or C1F (B), as determined using the Lysis II software programme, and represents the mean ± standard deviation of data derived from three separate experiments.

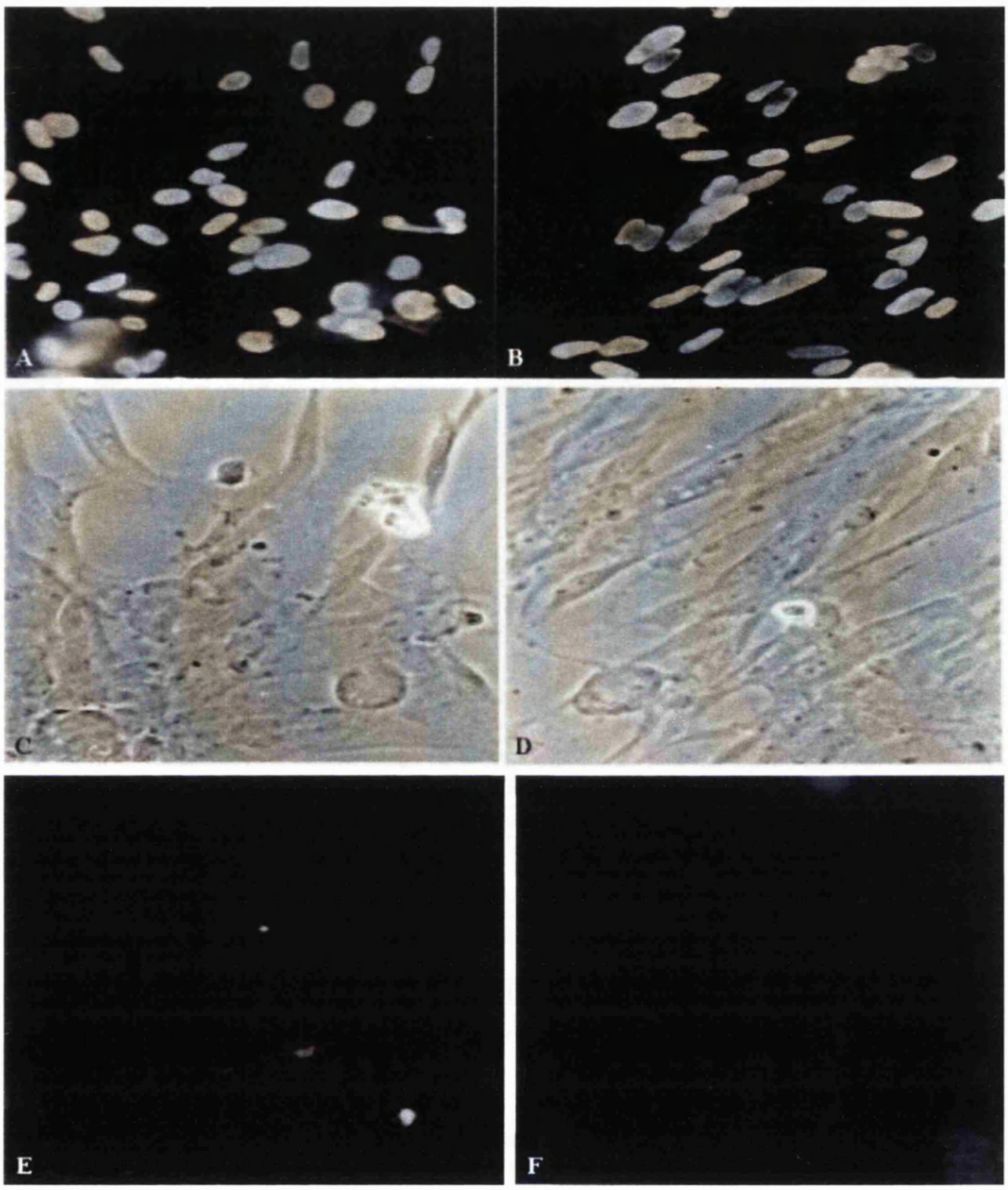


Figure 4.9. The detection of the CMV IE antigen, p72, at 24 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV IE antigen, p72, at 24 hours post-infection. The IE antigen is localized in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

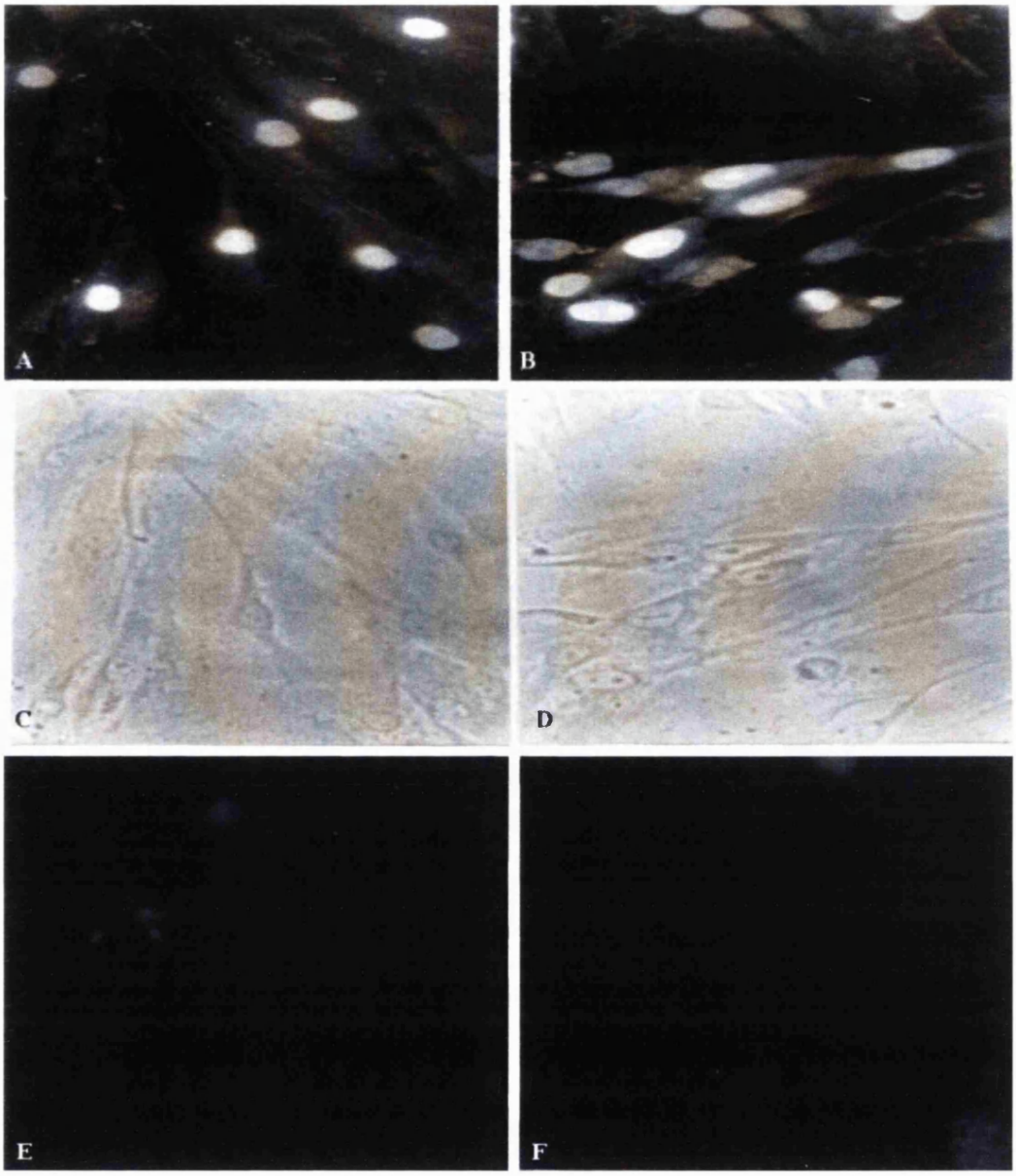


Figure 4.10. The detection of the CMV early antigen, pp65, at 24 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV early antigen, pp65, at 24 hours post-infection. The pp65 protein is localized in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

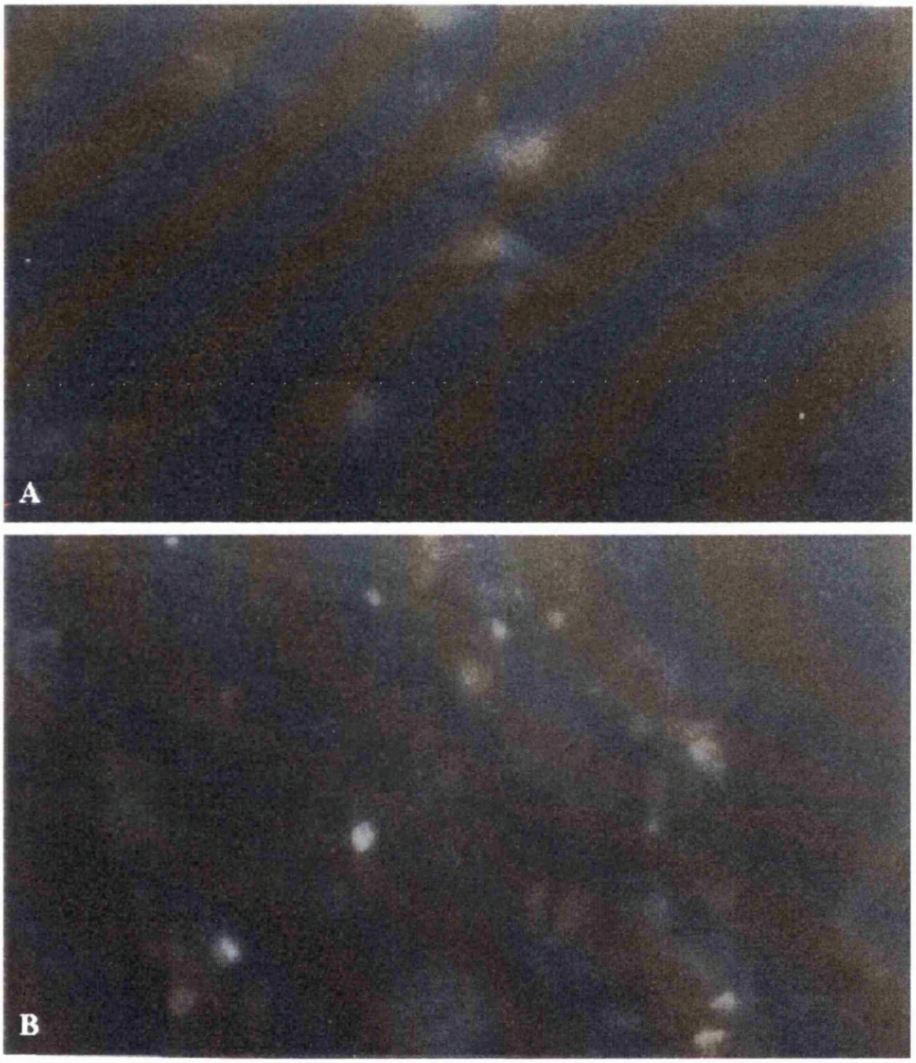


Figure 4.11. The detection of the CMV late antigen, glycoprotein B, at 24 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV late antigen, glycoprotein B, at 24 hours post-infection. Little or no detection of glycoprotein B was detected at this time point in either cell type. Magnification x1500.

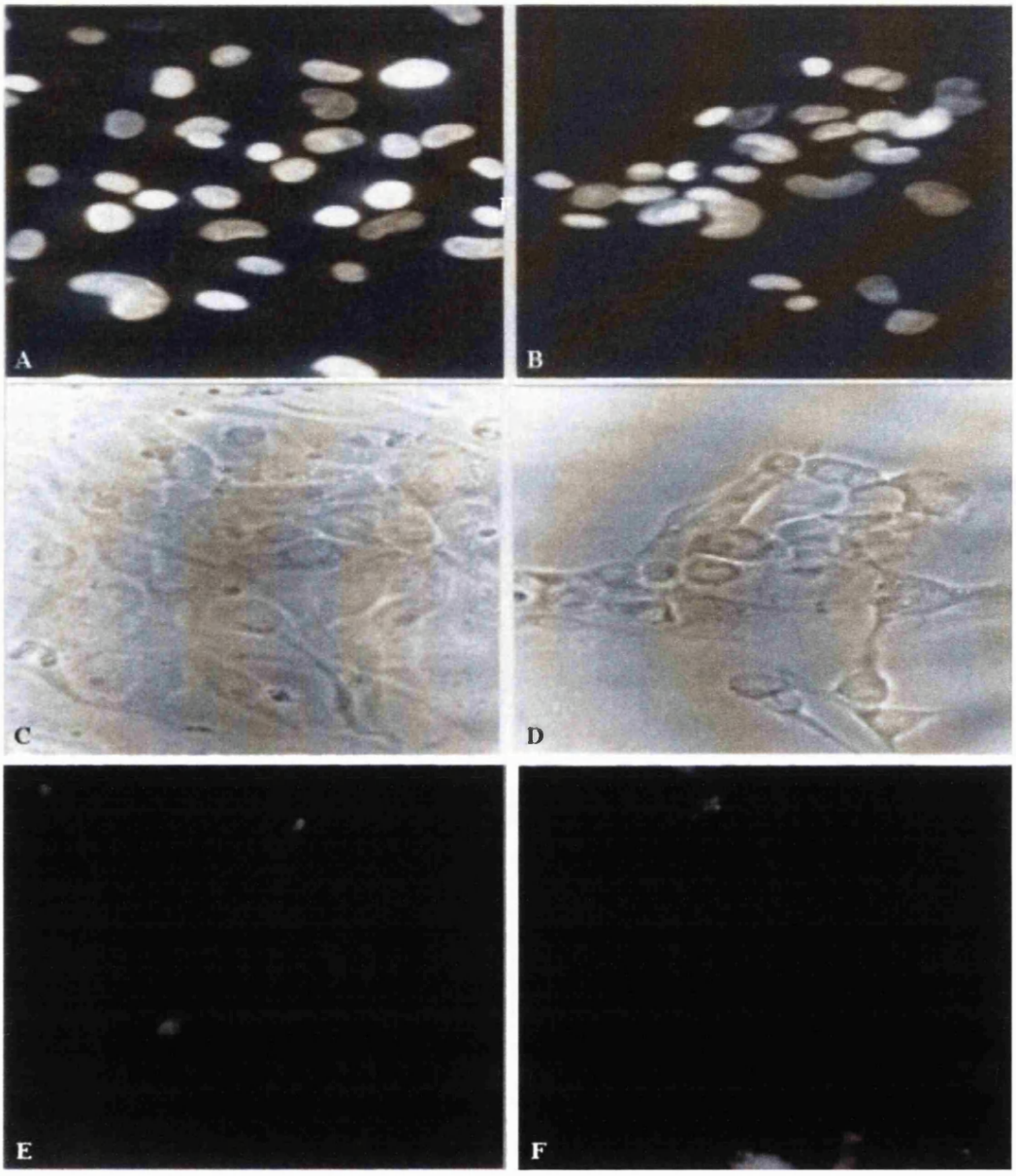


Figure 4.12. The detection of the CMV IE antigen, p72, at 72 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 72 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV IE antigen, p72, at 72 hours post-infection. The IE antigen is localized in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

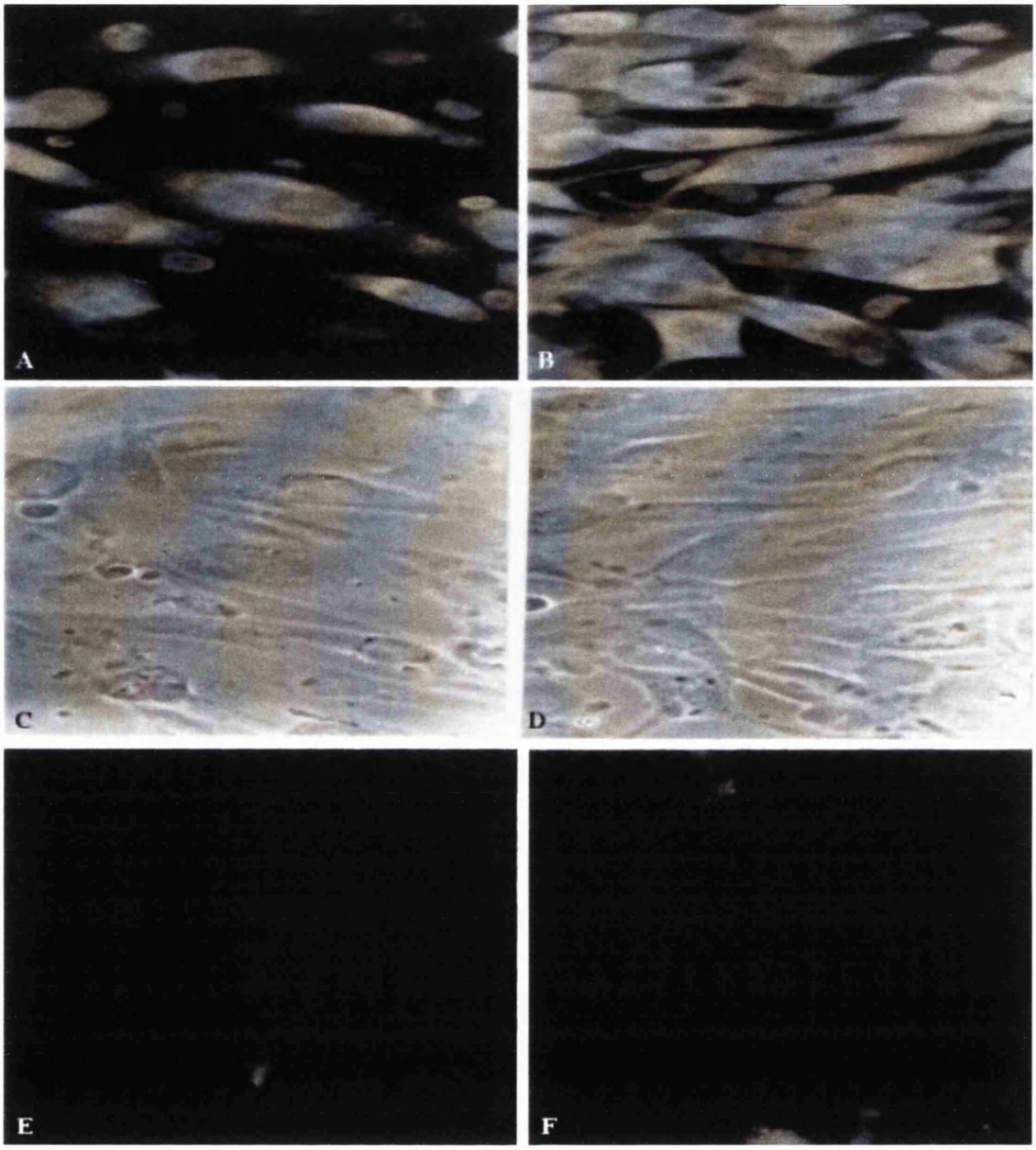


Figure 4.13. The detection of the CMV early antigen, pp65, at 72 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 72 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV early antigen, pp65, at 72 hours post-infection. The pp65 protein is localized in the cytoplasm as well as in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

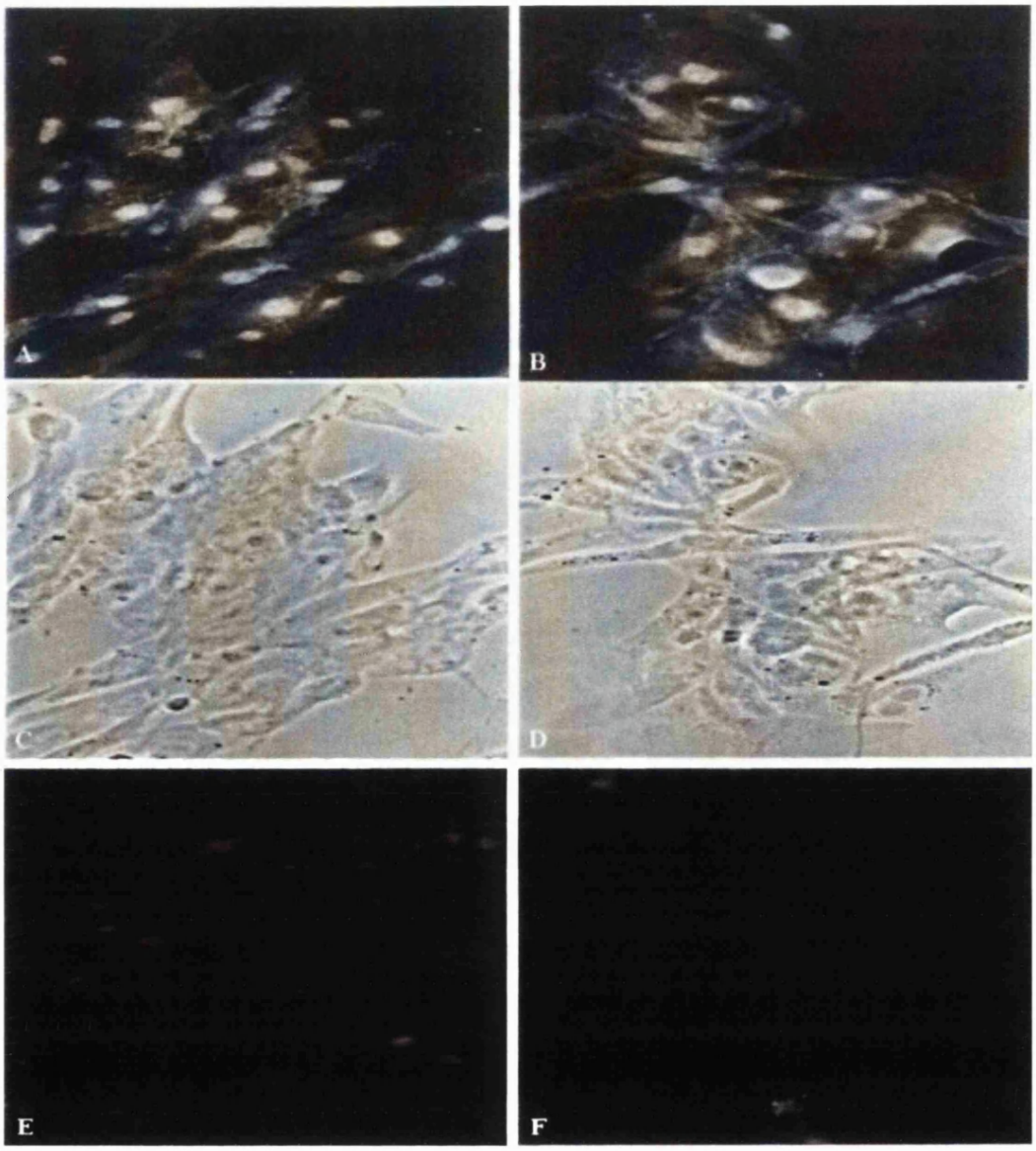


Figure 4.14. The detection of the CMV late antigen, glycoprotein B, at 72 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV late antigen, glycoprotein B, at 72 hours post-infection. The late antigen is localized in the perinuclear region of the infected cell in both cell types. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

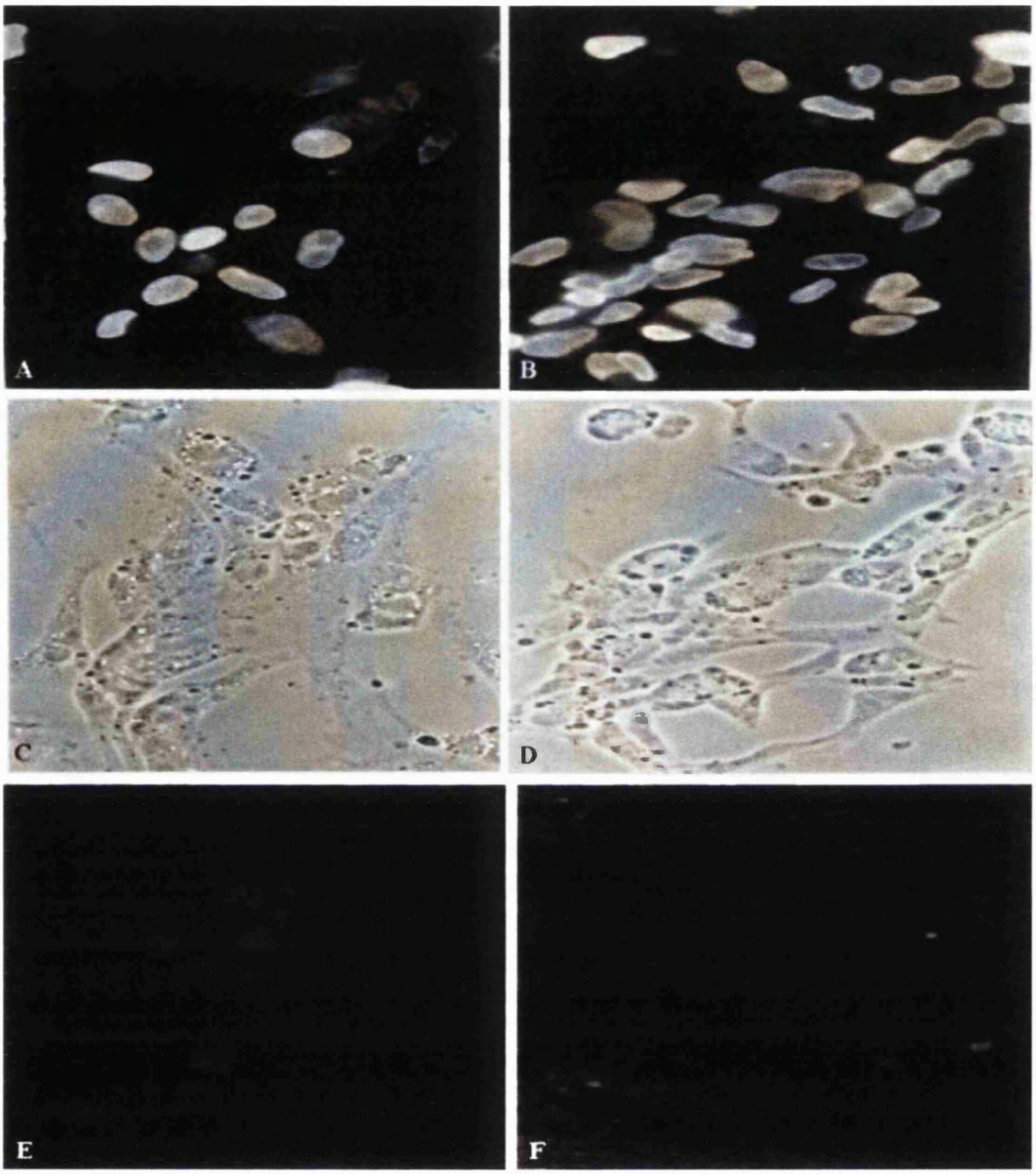


Figure 4.15. The detection of the CMV IE antigen, p72, at 120 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 120 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV IE antigen, p72, at 24 hours post-infection. The IE antigen is localized in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

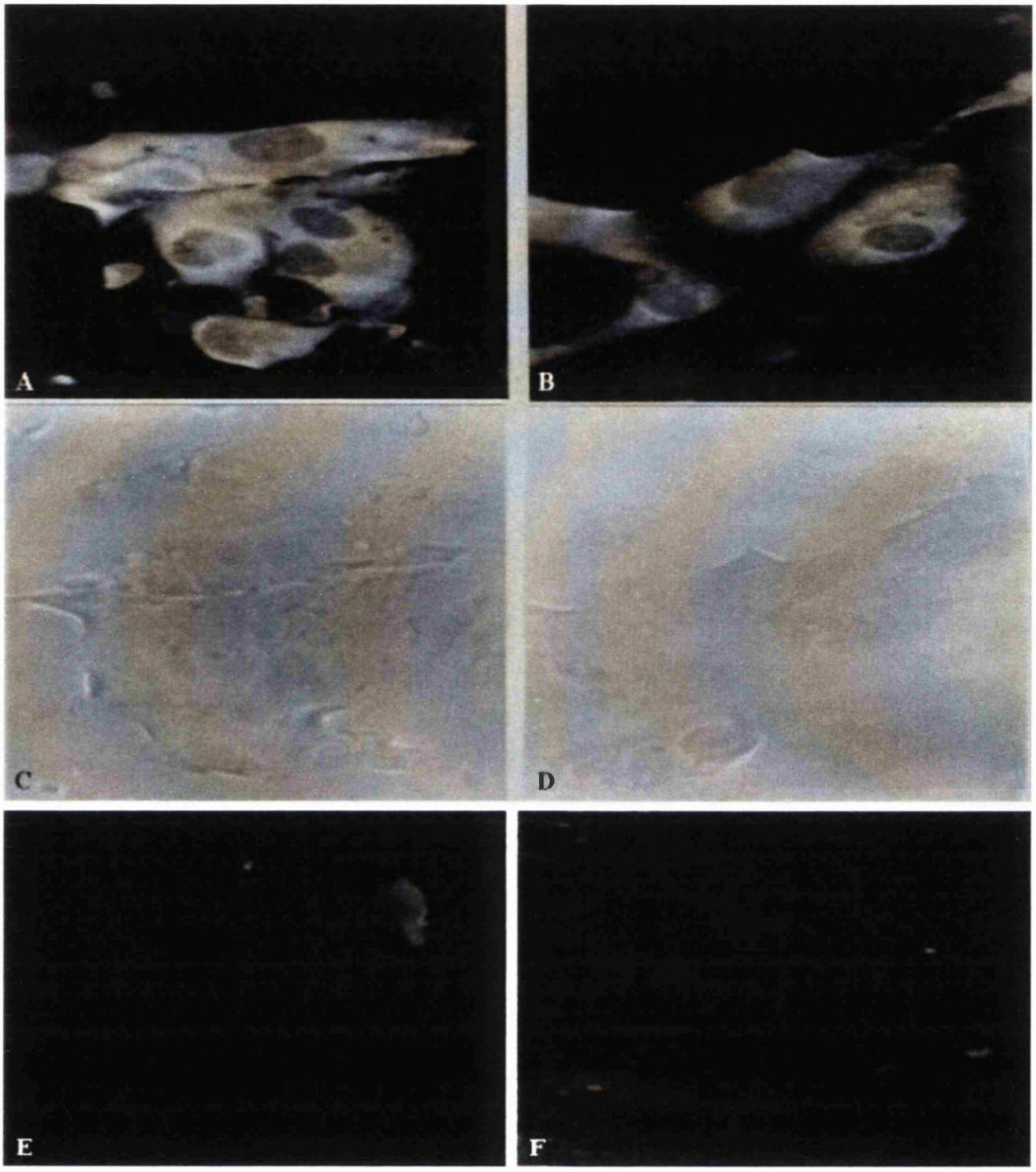


Figure 4.16. The detection of the CMV early antigen, pp65, at 120 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 120 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV early antigen, pp65, at 120 hours post-infection. The pp65 protein is localized in the cytoplasm as well as in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

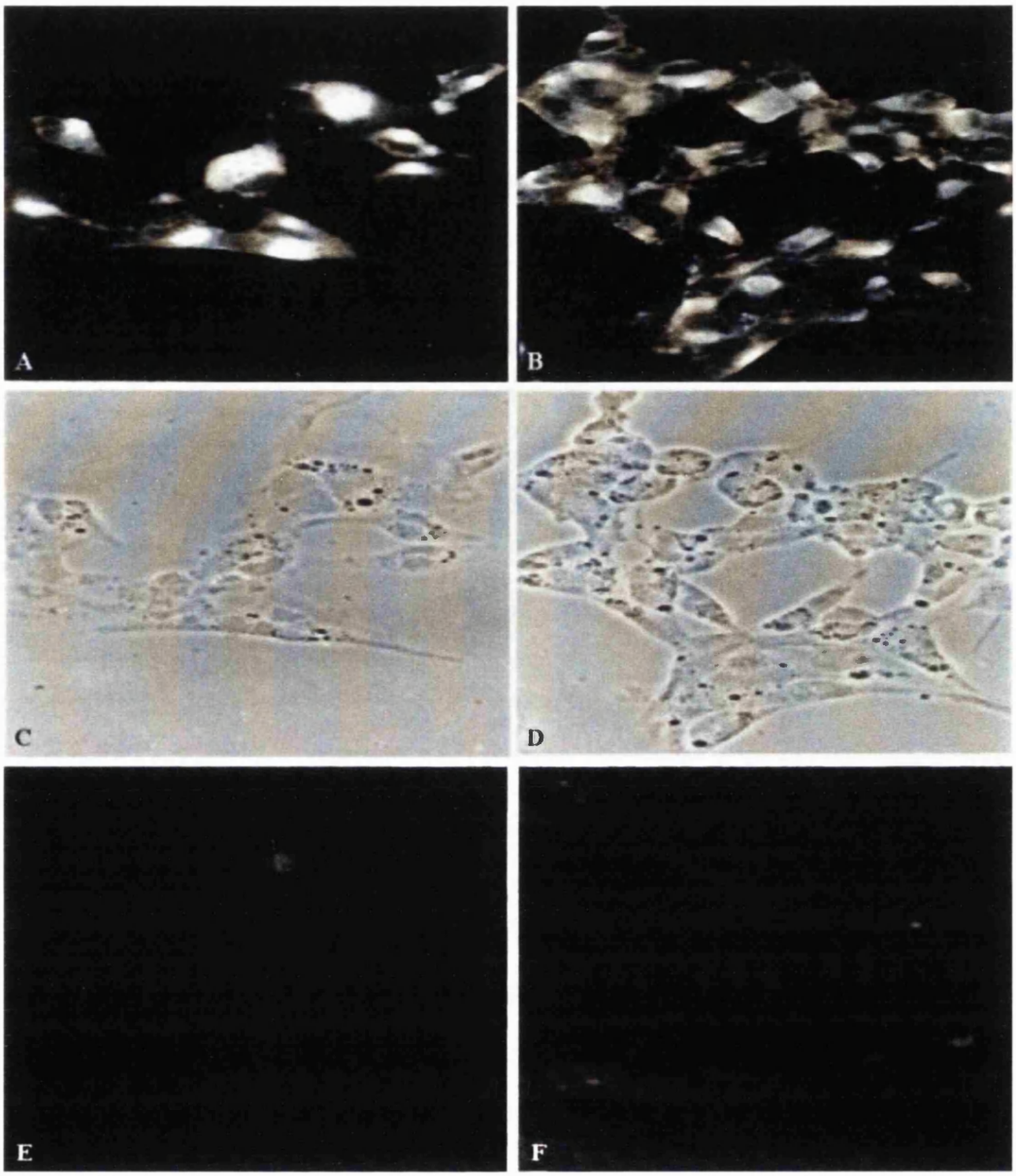


Figure 4.17. The detection of the CMV late antigen, glycoprotein B, at 120 hours post-infection by immunofluorescence in explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 120 hours after the initial seeding were infected with CMV strain AD169 at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV late antigen, glycoprotein B, at 120 hours post-infection. The late antigen is localized in the perinuclear region of the infected cell in both cell types. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Immunofluorescence staining of infected smooth muscle cells stained with an irrelevant isotype-matched control antibody, and uninfected cells stained for the expression of p72 are shown in (E) and (F), respectively. Magnification x800.

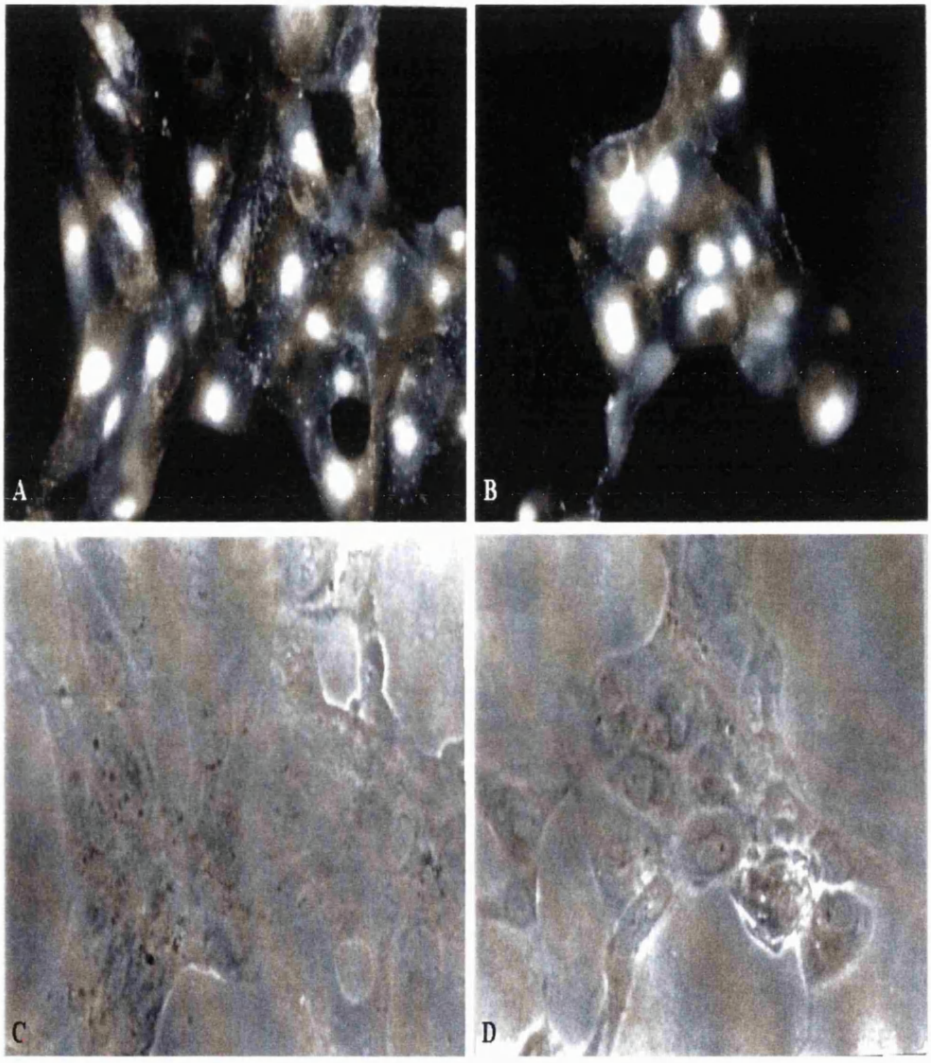


Figure 4.18. The detection of the CMV late antigen, glycoprotein B, at 120 hours post-infection by immunofluorescence in C1F-infected explant-migrated smooth muscle cells and fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were seeded into 8-well chamber slides, and 120 hours after the initial seeding were infected with CMV strain C1F at an MOI of 5. Shown are photomicrographs of explant-migrated smooth muscle cells (A) and human embryonic lung fibroblasts (B) stained by immunofluorescence for the expression of the CMV late antigen, glycoprotein B, at 120 hours post-infection. The late antigen is localized in the perinuclear region of the infected cell in both cell types. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Magnification x800.

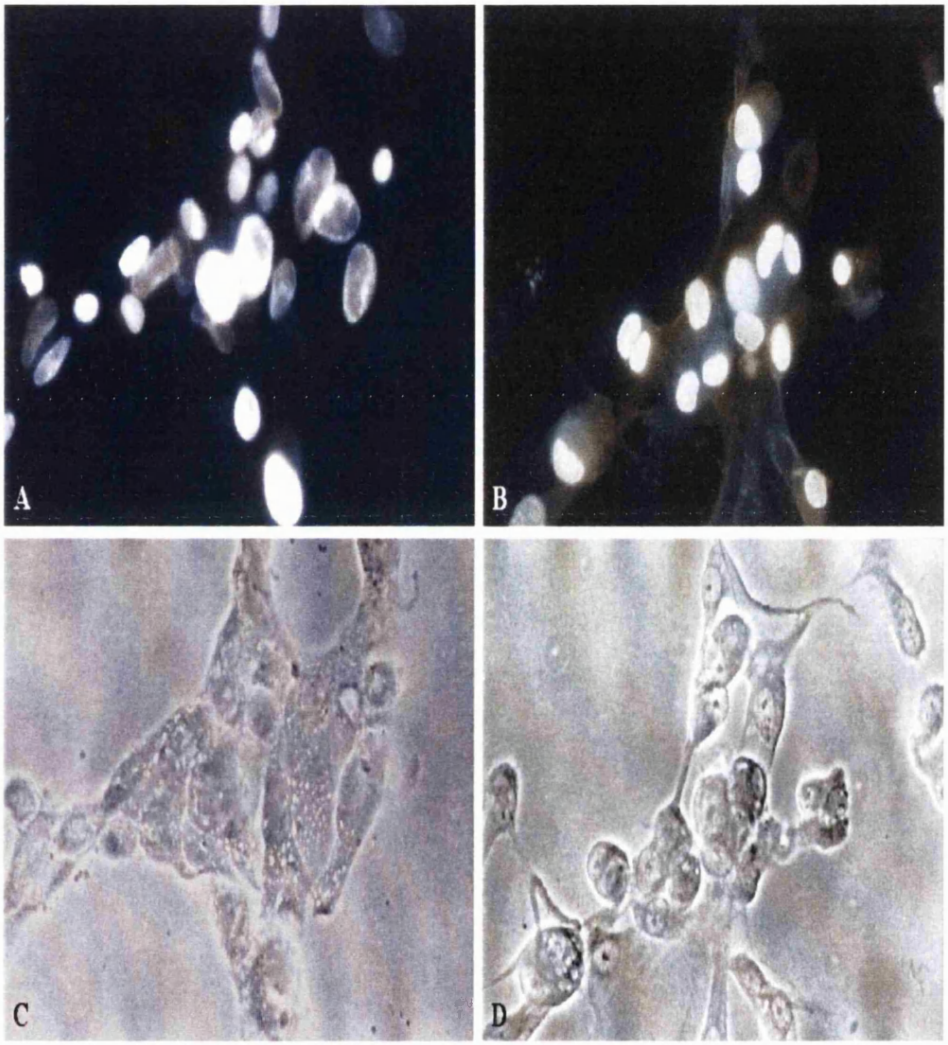


Figure 4.19. The detection of the CMV IE antigen, p72, at 120 hours post-infection by immunofluorescence in enzyme-dispersed and aortic smooth muscle cells.

Enzyme-dispersed and aortic smooth muscle cells at passage 3 and 24, respectively, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with the high passage CMV strain AD169 at an MOI of 5. Shown are photomicrographs of enzyme-dispersed-smooth muscle cells (A) and aortic smooth muscle cells (B) stained by immunofluorescence for the expression of the CMV IE antigen, p72, at 120 hours post-infection. The IE antigen is localized in the nuclei of infected cells. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for enzyme-dispersed-smooth muscle cells and aortic smooth muscle cells, respectively. Magnification x 800.

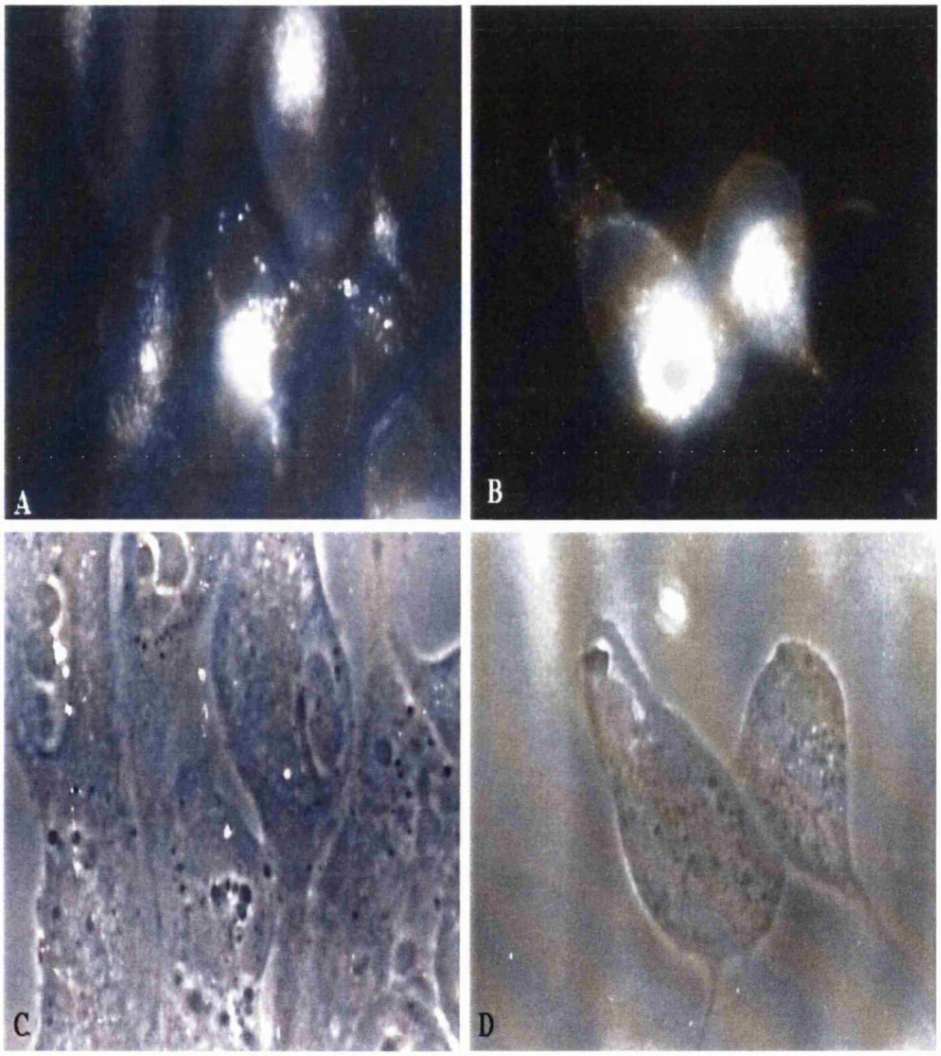


Figure 4.20. The detection of the CMV late antigen, glycoprotein B, at 120 hours post-infection by immunofluorescence in enzyme-dispersed and aortic smooth muscle cells

Enzyme-dispersed and aortic smooth muscle cells at passage 3 and 24, respectively, were seeded into 8-well chamber slides, and 24 hours after the initial seeding were infected with the high passage CMV strain AD169 at an MOI of 5. Shown are photomicrographs of enzyme-dispersed smooth muscle cells (A) and aortic smooth muscle cells (B) stained by immunofluorescence for the expression of the late CMV antigen, glycoprotein B, at 120 hours post-infection. The late antigen is localized in the perinuclear region of the infected cell in both cell types. Corresponding phase contrast photomicrographs of the cells are shown in (C) and (D) for explant-migrated smooth muscle cells and human embryonic lung fibroblasts, respectively. Magnification x1200.

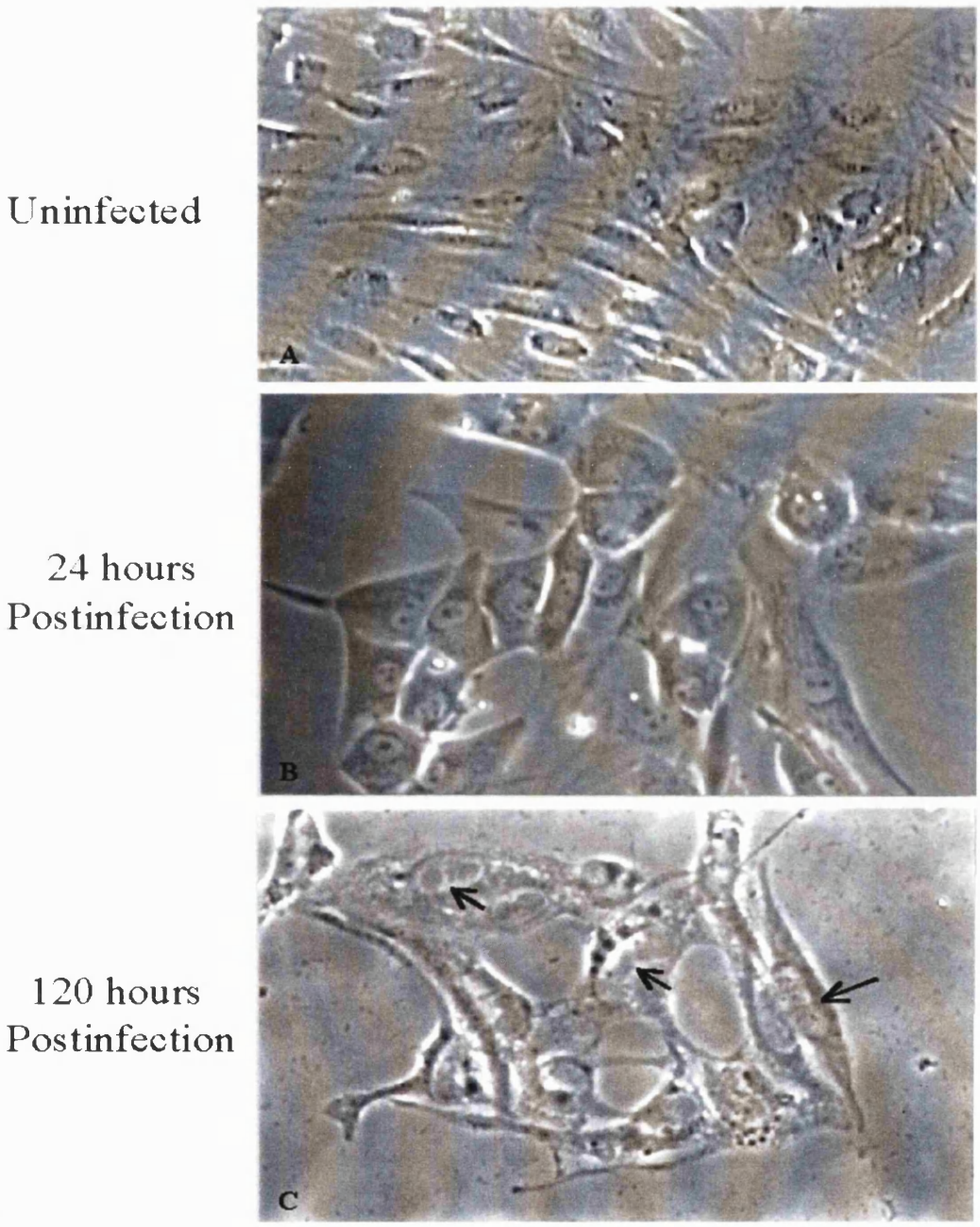


Figure 4.21. The ability of CMV strain AD169 to produce cytopathic effects in smooth muscle cells.

Explant-migrated smooth muscle cells were seeded into 8-well chamber slides and were either left uninfected or infected with CMV strain, AD169, at an MOI of 5. Cells were observed over a five-day period, using a light microscope. Shown are photomicrographs of cells left uninfected at 24 hours post-culture (A), or 24 hours post-infection (B) and 120 hours post-infection (C) with the high passage CMV strain AD169. At 24 hours post-infection, the apparent enlargement and clustering of cells can be seen, whilst at 120 hours post-infection, the nucleus of infected cells can be observed to adopt a kidney-shaped morphology (arrowed). Magnification x1000).

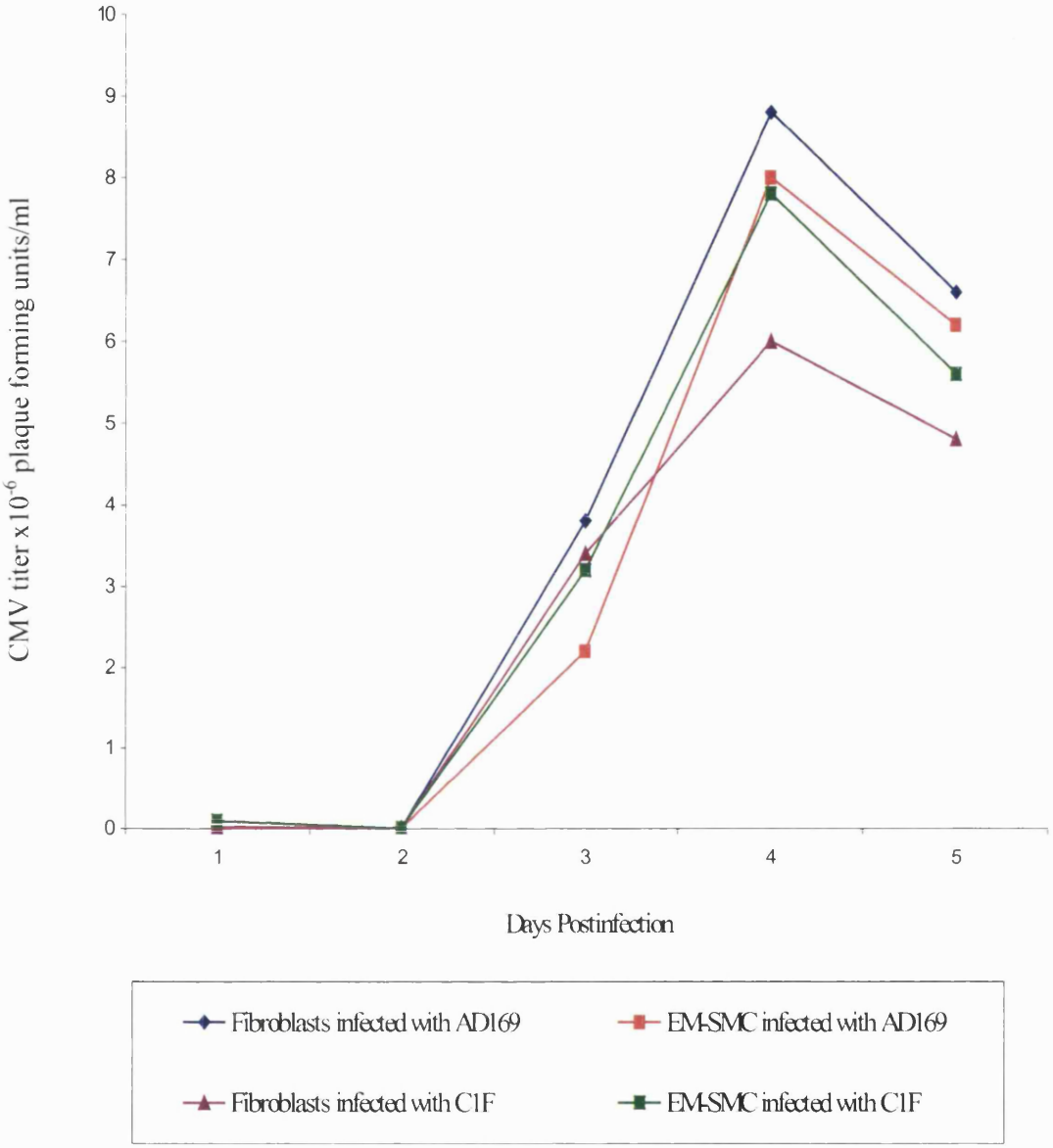


Figure 4.22. The production of infectious extracellular virus by explant-migrated smooth muscle cells infected with CMV compared to human embryonic lung fibroblasts.

Explant-migrated smooth muscle cells at passage 3, and human embryonic lung fibroblasts at passage 15, were infected with CMV strain AD169 or C1F at an MOI of 5. The supernatants from infected cultures were harvested at the times indicated, and extracellular virus titres were determined by plaque assay on human embryonic lung fibroblasts. The data shown represents the mean titre of extracellular virus released from smooth muscle and human fibroblast infected cultures from three separate experiments.

4.3 DISCUSSION

In the present study, the susceptibility of human smooth muscle cells isolated from various anatomical sources for CMV infection was investigated. The study initially focussed on the ability of infected smooth muscle cell cultures to initiate CMV replication by their ability to express IE antigen. The study proceeded to investigate whether the expression of viral antigens of all three temporal classes of CMV replication, the development of cytopathic effects, and the production of infectious virus progeny could be achieved in these cells. Smooth muscle cells used in this study included smooth muscle cells isolated from the saphenous vein, the umbilical cord and the human aorta as described in Chapter 3. In the first part of the study, all smooth muscle cell cultures were demonstrated to serve as targets for CMV infection following their infection with a high (AD169) or low (C1F) passage CMV strain. Flow cytometric detection of CMV-specific IE antigen in all smooth muscle cell cultures implied that the replicative cycle of CMV could be initiated in these cells. The percentage of smooth muscle cells expressing CMV IE antigen was not significantly different from that of human embryonic fibroblasts infected in parallel. In the second part of the study, immunofluorescence staining using monoclonal antibodies specific for antigens expressed at all three phases of CMV replication revealed that all smooth muscle cell cultures infected with either the AD169 strain or C1F strain expressed CMV-specific IE, early and late antigens. These observations were suggestive of the fact that a full CMV replicative cycle occurred in infected smooth muscle cell cultures. In addition, these observations showed that CMV infection of smooth muscle cells was indeed not latent or restricted to the expression of IE antigen, as had been previously suggested (Melnick et al., 1983). The ordered expression of the CMV-specific antigens was comparable to those observed in fully permissive fibroblasts. Thirdly, it was demonstrated that CMV infection resulted in a classically productive infection of human smooth muscle cells as characterized by the presence of cytopathic effects and the replication of input virus. The observed cytopathic effect was similar in morphological alterations, and in the kinetics of development, to that observed in CMV-infected fibroblasts. Productive infection was also confirmed by the ability to passage infectious virus from the supernatant of infected smooth muscle cell cultures to susceptible fibroblast cell monolayers. Growth curves constructed from assays of virus yield over time revealed that the kinetics of virus production in smooth muscle cells corresponded closely to that of fibroblasts. These studies demonstrated that the frequency of infected cells, the kinetics of viral antigen expression, the distribution of viral antigens, and the capability to support virus production within smooth muscle cells infected with

CMV strain AD169 or C1F were similar to those described for CMV-infected fibroblast cultures. There was no difference between smooth muscle cells isolated from the venous vasculature and the arterial smooth muscle cells in their ability to support CMV replication.

Contradictory to the above observations on the replication of CMV in smooth muscle cells, are results published by Benditt et al. 1983, Melnick et al. (1983) and Tumilowicz et al. (1985). Benditt et al. (1983) obtained smooth muscle cells from explants of abdominal and thoracic foetal aorta. The authors did not detect the CMV genome by *in situ* hybridisation, or CMV cytopathic effects, in these cells within the 48-hour experimental interval after inoculation with CMV strain AD169. In addition, the authors reported that the CMV genome was not detected in the abnormally thickened intima of the arteries from patients undergoing bypass surgery. Melnick et al. (1983) performed studies on smooth muscle cell cultures initiated from atheromatous and uninvolved arterial tissues of atherosclerotic patients. In more than 25% of the smooth muscle cell cultures, the authors detected immunofluorescent staining for CMV antigens using polyclonal antisera, though only a subpopulation (10-30%) of the cells in each positive culture expressed CMV antigen. CMV replication was not observed in any of their cultures by electron microscopy or by co-cultivation with permissive cells. The authors suggested that because they detected CMV antigen but not replicating CMV by electron microscopy, the artery wall may be a site of CMV latency. Tumilowicz et al. (1985) studied the replication of CMV strain AD169 in human smooth muscle cells derived from the umbilical arteries of two different donors, and in human lung fibroblasts. CMV antigens were detected by the indirect anti-complement immunofluorescence test with guinea-pig complement and anti-guinea pig complement C3 conjugated with fluorescein isothiocyanate. The authors reported both nuclear and cytoplasmic fluorescence, and the detection of enveloped CMV nucleocapsids by electron microscopy in human smooth muscle cells infected with CMV. It was reported that months after the destruction of the infected control fibroblast cultures, infectious virus in surviving smooth muscle cell cultures was demonstrated by transfer to permissive fibroblasts with ensuing cytopathic effects and CMV-specific immunofluorescence. The authors suggested that some smooth muscle cells are permissive for the replication of infectious CMV, and that the delayed nature of CMV-specific immunofluorescence, the presence of only nuclear fluorescence relatively late after infection in some smooth muscle cells, and the absence of fluorescence in other smooth muscle cells, suggested the possible existence of some non-permissive smooth muscle cells. It was concluded that CMV infection of smooth muscle cells relative to fibroblasts was delayed, prolonged and highly influenced by the

phenotypic diversity of the smooth muscle cell. In the present study, there was no evidence of the existence of non-permissive smooth muscle cells in the inoculated smooth muscle cells. The vast majority of smooth muscle cells from all three anatomical sources infected with either the AD169 strain or C1F strain were observed to initiate the synthesis of IE antigens as early as 24 hours post-infection. There were no differences in experimental strategy between the study reported by Tumilowicz et al. (1985), and the one presented here that could account for the discrepancy in the results obtained. However, the study presented here is more comprehensive and detailed in experimental design. For example, the CMV antigen detected in smooth muscle cells infected with CMV, and the specificity of the antibody used in the Tumilowicz study were not described.

In a recent study investigating the susceptibility of smooth muscle cells isolated from the vessels of the umbilical cords, Woodroffe et al. (1997) reported that the infection of these cells with CMV strain AD169 and a low passage clinical strain of CMV led to the expression of IE/early antigen in the inoculated cells, and virus release into the supernatant at various times post-infection. Differences in the replication of these two virus strains were observed between smooth muscle cells and fibroblasts. Whilst CMV strain AD169 was shown to infect fibroblasts efficiently, it showed a low infectivity profile for smooth muscle cells, whereas the low passage clinical isolate displayed comparable infectivity characteristics in both cell types. In contrast to the results of Woodroffe et al. (1997), the observations from the present study suggest that the infection of smooth muscle cells with CMV strain AD169 was equivalent to that observed in fibroblasts. A slight (but not statistically significant) difference in the number of cells expressing IE antigen was observed at high multiplicities of infection between smooth muscle cells and fibroblasts infected with CMV strain AD169. However, over the range of multiplicities of infection examined, there was no apparent difference in the number of cells expressing IE antigen between smooth muscle cells and fibroblasts infected with CMV strain AD169, and no statistically significant difference. In support of the observations of Woodroffe et al. (1997), a comparable level of infection between smooth muscle cells and fibroblasts infected with a low passage clinical isolate of CMV was observed. The similarity in the present study between the number of cells expressing IE antigens between smooth muscle cells in culture and fibroblasts infected with CMV strain AD169 or C1F, highlights the close relationship between these two mesenchymal cell types. The similarity in infectivity between these cell types could also be attributed to the phenomenon of phenotypic modulation, which collectively describes the changes in the morphological, cellular, and biochemical properties of the smooth muscle cell to become more fibroblast-like. This

phenomenon is characteristic of smooth muscle cells isolated from the blood vessel wall and maintained in culture. Further support for the observations in the present study, are observations made by others from studies using aortic smooth muscle cells, in which CMV infection with strain AD169 was demonstrated to be permissive for at least the early events of infection. Virtually 100% of the nuclei of the smooth muscle cells from this source were observed to be stained with a monoclonal antibody specific for CMV IE antigen. However, productive CMV replication in these cells was not investigated (Hosenpud et al., 1991).

CMV infection of the other predominant cell type found in the blood vessel wall, the endothelial cell, has been well documented. It has been demonstrated that CMV strain AD169 has a restricted capacity to infect endothelial cells *in vitro*, with <5% of inoculated cells expressing CMV-specific IE antigens (Friedman et al., 1981; Ho et al., 1984; MacCormac & Grundy, 1999). This is in contrast to the observations in the present study where CMV infection of smooth muscle cells with strain AD169 resulted in approximately 90% of the inoculated cells expressing IE antigen. The infectivity for endothelial cells with CMV strain AD169 could be increased by treatment of the cells with sodium butyrate (MacCormac & Grundy, 1999). The fact that sodium butyrate could enhance CMV replication in endothelial cells after virus inoculation, suggests that sodium butyrate could affect events subsequent to virus entry into the cell, such as the induction of a protein necessary for viral replication, or the removal of an inhibitor of viral replication.

The infectivity of endothelial cells with CMV strain AD169 could be further increased by low speed centrifugal inoculation of the virus (MacCormac & Grundy, 1999). The combination of low speed centrifugal inoculation plus sodium butyrate treatment resulted in optimal infectivity of endothelial cells for CMV strain AD169 (MacCormac & Grundy, 1999). In contrast, CMV infection of endothelial cells with low passage CMV strains has been found to be more productive, with increased numbers of cells displaying CMV IE antigen than with CMV strain AD169. Thus in endothelial cells, only low passage strains of CMV replicate efficiently, whereas the replication of the high passage AD169 strain and the low passage C1F strain in smooth muscle cells was virtually equivalent, as demonstrated in the present study. Chemical induction with sodium butyrate and low speed centrifugation were not required for productive infection of smooth muscle cells with either the AD169 strain or low passage C1F strain.

The difference in the susceptibility of endothelial cells to virus strains has been suggested to arise as a result of the adaptation of CMV strain AD169 to fibroblasts. CMV strain AD169 has been propagated in fibroblasts for nearly 30 years, and it is believed that the continuous propagation of this strain in fibroblasts has resulted in changes in the CMV

DNA, which might alter its infectivity for other cell types (Furukawa, 1984). This might also explain the reduced permissivity of epithelial cells to infection with CMV strain AD169. In addition, since the envelope constituents of the CMV virion is obtained from virally modified cell components, and the viral envelope determines the binding and the interaction of the virus with the host cell, such continuous propagation of the virus might affect the properties of the resulting virus progeny. The difference in the susceptibility of endothelial cells to infection with CMV strain AD169 and strain C1F could also be attributed to the recent observation that one low passage clinical isolate of CMV contains at least 19 genes which are absent from the predicted sequence of the high passage CMV strain AD169. It is believed that this substantial amount of genetic information encoded by the viral genome must have been lost during long-term passage of the virus in cell culture (Cha et al., 1996).

It has been reported that the replication of CMV strain AD169 in human epithelial thyroid papillary carcinoma cells is restrictive, with approximately 10 cells per 3 x 10^5 of inoculated cells expressing IE antigen at 24 hours post-infection (Tanaka et al., 1991). This data is similar to the situation in other epithelial cell cultures (St. Jeor & Rapp, 1973; Michelson-Fiske et al., 1975; Knowles, 1976). However, it has been demonstrated that the pre-treatment of human epithelial thyroid papillary carcinoma cells with sodium butyrate for 24 hours before infection with CMV enhanced the yield of infectious virus to a similar level to that seen in human embryonic lung fibroblasts. There is one report that contradicts reports demonstrating the non-permissiveness of epithelial cells to CMV infection *in vitro*. Detrick et al. (1996) evaluated the ability of CMV to initiate replication in human retinal pigment epithelial cells, and compared this system with CMV replication in human fibroblasts. Human retinal pigment epithelial cells were obtained from donor eyes and propagated *in vitro*. Cells were infected with CMV strain AD169, and CMV replication was evaluated by detecting viral antigens by immunofluoresence and flow cytometry, virus-induced cytopathic effects, and the production of infectious virus. CMV replication was detected in human retinal pigment epithelial cells without the treatment of these cells with sodium butyrate. There were a number of differences in CMV replication in retinal pigment epithelial cells compared to replication in human fibroblasts. For example, virus-induced cytopathic effects and the production of infectious virus in retinal pigment epithelial cells were delayed when compared to virus infection in human fibroblasts. These studies suggest that in contrast to smooth muscle cells infected with CMV strain AD169 which are virtually equivalent to fibroblasts in their ability to support CMV replication, infection of epithelial cells with the AD169 strain is relatively slower

In conclusion, the results of the present study extend previous findings by demonstrating that, under the given culture conditions, CMV inoculation of smooth muscle cells can result in essentially greater than 90% of the inoculated cells expressing CMV viral antigens of all three temporal classes of CMV replication. In addition smooth muscle cells had the capability to support virus growth. The present study shows that CMV replication with CMV strains AD169 or C1F in smooth muscle cells proceeds in a fashion comparable to CMV replication in human embryonic lung fibroblasts. This suggest that smooth muscle cells may be a host for replication of the virus *in vivo*, and that such infection is likely to be significant in the pathogenesis of vascular diseases.

CHAPTER 5

The effect of CMV infection of smooth muscle cells on the cell surface expression of adhesion molecules and major histocompatibility antigens

5.1 INTRODUCTION

It is well established that interactions between endothelial cells and leukocytes via cell adhesion molecules play an important role in leukocyte recruitment in atherogenesis (Ross, 1993; Jang et al., 1994). Besides selectins, the adhesion molecules of the immunoglobulin family, ICAM-1 and VCAM-1 contribute to the adhesion of leukocytes to activated endothelium (Smith, 1993). ICAM-1 on the endothelial cell binds to LFA-1 (CD18/CD11a) or Mac-1 (CD18/CD11b) on leukocytes, and mediates the adhesion of monocytes, lymphocytes and neutrophils to endothelial cells (van de Stolpe & van der Saag, 1996). VCAM-1 on the endothelial cell binds to VLA-4 (integrin $\alpha_4\beta_1$) on lymphocytes and monocytes (Faruqi & Dicorleto, 1993). These adhesion molecules play a significant role in cell signalling and in leukocyte trafficking, thus alterations in their expression might affect the activation state or distribution of leukocytes in the body.

There has been considerable interest in recent years into the alteration of adhesion molecules in cells infected with CMV. It is proposed that the interaction between leukocytes and virus-infected cells may contribute to virus-induced inflammation. It has been reported that CMV infection induces the up-regulation of ICAM-1 (Grundy & Downes, 1993; Ito et al., 1995) and LFA-3 (Grundy & Downes, 1993) on the surface of fibroblasts. Similarly, it has been demonstrated in cultured endothelial cells that CMV infection induces the expression of ICAM-1, although it does not alter the constitutive expression of VCAM-1 on these cells (Sedmak et al., 1994). There are however, no studies to date investigating the effect of CMV infection on the expression of adhesion molecules on smooth muscle cells *in vitro*. CMV infection might affect the expression of adhesion molecules on the surface of smooth muscle cells, facilitating the accumulation of transmigrated leukocytes within the vascular wall. In the present study, smooth muscle cells were infected with CMV strain AD169 or strain C1F, and the effect of such infection on the expression of the adhesion molecules ICAM-1, VCAM-1 and LFA-3 was determined by flow cytometry. Recent studies suggest that the vitronectin receptor (integrin $\alpha_v\beta_3$) is a critical molecule in several processes involved in atherosclerotic progression and smooth muscle cell migration (Hoshiga et al., 1995; Dufourcq et al., 1997). Given that CMV infection of smooth muscle cell is postulated to contribute to atherosclerotic development, an investigation into the effects of CMV infection on the expression of the vitronectin receptor on the surface of smooth muscle cells was also studied.

Class I and class II MHC antigens are crucial in directing the adaptive immune response to foreign antigens. Activation of $CD8^+$ and $CD4^+$ lymphocytes requires the

recognition of viral antigens as short peptides bound to class I and class II MHC antigens, respectively, on the surface of antigen presenting cells (Hengel & Koszinowski, 1997; Miller & Sedmak, 1999). Thus, a decrease in the expression of class I or class II MHC antigens during the course of viral infection could prevent the effective clearance of the virus from the host, facilitating its persistence. On the other hand, an increase in the cell surface expression of class I or class II MHC antigens has been suggested to contribute to the development of transplant-associated atherosclerosis, the primary manifestation of chronic rejection in heart transplant recipients (Grattan et al., 1989). Several lines of evidence suggest that CMV infection plays at least a contributory role in the development of these processes by altering the expression of MHC antigens in the host cell (Tuder et al., 1994).

There are several conflicting reports on the alteration of expression of class I MHC antigens in a variety of cells infected with CMV. There have been reports demonstrating an increase in class I MHC expression in epithelial cells (van Dorp et al., 1993) and endothelial cells (Tuder et al., 1991), and also reports demonstrating the down-regulation of cell surface class I MHC antigens in fully permissive fibroblasts (Barnes & Grundy, 1993; Steinmassl & Hamprecht, 1994; Yamashita et al., 1993). In CMV-infected smooth muscle cells, an increase in the level of expression of class I MHC antigens has been demonstrated (Hosenpud et al., 1991; Arkonac et al., 1997). Hence, in the present study, the effect of CMV infection on the expression of class I MHC antigens on smooth muscle cells was studied. In addition, smooth muscle cells not only constitutively express class I MHC antigens, but can be induced to express class II MHC antigens following stimulation with a variety of cytokines such as interferon-γ (IFN-γ) (Hosenpud et al., 1991). In endothelial cells, it has been demonstrated that CMV can down-regulate the expression of class II MHC induced by IFN-γ (Sedmak et al., 1994). In fibroblasts, IFN-β released from infected cells, has been reported to be responsible for the up-regulation of class I MHC on uninfected cells in a mixed population of CMV-infected and uninfected fibroblasts (Grundy et al., 1988). Hence, an investigation was carried out to deduce the effects of CMV infection on the expression of class II MHC antigens, and on the ability of IFN-γ to alter the cell surface expression of class I and class II MHC antigens on smooth muscle cells.

The effects of CMV infection on the alteration of expression of adhesion molecules and MHC antigens were exclusively performed on explant-migrated smooth muscle cells. The latter source of smooth muscle cells were used in this part of the study primarily because of the ease in obtaining the cell numbers required to perform such

experiments. In addition, all studies were performed using both the high (AD169) and the low (C1F) passage strains of CMV.

5.2 RESULTS

5.2.1 The effect of CMV infection on the cell surface expression of ICAM-1 on vascular smooth muscle cells.

The effect of CMV infection on the cell surface expression of ICAM-1 on vascular smooth muscle cells was studied *in vitro*. Explant-migrated smooth muscle cells were seeded into culture plates, and were either left uninfected, or infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10. The experiments were conducted in conditions in which the vast majority of cells were infected, so that only the effects on the infected cells were studied. The percentage of cells expressing the CMV-specific IE antigen was determined by flow cytometry, using the monoclonal antibody, E13 (see section 4.2.1). A high percentage of infection was achieved with both CMV strains AD169 and C1F with approximately 85% of cells expressing the CMV IE antigen at 24 hours post-infection and greater than 95% in all cases examined by days 2-5 post-infection. The expression of ICAM-1 on the surface of infected and uninfected cells was then analysed at various days post-infection by flow cytometry. Figure 5.1 shows representative flow cytometric profiles of the expression of ICAM-1 on the surface of smooth muscle cells infected with CMV strain AD169. As shown, CMV strain AD169 induced a marked increase in the cell surface expression of ICAM-1. The level of expression of ICAM-1 was observed to be elevated from days 1-4 post-infection, with maximum expression observed on day 2 post-infection. Figure 5.2 shows representative flow cytometric profiles of the expression of ICAM-1 on the surface of smooth muscle cells infected with CMV strain C1F. When smooth muscle cells were infected with CMV strain C1F, it was observed that alterations in the level of expression of ICAM-1 were similar to those observed on cells infected with CMV strain AD169.

The logarithmically acquired data from three separate experiments was converted to a linear form, and subsequently to fluorescence intensity units as described in section 2.8.5. The fluorescence intensity units served to relate the level of expression of a particular adhesion molecule on infected cells to their corresponding level of expression on uninfected cells at the same time point. Using the data expressed in fluorescence intensity units, the peak expression of ICAM-1 on AD169-infected explant-migrated smooth muscle cells was found to be approximately 2.5-fold that seen on uninfected cells (Figure 5.3). The data also revealed that the peak expression of ICAM-1 on C1F-infected smooth muscle cells was approximately 2.2-fold that seen on uninfected cells. By day 5, when cells were undergoing extensive cytopathic effects, the expression of ICAM-1 on

cells infected with either CMV strain had returned to the levels seen on uninfected cells. The Mann-Whitney t-test was used to compare the significance of the differences between the levels of expression of ICAM-1 on infected and uninfected smooth muscle cells. The data from three separate experiments were examined, and it was deduced that there was a significant difference at each time point in the level of expression of ICAM-1 antigens expressed in fluorescence intensity units between infected and uninfected cells at days 1-3 post-infection ($P < 0.05$). There was no significant difference in the level of ICAM-1 expression on smooth muscle cells infected with CMV strain AD169 and strain C1F at any time point examined ($P > 0.05$).

The data therefore showed that CMV infection of smooth muscle cells led to a significant increase in the constitutive expression of ICAM-1 on the surface of smooth muscle cells.

5.2.2 The effect of CMV infection on the cell surface expression of LFA-3 on vascular smooth muscle cells.

To investigate the effect of CMV infection on the cell surface expression of LFA-3 molecules, explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10, and subsequently analysed by flow cytometry as described above for ICAM-1. A high percentage of infection was achieved with approximately 95-100% of cells expressing the CMV immediate-early antigen by day 2 post-infection, as determined by flow cytometry using the monoclonal antibody, E13. Figure 5.4 shows representative flow cytometric profiles of the expression of LFA-3 on the surface of smooth muscle cells infected with CMV strain AD169. As shown, CMV strain AD169 induced a marked increase in the cell surface expression of LFA-3. The levels of expression of LFA-3 were observed to be elevated from days 1-5 post-infection, with the maximum expression observed on day 2 post-infection. Figure 5.5 shows representative flow cytometric profiles of the cell surface expression of LFA-3 molecules on smooth muscle cells infected with CMV strain C1F. When smooth muscle cells were infected with CMV strain C1F, it was observed that there was only a very slight, if any, increase in the level of expression of LFA-3 on the surface of infected cells above the level of expression observed on uninfected cells.

The logarithmically acquired data from three separate experiments were converted to fluorescence intensity units and the relative levels of expression of LFA-3 on CMV-

infected cells to their corresponding level of expression on uninfected cells at the same time point were deduced. The infection of smooth muscle cells with CMV strain AD169 induced a marked increase in the level of expression of LFA-3 molecules. The level of expression of LFA-3 expressed in fluorescence intensity units was observed to be elevated from days 1-3 post-infection with the maximum expression observed on day 2 post-infection. The level of expression of LFA-3 was approximately 2.7-fold of that seen on uninfected cells at day 2 post-infection (Figure 5.6). Similarly to the observed expression of ICAM-1 molecules on smooth muscle cells, the level of expression of LFA-3 returned to the base-line levels observed on uninfected cells by day 5 post-infection. On the other hand, the data expressed in fluorescence intensity units revealed that the infection of smooth muscle cells with CMV strain C1F failed to alter the level of expression of LFA-3 molecules compared to the level of expression on uninfected cells. The Mann-Whitney t-test was used to compare the significance of the differences at each time point between the level of expression of LFA-3 on infected and uninfected smooth muscle cells. Using the data from three separate experiments, it was deduced that there was a significant difference in the level of expression of LFA-3 antigens expressed in fluorescence intensity units between AD169-infected and uninfected cells at days 1-3 post-infection ($P< 0.05$). There was also a significant difference between the level of expression of LFA-3 between AD169 and C1F-infected smooth muscle cells ($P> 0.05$), consistent with the observation that the level of expression of LFA-3 on C1F-infected smooth muscle cells was comparable to, and not significantly different from, that on uninfected cells.

Thus, in contrast to the results observed for ICAM-1, infection of explant-migrated smooth muscle cells with CMV strain AD169 induced an increase in the expression of LFA-3 molecules, whilst infection with CMV strain C1F had no effect on the level of expression of LFA-3.

5.2.3 The effect of CMV infection on the cell surface expression of VCAM-1 on vascular smooth muscle cells.

To investigate the effect of CMV infection on the cell surface expression of VCAM-1 molecules, explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10, and subsequently analysed by flow cytometry as described above for ICAM-1 and LFA-3. A high percentage of infection was achieved, with approximately 95-100% of cells expressing the CMV immediate-early antigen by day 2 post-infection. The representative cytometric profiles of

the expression of VCAM-1 on the surface of smooth muscle cells infected with CMV strain AD169 and strain C1F are shown in Figures 5.7 and 5.8, respectively. When explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F, it was observed that the expression of VCAM-1 on the surface of infected cells was undetectable. The level of expression of VCAM-1 on CMV-infected cells were similar to those observed on uninfected cells or on cells treated with an isotype-matched control antibody (data not shown). Human umbilical vein endothelial cells stimulated for 24 hours with 10 μg/ml tumour necrosis factor served as a positive control for the expression of VCAM-1 (Figure 5.7).

The logarithmically acquired data from three separate experiments were converted to fluorescence intensity units. Using the data expressed as fluorescence intensity units, it was observed that the level of expression of VCAM-1 on CMV-infected cells and uninfected cells at all time points examined were comparable (Figure 5.9). The Mann-Whitney t-test was used to compare the significance of the differences at each time point between the level of expression of VCAM-1 on infected and uninfected smooth muscle cells. It was deduced that there was no significant difference between the fluorescence intensity units of VCAM-1 antigens expressed on CMV-infected and uninfected cells at days 1-5 post-infection ($P > 0.05$). There was no difference in the level of expression of VCAM-1 between CMV strain AD169 and strain C1F-infected smooth muscle cell cultures ($P > 0.05$). The level of expression of VCAM-1 molecules in infected and uninfected smooth muscle cells were comparable to the levels of expression obtained with an IgG isotype matched antibody.

Thus, in contrast to the results observed for ICAM-1, infection of explant-migrated smooth muscle cells with CMV strain AD169 or strain C1F did not induce the expression of VCAM-1 molecules on the surface of smooth muscle cells.

5.2.4 The effect of CMV infection on the cell surface expression of the vitronectin receptor on vascular smooth muscle cells.

To investigate the effect of CMV infection on the cell surface expression of the vitronectin receptor (integrin $\alpha_V\beta_3$), explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10, and subsequently analysed by flow cytometry as described above. A high percentage of infection was achieved with approximately 95-100% of cells expressing the CMV immediate-early antigen by day 2 post-infection. The representative cytometric profiles of

the expression of the vitronectin receptor on the surface of smooth muscle cells infected with CMV strain AD169 and strain C1F are shown in Figures 5.10 and 5.11, respectively. When explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F, it was observed that the expression of the vitronectin receptor on the surface of infected cells was undetectable. The level of expression of the vitronectin receptor on infected smooth muscle cells was similar to that observed on uninfected cells, or on cells treated with an irrelevant isotype-matched control antibody (data not shown). EBV-transformed B cells served as a positive control for the expression of the vitronectin receptor (Figure 5.10).

The logarithmically acquired data from three separate experiments was converted to fluorescence intensity units. Using the data expressed as fluorescence intensity units, it was observed that the level of expression of the vitronectin receptor on CMV-infected cells and uninfected cells at all time points examined were comparable (Figure 5.12). The Mann-Whitney t-test was used to compare the significance of the differences at each time point between the level of expression of the vitronectin receptor on infected and uninfected smooth muscle cells. It was deduced that there was no significant difference between the fluorescence intensity units of the vitronectin receptor expressed on CMV-infected and uninfected cells at days 1-5 post-infection ($P > 0.05$). There was no difference in the level of expression of the vitronectin receptor between CMV strain AD169 and strain C1F-infected smooth muscle cells. The level of expression of the bitronectin receptor in infected and uninfected smooth muscle cells were comparable to the levels of expression obtained with an IgG isotype matched antibody.

Thus, the vitronectin receptor was undetectable in explant-migrated smooth muscle cells, and infection with CMV strain AD169 or strain C1F did not induce its expression on the surface of the smooth muscle cell.

5.2.5 The effect of CMV infection on the cell surface expression of class I MHC antigens on vascular smooth muscle cells.

In order to investigate the effect of CMV infection on the cell surface expression of class I MHC molecules, explant-migrated smooth muscle cells were infected with CMV strain AD169 or strain C1F at a multiplicity of infection of 10. They were then analysed for the expression of class I MHC antigens at various days post-infection by flow cytometry. A high percentage of infection, approximately 95-100% was achieved in this cell type as determined by flow cytometry using the CMV IE-antigen specific monoclonal

antibody, E13. Flow cytometry for the detection of class I MHC antigens was performed with the monoclonal antibody PA.26, which is specific for a conformational determinant on the class I HLA heterodimer. The expression of class I MHC on the surface of the infected and uninfected cells was analysed at days 1 through to 5 post-infection. Figure 5.13 shows representative flow cytometric profiles of the expression of class I MHC antigens on the surface of smooth muscle cells infected with CMV strain AD169 relative to uninfected cells. As shown, infection with CMV strain AD169 resulted in a marked and progressive decrease in the level of expression of class I MHC antigens. The level of expression of class I MHC antigens was observed to be reduced from days 2-5 post-infection. Figure 5.14 shows representative flow cytometric profiles of the expression of class I MHC antigens on the surface of explant-migrated smooth muscle cells infected with CMV strain C1F, relative to uninfected cells. The infection of smooth muscle cells with CMV strain C1F was also shown to alter the level of class I MHC antigens with a profile similar to that observed with CMV strain AD169.

The logarithmically acquired data from three separate experiments was converted to fluorescence intensity units, and the relative level of expression of class I MHC antigens on CMV-infected cells compared to the corresponding level of expression on uninfected cells at the same time point was deduced. The data expressed in fluorescence intensity units revealed that at day 2 post-infection, where the maximal expression of ICAM-1 and LFA-3 was observed on infected smooth muscle cells, class I MHC antigens on cells infected with CMV strain AD169 were expressed at only 38% of the level found on uninfected cells (Figure 5.15). The expression of class I MHC antigens continued to fall on cells infected with CMV strain AD169, such that it was only 15% of the uninfected cell level on day 5. It was evident from the data obtained that the progressive decline in the level of expression of class I MHC was independent of the virus strain used to infect the cells as similar reductions in class I expression were observed in smooth muscle cells infected with CMV strain C1F. The Mann-Whitney t-test was used to compare the significance of the difference at each time point between the levels of expression of class I MHC antigens on infected and uninfected smooth muscle cells. The data from three separate experiments was examined, and it was deduced that there was a significant difference between the level of expression of class I MHC antigens expressed on infected and uninfected cells at days 1-5 post-infection ($P < 0.05$) for both virus strains. There was no significant difference in the decrease in the expression of class I MHC antigens between CMV strain AD169-infected and C1F-infected explant-migrated smooth muscle cells ($P > 0.05$).

Thus, as found previously in fibroblasts which are also fully permissive to CMV infection, CMV infection of explant-migrated smooth muscle cells led to a marked decrease in the cell surface expression of class I MHC antigens.

5.2.6 The effect of CMV infection on the cell surface expression of class II MHC antigens on vascular smooth muscle cells.

The effect of CMV infection on the cell surface expression of class II MHC antigens was investigated. Explant-migrated smooth muscle cells were infected with CMV strains AD169 or strain C1F at a multiplicity of infection of 10. A high percentage of infection, approximately 95-100%, was achieved in this cell type. The level of expression of class II MHC antigens was then analysed using a monoclonal antibody specific for a non-polymorphic determinant of the HLA-DR molecule. Figure 5.16 shows representative flow cytometric profiles of the expression of class II MHC antigens on the surface of smooth muscle cells infected with CMV strain AD169 relative to uninfected cells. As shown, the analysis of the cell surface expression of class II MHC antigens on mock-infected and infected smooth muscle cells revealed that class II MHC antigens were not expressed on these cells. The expression of class II MHC antigens on the surface of Epstein-Barr virus transformed B cells served as a positive control for the expression of class II MHC antigens in these experiments (Figure 5.16). Figure 5.17 shows representative flow cytometric profiles of the expression of class II MHC antigens on the surface of smooth muscle cells infected with CMV strain C1F, relative to uninfected cells. The infection of smooth muscle cells with CMV strain C1F was shown to have no effect on the level of expression of class II MHC antigens, as observed for cells infected with CMV strain AD169.

The logarithmically acquired data from three separate experiments was converted to fluorescence intensity units and the relative level of expression of class II MHC antigens on CMV-infected cells compared to the corresponding level of expression on uninfected cells at the same time point was deduced. The data expressed in fluorescence intensity units, revealed that there was no expression of class II MHC antigens observed on the surface of either CMV-infected or on uninfected cells (Figure 5.18). These observations were obtained regardless on virus strain used to infect the cells. The Mann-Whitney t-test was used to compare the significance of the differences at each time point between the level of expression of class II MHC antigens on infected and uninfected smooth muscle cells. The data showed that there was no significant difference between the level of expression of class II MHC antigens expressed in fluorescence intensity units

on infected and uninfected cells at days 1-5 post-infection ($P > 0.05$). There was no significant difference in the level of expression of class II MHC antigens between CMV strain AD169-infected and C1F-infected explant-migrated smooth muscle cells ($P > 0.05$).

Hence, no expression of class II MHC antigens was observed on the cell surface of explant-migrated smooth muscle cells. CMV infection did not induce the expression of class II MHC antigens on these cells.

5.2.7 The effect of CMV infection on the ability of interferon-γ to stimulate the cell surface expression of class I MHC antigens on vascular smooth muscle cells.

In section 5.2.5, it was demonstrated that CMV infection resulted in a marked and progressive decrease in the expression of class I MHC antigens. In order to investigate the effect of CMV infection on the ability of IFN-γ to overcome the decrease in class I MHC expression, explant-migrated smooth muscle cells were either infected with CMV strain AD169 at a multiplicity of infection of 10, or left uninfected. Infected and uninfected cells were either treated with 10μg/ml IFN-γ or left untreated. They were then analysed at various days post-infection by flow cytometry for the expression of class I MHC antigens using the monoclonal antibody PA.26 as described above. Figure 5.19 shows representative flow cytometric profiles of the expression of class I MHC antigens on the surface of smooth muscle cells infected with CMV strain AD169, relative to uninfected cells, that were either treated with IFN-γ or left untreated. As shown, IFN-γ stimulation of uninfected smooth muscle cells induced an increase in the expression of class I MHC antigens above the baseline levels of uninfected-unstimulated smooth muscle cells. In CMV-infected smooth muscle cells, IFN-γ stimulation had no effect on the CMV-induced down-regulation of class I MHC expression. There was no apparent difference in the median channel fluorescence between infected-unstimulated and infected-stimulated smooth muscle cells.

The effect of IFN-γ stimulation was assessed by determining the percentage change in the level of expression of class I MHC antigen between uninfected-stimulated and uninfected-unstimulated smooth muscle cells. The data from three separate experiments revealed that the level of expression of class I MHC on uninfected cells was elevated from day 1-5 post-stimulation, with the maximum expression observed at day 3 post-stimulation (Figure 5.20). Statistical analysis, using the Mann-Whitney t-test revealed, that there was a significant difference in the level of expression of class I MHC between uninfected-stimulated and uninfected-unstimulated smooth muscle cells from

days 1-5 post-stimulation ($P < 0.05$). In contrast, there was no significant difference at any time point in the level of expression of class I MHC antigen between stimulated and unstimulated infected smooth muscle cell cultures ($P > 0.05$).

Thus, CMV infection of smooth muscle cells prevented the increase of class I MHC antigens induced by IFN-γ. Furthermore, the down-regulation of class I MHC antigens as a result of CMV infection was not prevented by IFN-γ stimulation. The effect of infection with CMV strain C1F on the ability of interferon-γ to stimulate the cell surface expression of class I MHC antigens on vascular smooth muscle cells was not investigated.

5.2.8 The effect of CMV infection on the ability of interferon-γ to stimulate the cell surface expression of class II MHC antigens on vascular smooth muscle cells.

The effect of IFN-γ stimulation of CMV-infected smooth muscle cells was examined in order to assess the ability of IFN-γ to induce class II MHC antigens on infected smooth muscle cells. Explant-migrated smooth muscle cells were either infected with CMV strain AD169 at a multiplicity of infection of 10, or left uninfected. Infected and uninfected cells were either treated with 10μg/ml IFN-γ or left untreated. They were then analysed at various days post-infection by flow cytometry for the expression of class II MHC antigens, using a monoclonal antibody reactive with a non-polymorphic determinant on HLA-DR class II molecules. Figure 5.21 shows representative flow cytometric profiles of the expression of class II MHC antigens on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169, relative to uninfected cells, that were either treated with IFN-γ or left untreated. As shown, IFN-γ stimulation of uninfected smooth muscle cells failed to induce the expression of class II MHC antigens on these cells. There was no difference in the flow cytometric profiles between uninfected-stimulated and uninfected-unstimulated smooth muscle cells. Similarly, it was observed that IFN-γ stimulation of CMV-infected smooth muscle cells failed to induce the expression of class II MHC antigens on these cells. There was no difference in the flow cytometric profiles between infected-stimulated and infected-unstimulated smooth muscle cells.

Thus in contrast to the increased level of expression of class I MHC antigens seen after IFN-γ-stimulation of uninfected smooth muscle cells, IFN-γ treatment failed to induce the expression of class II MHC antigens on the cell surface of either uninfected or infected smooth muscle cells.

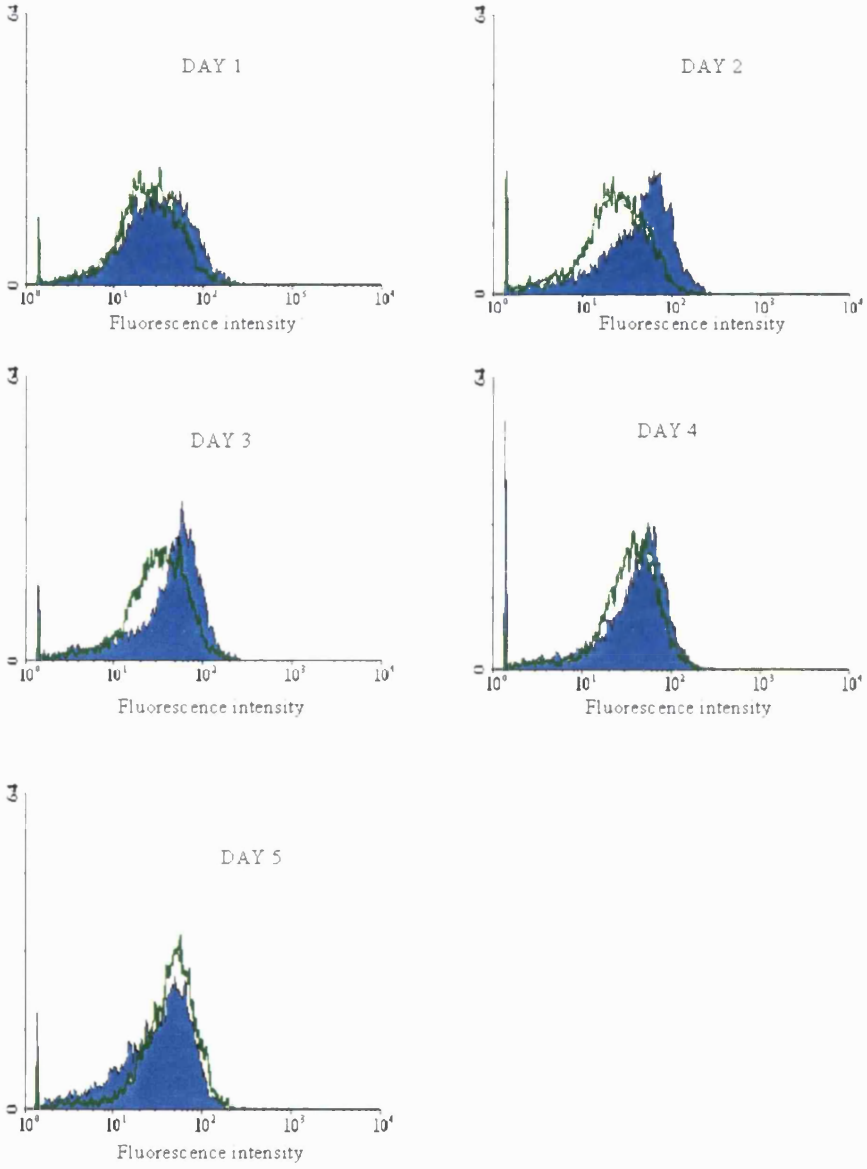


Figure 5.1. Flow cytometric profiles of the cell surface expression of ICAM-1 on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain AD169 at an MOI of 10, and the cell surface expression of ICAM-1 was determined by flow cytometry at various times post-infection. The levels of expression of ICAM-1 on infected cells (blue) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

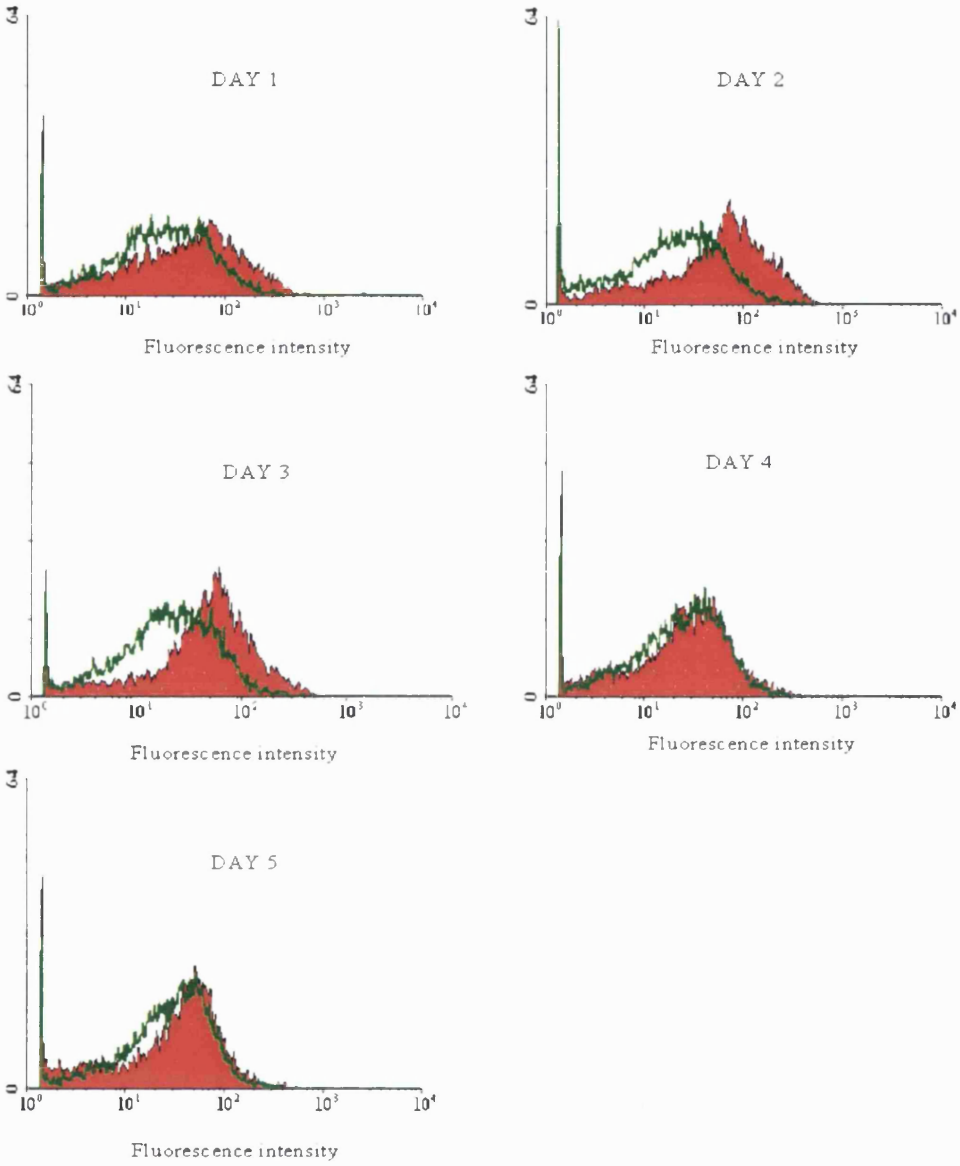


Figure 5.2. Flow cytometric profiles of the cell surface expression of ICAM-1 on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain C1F at an MOI of 10, and the cell surface expression of ICAM-1 was determined by flow cytometry at various times post-infection. The levels of expression of ICAM-1 on infected cells (red) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

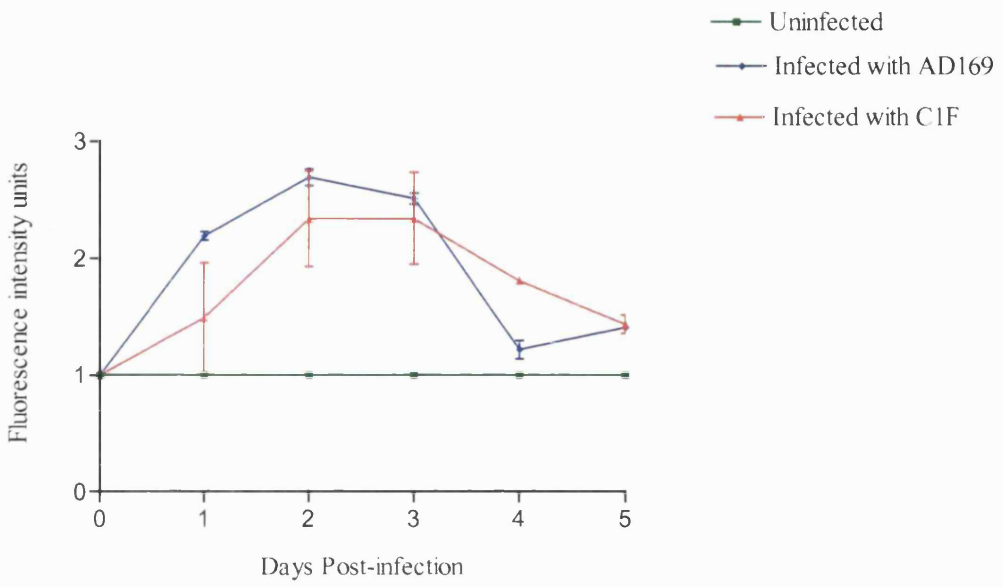


Figure 5.3. The expression of ICAM-1 on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strains AD169 or C1F at an MOI of 10, and the expression of ICAM-1 on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of ICAM-1 on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of ICAM-1 expression on uninfected cells.

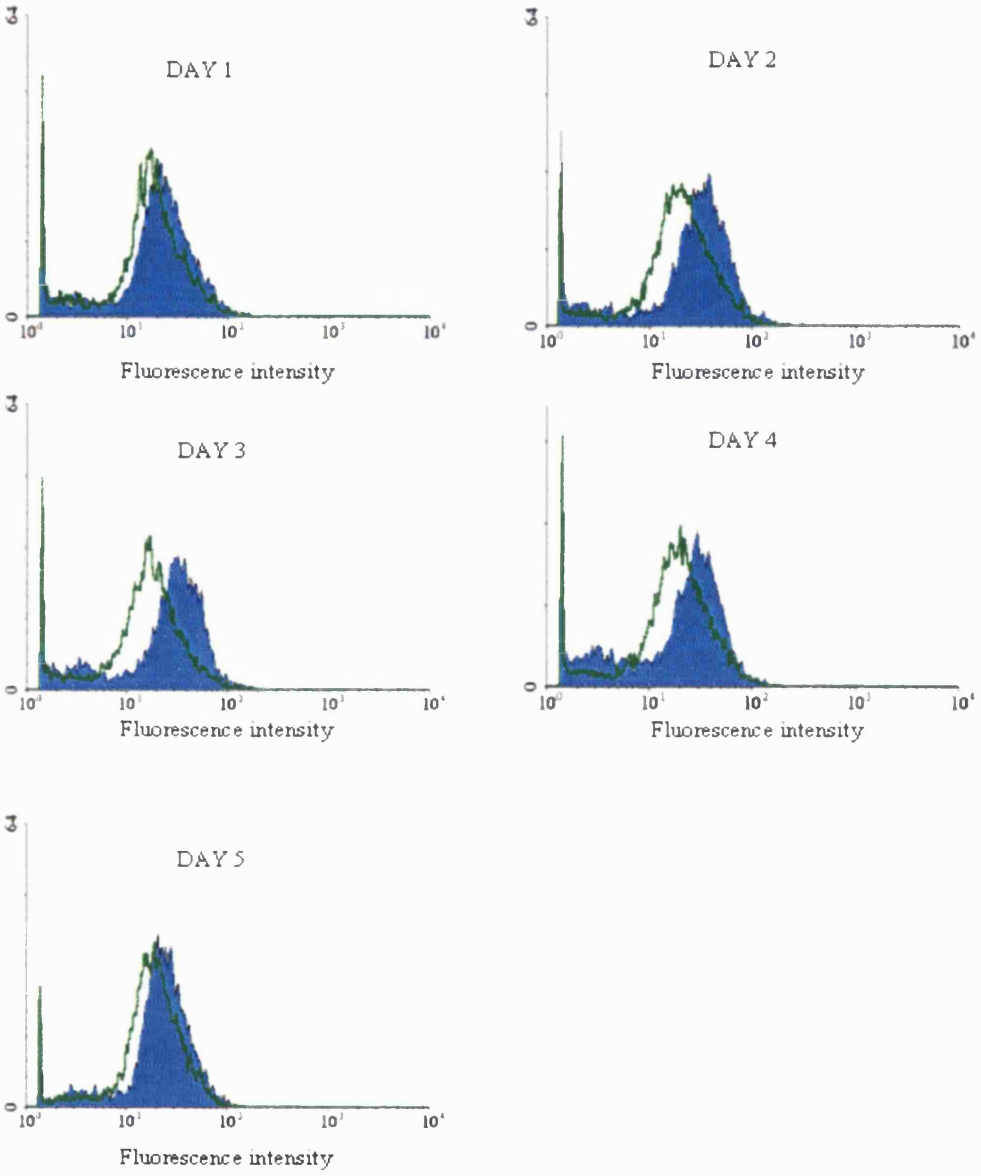


Figure 5.4. Flow cytometric profiles of the cell surface expression of LFA-3 on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain AD169 at an MOI of 10, and the cell surface expression of LFA-3 was determined by flow cytometry at various times post-infection. The levels of expression of LFA-3 on infected cells (blue) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

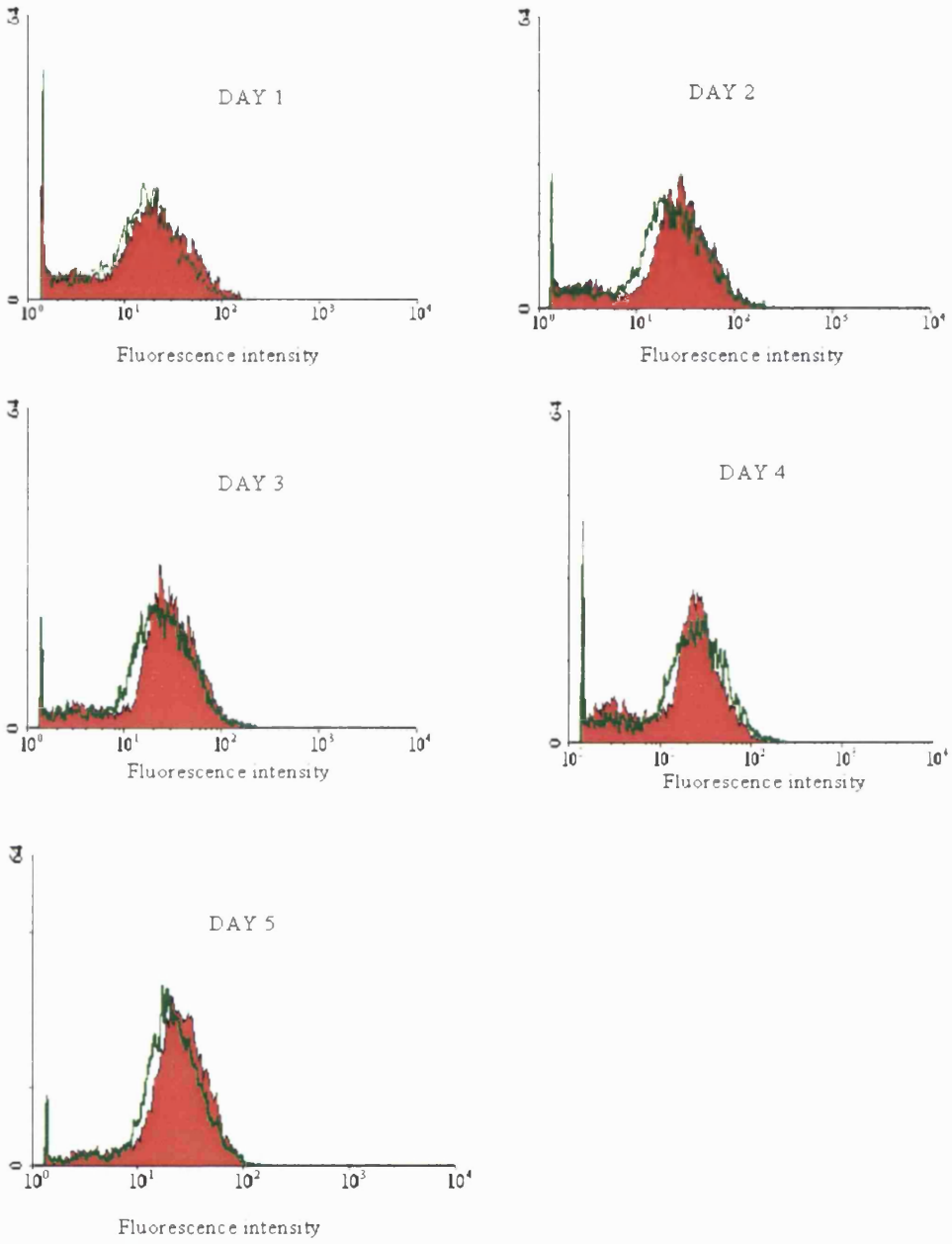


Figure 5.5. Flow cytometric profiles of the cell surface expression of LFA-3 on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain C1F at an MOI of 10, and the cell surface expression of LFA-3 was determined by flow cytometry at various times post-infection. The levels of expression of LFA-3 on infected cells (red) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

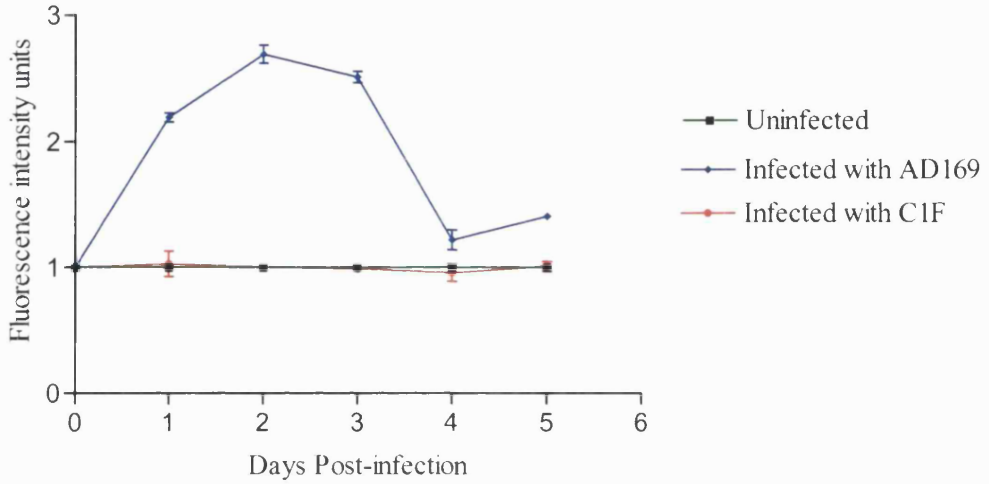


Figure 5.6. The expression of LFA-3 on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strains AD169 or C1F at an MOI of 10, and the expression of LFA-3 on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of LFA-3 on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of LFA-3 expression on uninfected cells.

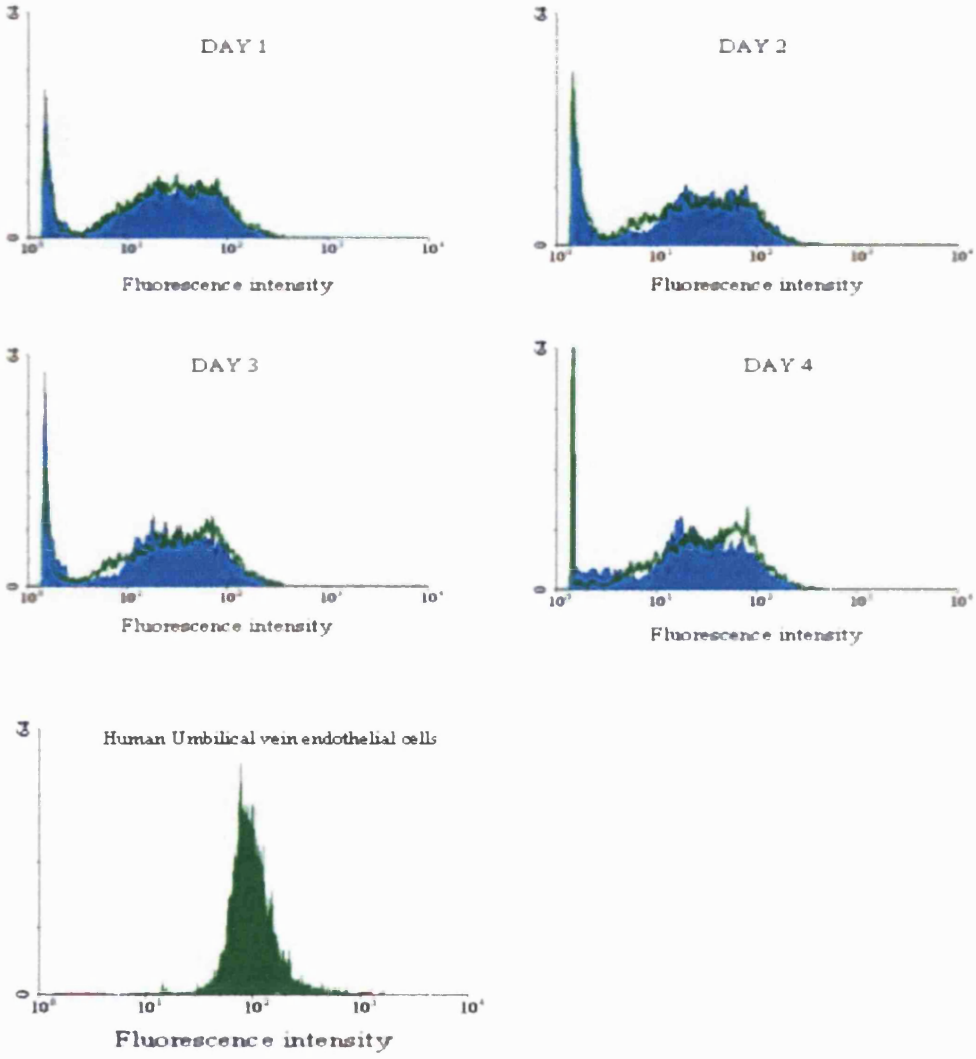


Figure 5.7. Flow cytometric profiles of the cell surface expression of VCAM-1 on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain AD169 at an MOI of 10, and the cell surface expression of VCAM-1 was determined by flow cytometry at various times post-infection. The levels of expression of VCAM-1 on infected cells (blue) compared to uninfected cells (green) at days 1-4 post-infection are shown. The expression of VCAM-1 on the surface of human umbilical vein endothelial cells stimulated for 24 hours with 10 μg/ml tumour necrosis factor served as a positive control for the expression of VCAM-1, and is shown as indicated in the bottom left panel. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

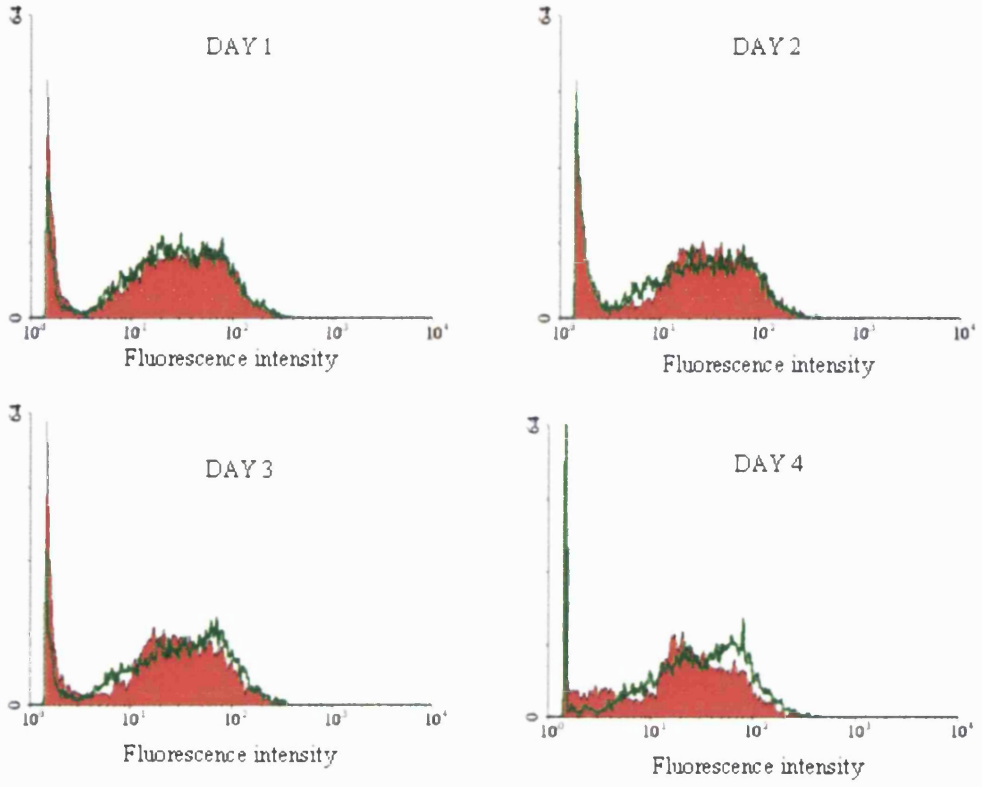


Figure 5.8. Flow cytometric profiles of the cell surface expression of VCAM-1 on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain C1F at an MOI of 10, and the cell surface expression of VCAM-1 was determined by flow cytometry at various times post-infection. The levels of expression of VCAM-1 on infected cells (red) compared to uninfected cells (green) at days 1-4 post-infection are shown. Positive control for VCAM-1 staining is shown in Figure 5.7. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

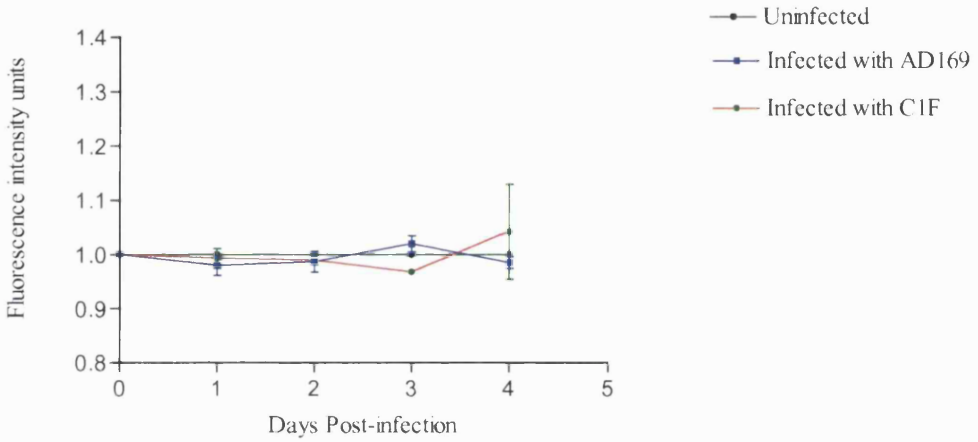


Figure 5.9. The expression of VCAM-1 on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strains AD169 or strain C1F at an MOI of 10, and the expression of VCAM-1 on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of VCAM-1 on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of VCAM-1 expression on uninfected cells.

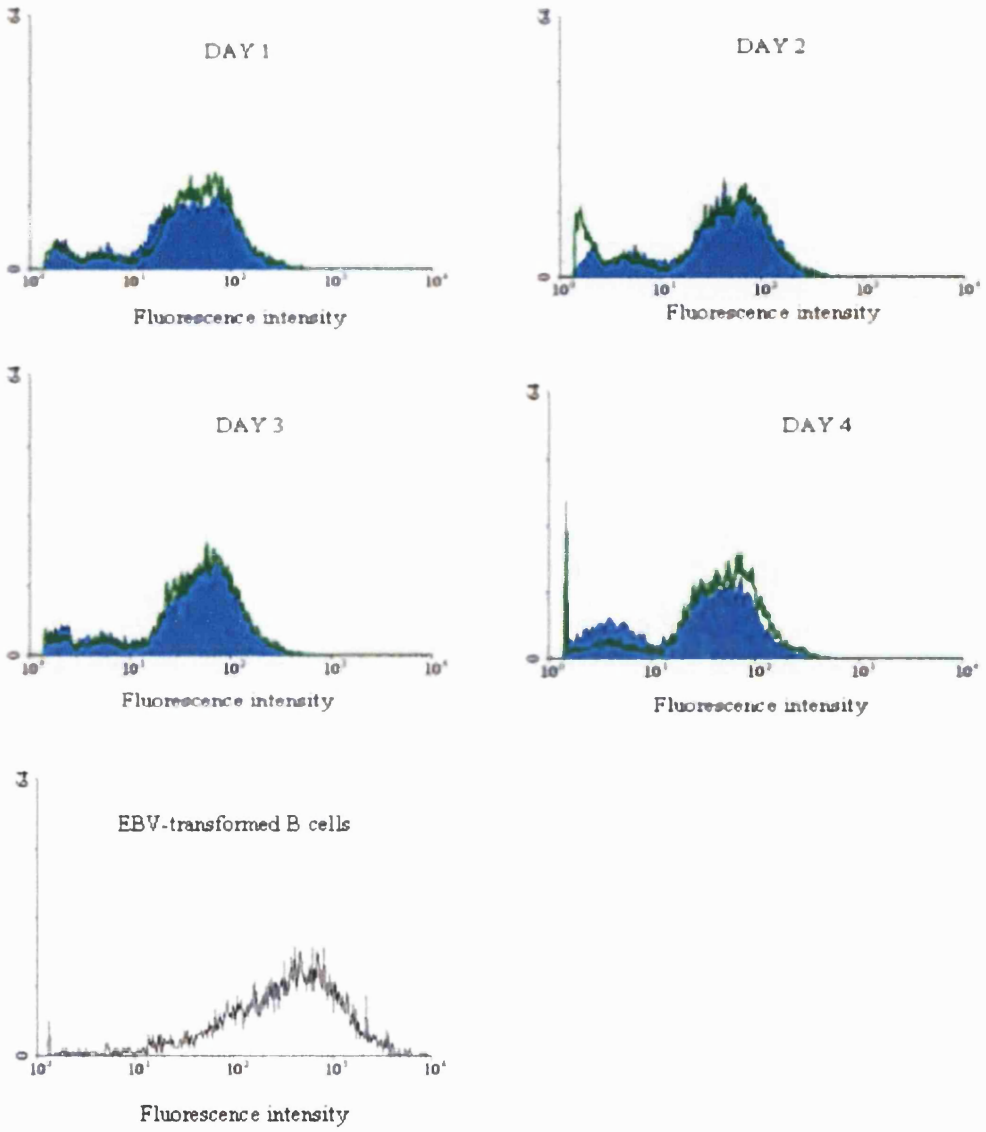


Figure 5.10. Flow cytometric profiles of the cell surface expression of the vitronectin receptor on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain AD169 at an MOI of 10, and the cell surface expression of the vitronectin receptor was determined by flow cytometry at various times post-infection. The levels of expression of the vitronectin receptor on infected cells (blue) compared to uninfected cells (green) at days 1-4 post-infection are shown. The expression of the vitronectin receptor on the surface of EBV-transformed B cells served as a positive control for the expression of the vitronectin receptor, and is shown as indicated in the bottom left panel. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

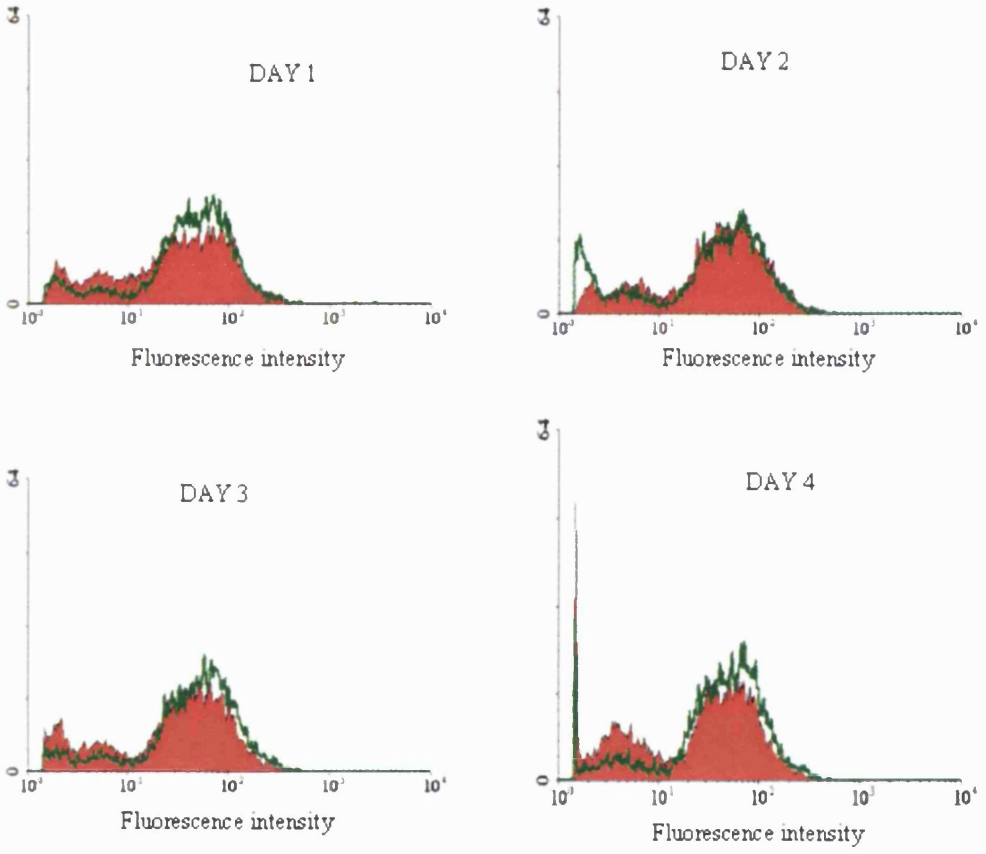


Figure 5.11. Flow cytometric profiles of the cell surface expression of the vitronectin receptor on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strain C1F at an MOI of 10, and the cell surface expression of the vitronectin receptor was determined by flow cytometry at various times post-infection. The levels of expression of the vitronectin receptor on infected cells (red) compared to uninfected cells (green) at days 1-4 post-infection are shown. Positive control for the vitronectin receptor staining is shown in Figure 5.10. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

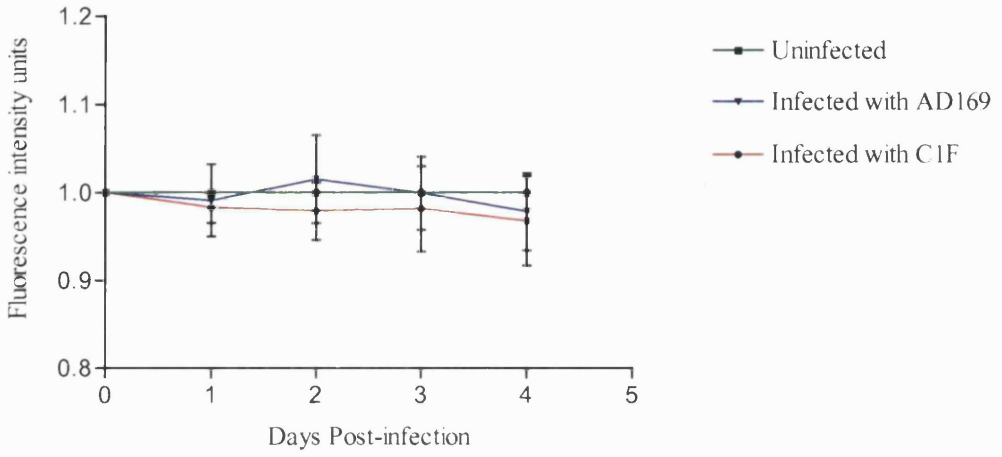


Figure 5.12. The expression of the integrin vitronectin receptor on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected with CMV strains AD169 or C1F at an MOI of 10, and the expression of the vitronectin receptor on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of the vitronectin receptor on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of the vitronectin receptor expression on uninfected cells.

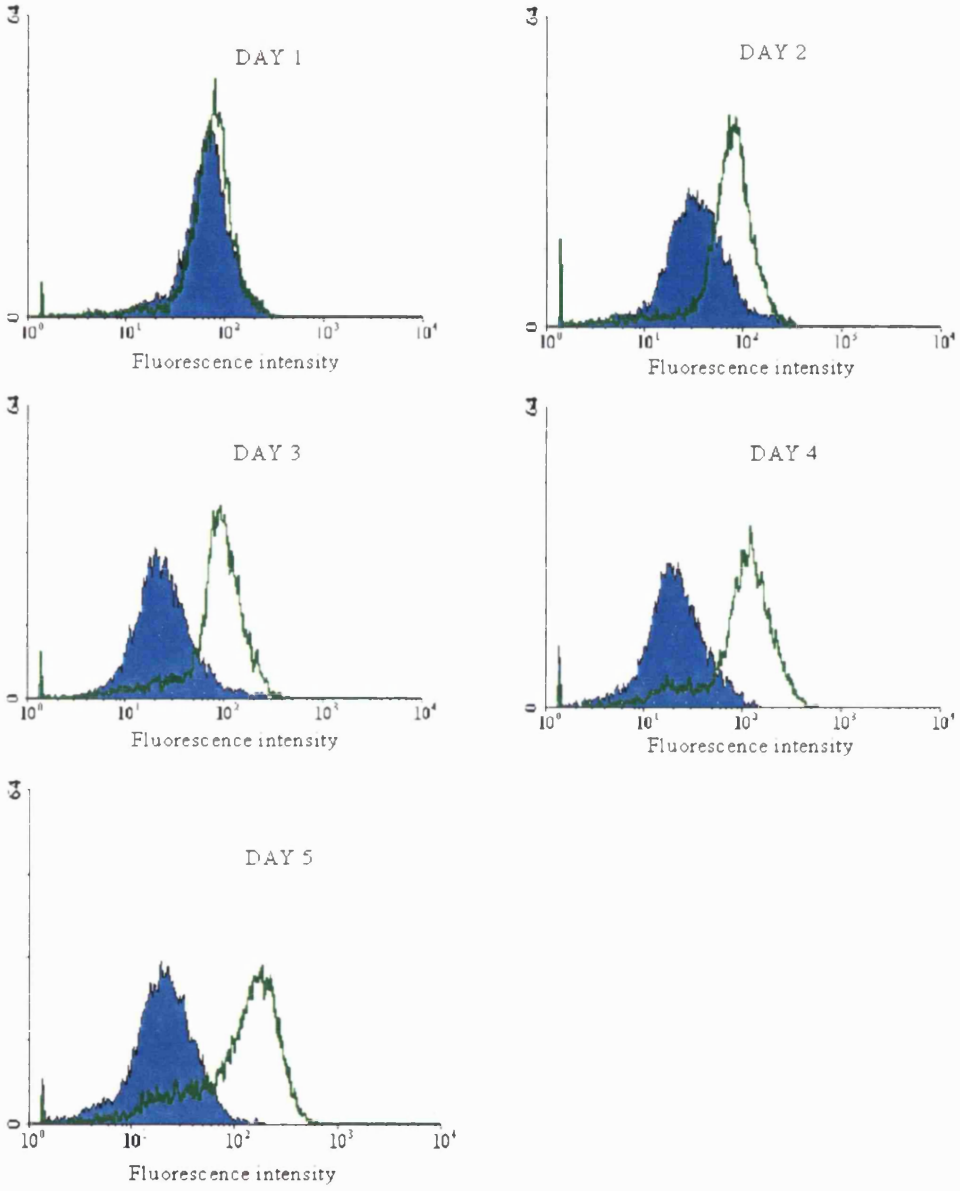


Figure 5.13. Flow cytometric profiles of the cell surface expression of class I MHC antigens on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strain AD169, and the cell surface expression of class I MHC antigens was determined by flow cytometry at various times post-infection. The levels of expression of class I MHC antigens on infected cells (blue) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

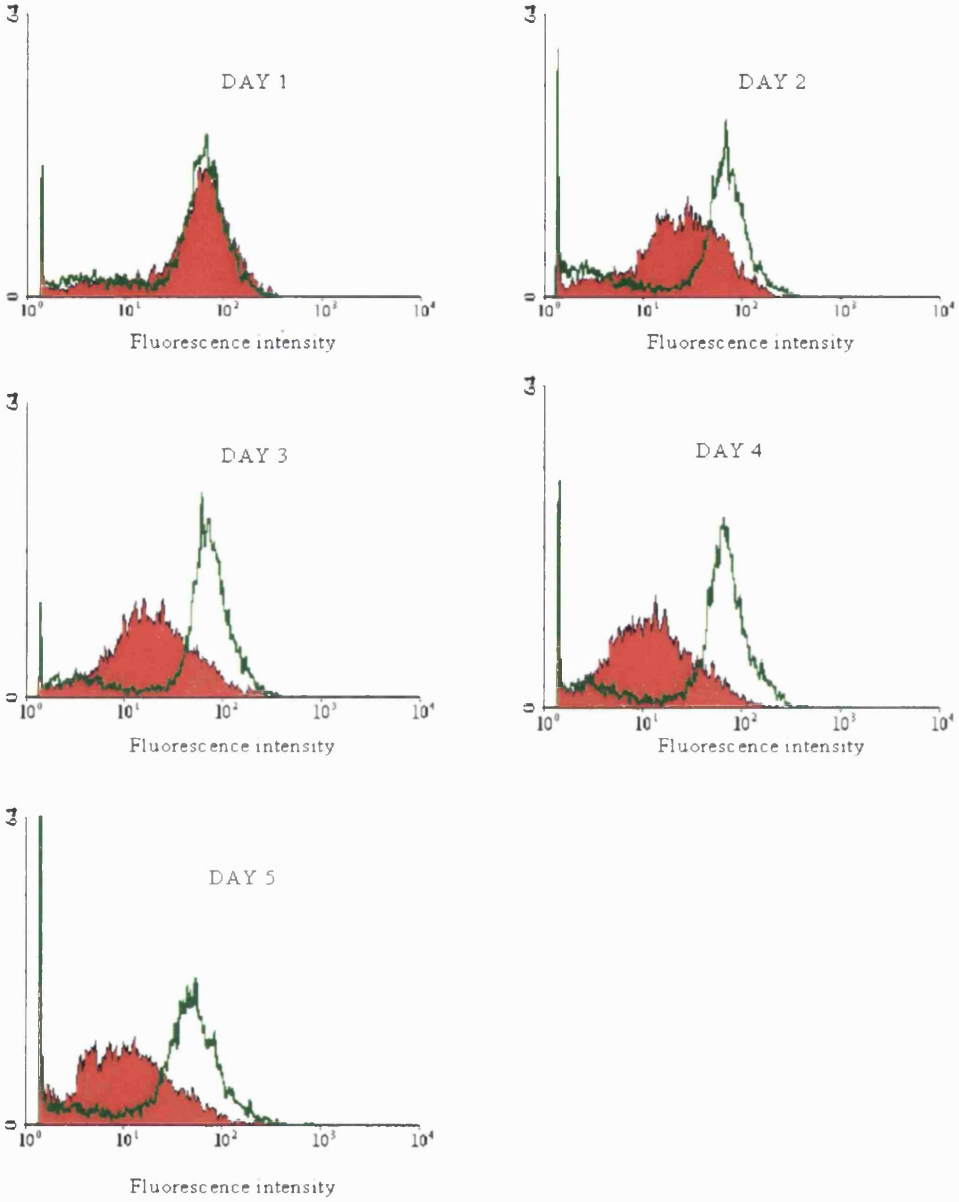


Figure 5.14. Flow cytometric profiles of the cell surface expression of class I MHC antigens on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strain C1F, and the cell surface expression of class I MHC antigens was determined by flow cytometry at various times post-infection. The levels of expression of class I MHC antigens on infected cells (red) compared to uninfected cells (green) at days 1-5 post-infection are shown. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

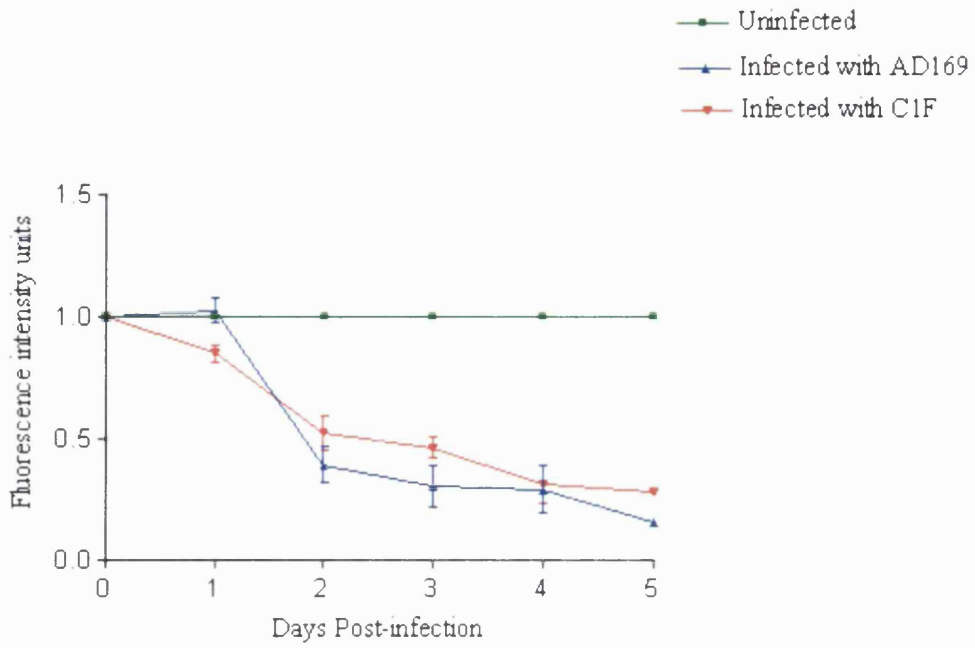


Figure 5.15. The expression of class I MHC antigens on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explanted migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strains AD169 or C1F, and the expression of class I MHC antigens on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of class I antigens on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of class I antigens expression on uninfected cells.

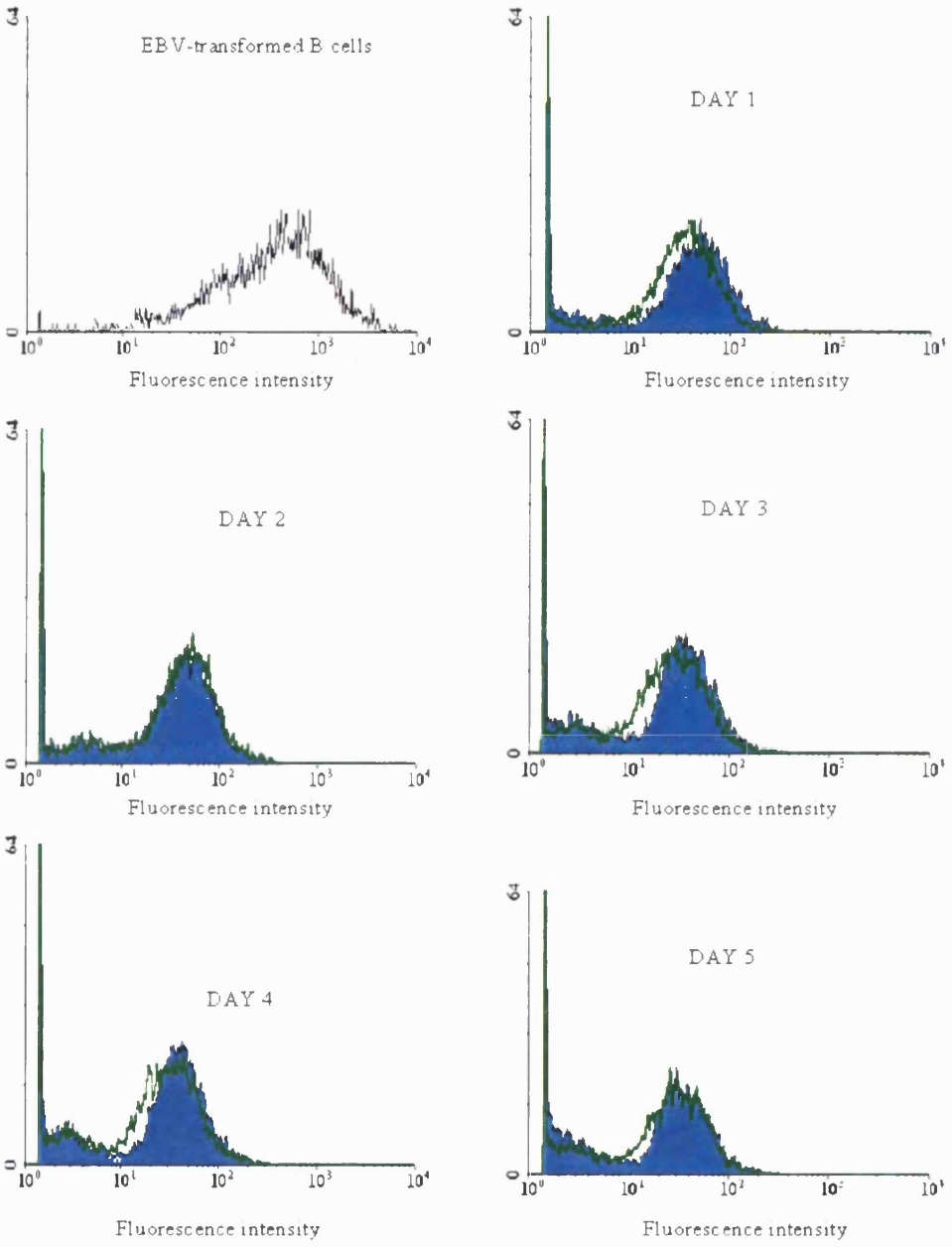


Figure 5.16. Flow cytometric profiles of the cell surface expression of class II MHC antigens on explant-migrated smooth muscle cells infected with CMV strain AD169.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strain AD169, and the cell surface expression of class II MHC antigens was determined by flow cytometry at various times post-infection. The levels of expression of class II MHC antigens on infected cells (blue) compared to uninfected cells (green) are shown. The expression of class II MHC antigens on the surface of EBV-transformed B cells served as a positive control for class II MHC antigen expression, and is shown as indicated in the top left panel. The number of cells is shown on the y-axis on a linear scale, and the median fluorescence intensity on the x-axis on a logarithmic scale.

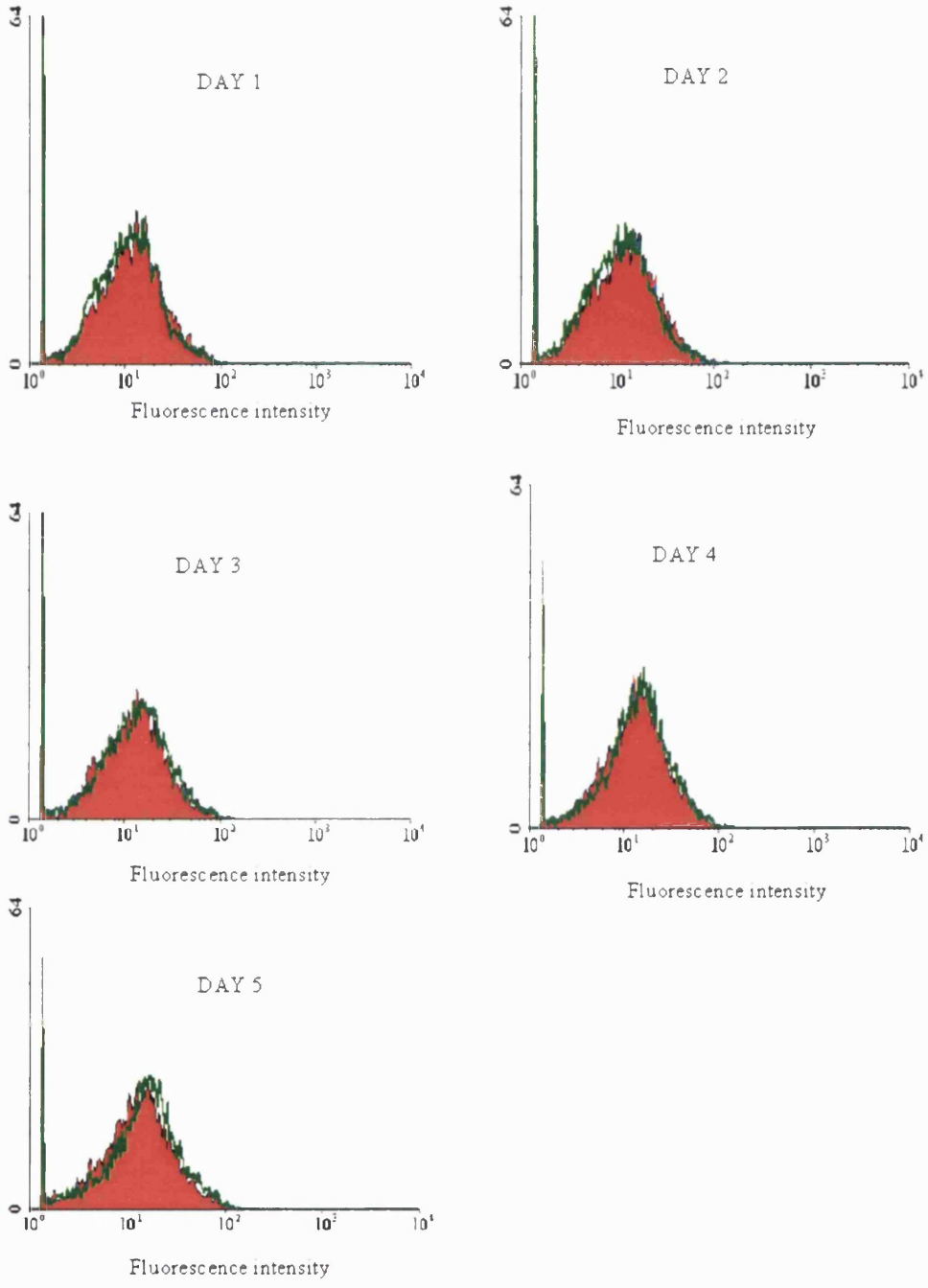


Figure 5.17. Flow cytometric profiles of the cell surface expression of class II MHC antigens on explant-migrated smooth muscle cells infected with CMV strain C1F.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strain C1F, and the cell surface expression of class I MHC antigens was determined by flow cytometry at various times post-infection. The levels of expression of class II MHC antigens on infected cells (red) compared to uninfected cells (green) at days 1-5 post-infection are shown. Positive control for class II staining is shown in Figure 5.16. The number of cells is shown on the y-axis on a linear scale and the median fluorescence intensity on the x-axis on a logarithmic scale.

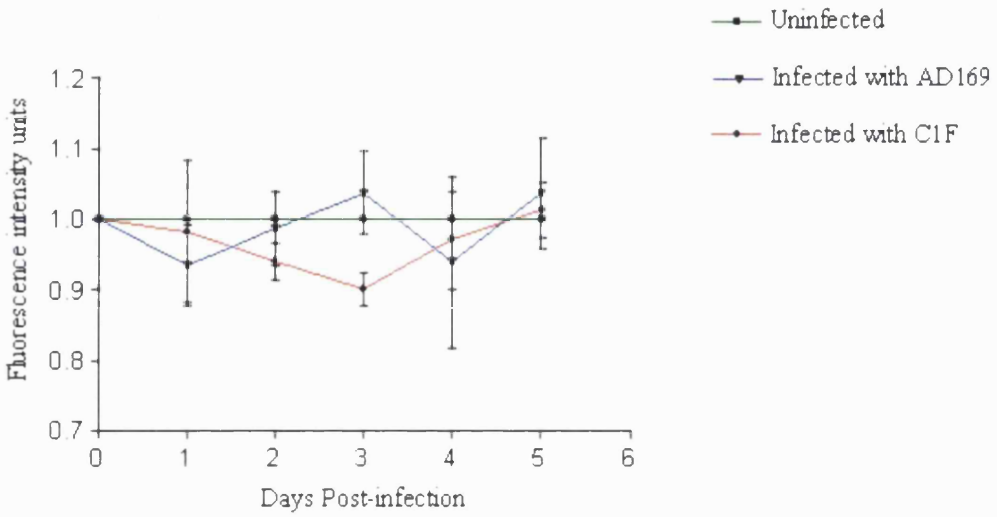


Figure 5.18. The expression of class II MHC antigens on the surface of explant-migrated smooth muscle cells infected with CMV strain AD169 or strain C1F versus uninfected cells at various times post-infection.

Explanted migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strains AD169 or C1F, and the expression of class II MHC antigens on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the levels of expression of class II antigens on infected and uninfected cells expressed as the mean ± standard deviation of fluorescence intensity units derived from three separate experiments at days 1-5 post-infection. The green line represents the baseline level of class II antigens expression on uninfected cells.

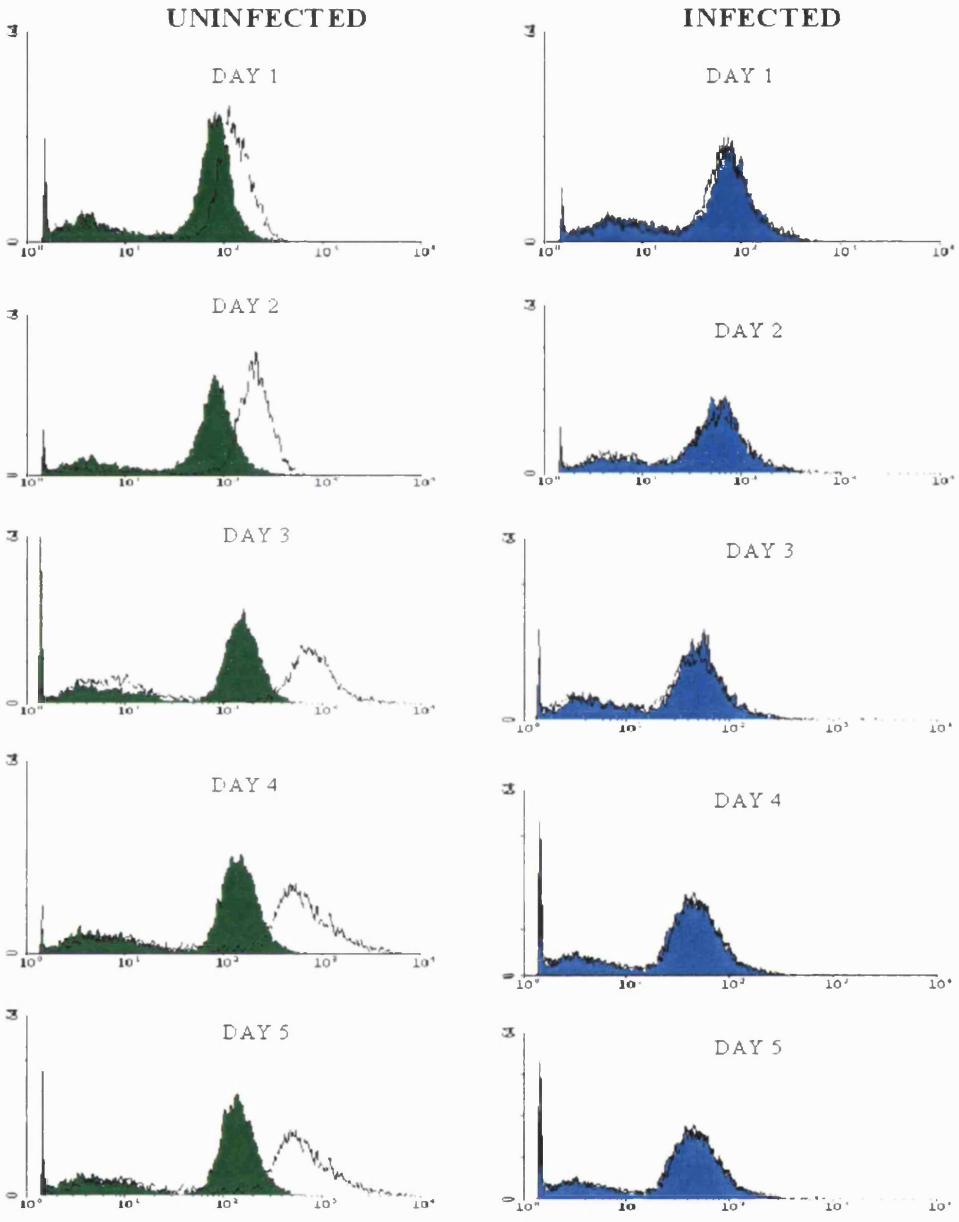


Figure 5.19. Representative flow cytometric profiles showing the effect of CMV infection on the ability of IFN-γ to stimulate the expression of class I MHC antigens on the surface of explant-migrated smooth muscle cells.

Explant-migrated smooth muscle cells at passage 3 were either left uninfected, or infected at an MOI of 10 with CMV strain AD169. Infected and uninfected cells were then either treated with 10 μg/ml IFN-γ or left untreated. The level of expression of class I MHC antigens was determined by flow cytometry at various times post-infection. The levels of expression of class I MHC antigens on untreated infected cells (blue) and untreated uninfected cells (green) compared to the expression of class I MHC antigens on cells treated with IFN-γ (open-black) for both uninfected and infected cells are shown. The number of cells is shown on the y-axis on a linear scale and the median fluorescence intensity on the x-axis on a logarithmic scale.

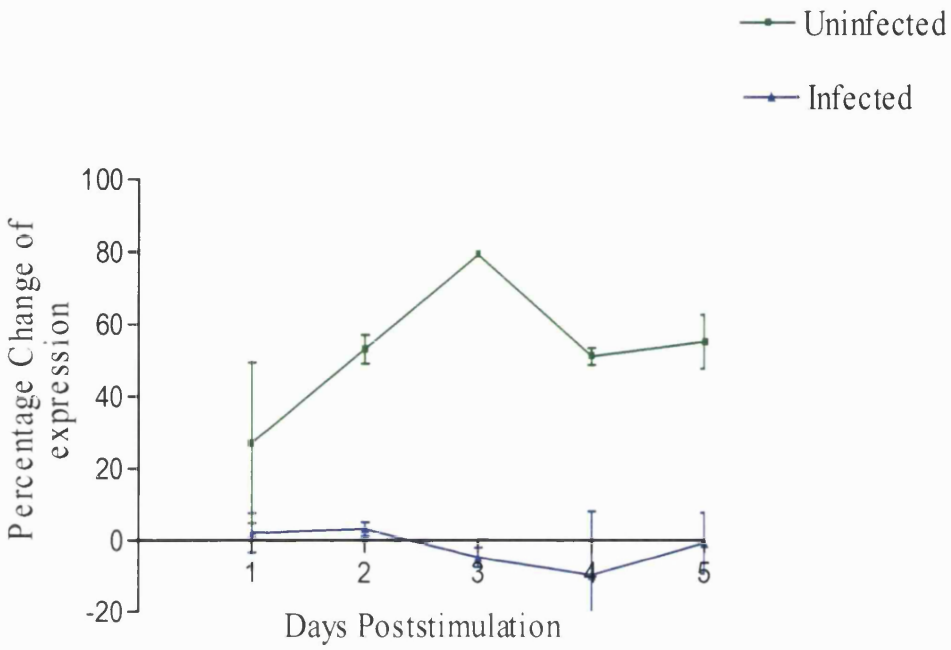


Figure 5.20. The ability of IFN-γ treatment to stimulate the expression of class I MHC antigens on the surface of smooth muscle cells.

Explant-migrated smooth muscle cells at passage 3 were either infected with CMV strain AD169 at an MOI of 10 or left uninfected. Infected and uninfected cells were either stimulated with 10 μg/ml IFN-γ or left unstimulated. The level of expression of class I MHC antigens on the cell surface was determined by flow cytometry at various times post-infection. The data shown represents the mean ± standard deviation of the median fluorescence intensity values from three separate experiments from which the percentage change of class I MHC antigen expression between uninfected-stimulated and infected-stimulated smooth muscle cells has been calculated.

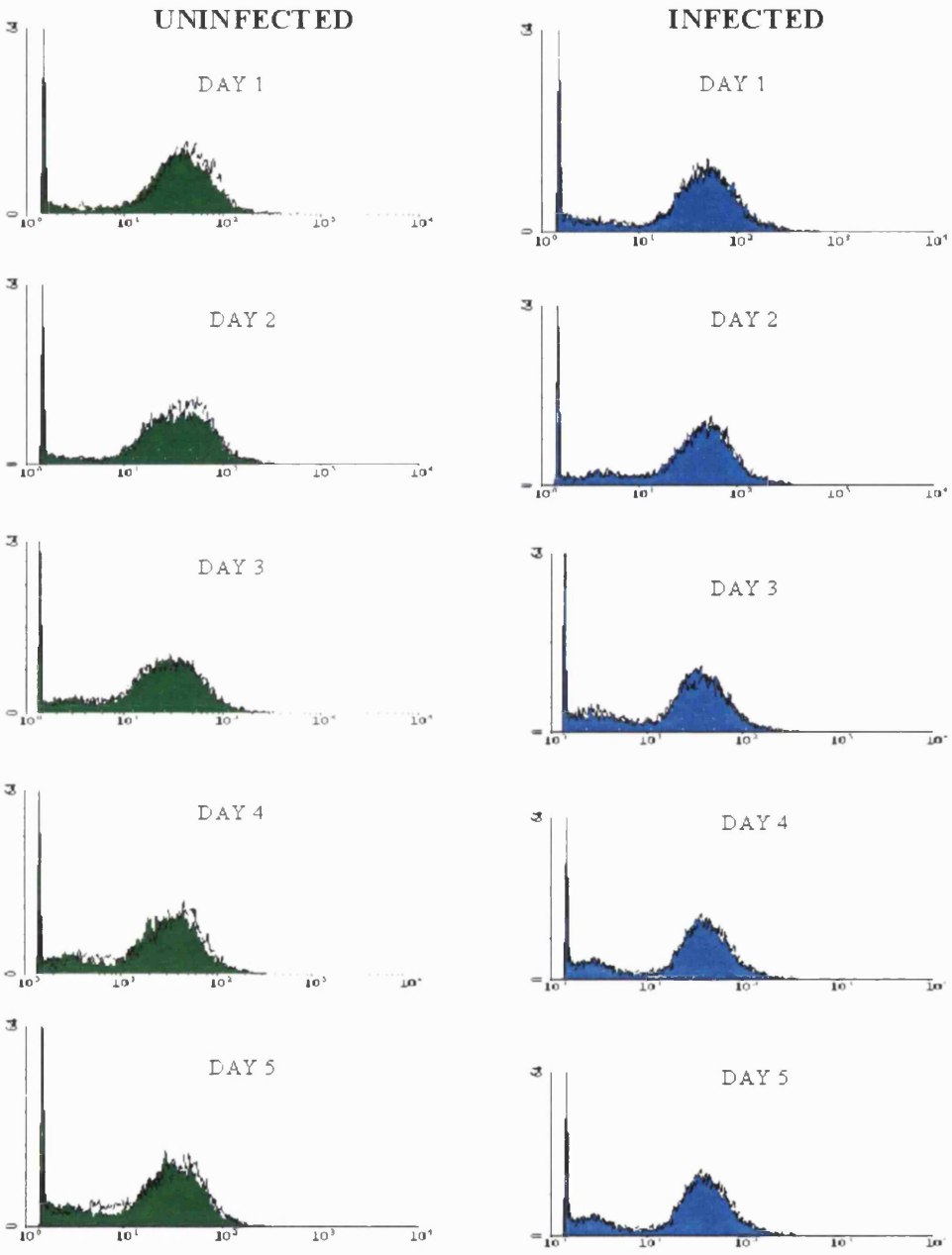


Figure 5.21. Representative flow cytometric profiles showing the effect of CMV infection on the ability of IFN-γ to stimulate the expression of class II MHC antigens on the surface of explant-migrated smooth muscle cells.

Explant-migrated smooth muscle cells at passage 3 were either infected at an MOI of 10 with CMV strain AD169 or left uninfected. Infected and uninfected cells were either then treated with 10 μg/ml IFN-γ or left untreated. The level of expression of class II MHC antigens was determined by flow cytometry at various times post-infection. The level of expression of class II MHC antigens on untreated infected cells (blue) and untreated uninfected cells (green) compared to the expression of class II MHC antigens in cells treated with IFN-γ (open-black) for both uninfected and infected cells are shown. The number of cells is shown on the y-axis on a linear scale and the median fluorescence intensity on the x-axis on a logarithmic scale.

5.3 DISCUSSION

In the present study, the cell surface expression of ICAM-1 on smooth muscle cells was shown to be significantly increased following the infection of these cells with either a high (AD169) or low (C1F) passage strain of CMV. Similarly, the level of expression of LFA-3 was observed to be significantly up-regulated on the surface of smooth muscle cells infected with CMV strain AD169. However, infection of smooth muscle cells with CMV strain C1F had no significant effect on the level of expression of LFA-3 molecules. The kinetics of ICAM-1 up-regulation and, in the case of strain AD169, LFA-3 up-regulation, in CMV-infected smooth muscle cells, was observed to be similar for both molecules, with increased expression seen on days 1-3, and peak levels of expression on day 2 post-infection. In contrast, no expression of the VCAM-1 adhesion molecule and the vitronectin receptor (integrin $\alpha_v\beta_3$) was found on smooth muscle cells, and CMV infection with either a high or low passage strain of CMV failed to induce the expression of these molecules on the cell surface.

The level of expression of ICAM-1 and LFA-3 has been reported to be significantly increased in fibroblasts infected with CMV strain AD169 (Grundy & Downes, 1993). The kinetics of the up-regulation of these molecules in fibroblasts was similar to that observed here with smooth muscle cells. It has also been reported that the constitutive expression of ICAM-1 is increased on the cell surface of epithelial cells (van Dorp et al., 1993), and endothelial cells (Scholz et al., 1992) infected with CMV strain AD169. In endothelial cells, it has been reported that CMV infection resulting in only about 10% of total cell population infected, leads to an increase in the expression of the adhesion molecule ELAM-1 (Span et al., 1991). The authors of the latter study concluded that the increased ELAM-1 expression was due to a bystander effect of interleukin-1 released from infected cells acting on uninfected cells, which comprised the majority of the cells in the culture. In addition, it has been shown that a number of cytokines up-regulate the expression of ICAM-1, namely interferon-gamma, tumour necrosis factor and interleukin-1 (Barath et al., 1990; Moyer et al., 1991; Wang et al., 1995). Thus, it is important to conduct experiments on the effects of CMV infection on adhesion molecule expression in conditions in which near 100% infection of cells is achieved, so that the alterations in adhesion molecule expression on the infected cell itself, and not the actions of cytokines on bystander uninfected cells, are studied. Such conditions were satisfied in the present study, as verified by determining the percentage of cells expressing IE antigen following the infection of smooth muscle cells with both strains of CMV. The percentage of cells expressing CMV-specific IE antigen was of the order of approximately 95% from days 2-5 post-infection, suggesting that the up-regulation of ICAM-1 by CMV was a direct viral effect. This conclusion is supported by

studies in fibroblasts, where the effect of CMV infection on the up-regulation in the level of expression of ICAM-1 was demonstrated to result from the direct effect of the virus, and not from the effects of inducible cytokines released from the infected cells, or present initially in the virus inoculum (Craigen & Grundy, 1996). In addition, virus-free supernatant or ultraviolent-inactivated virus was shown to be incapable of up-regulating the expression of ICAM-1 in fibroblasts (Craigen & Grundy, 1996), suggesting that infectious virus and/or the initiation of a viral replicative cycle were required.

It was observed that the infection of smooth muscle cells with CMV strain AD169 led to an up-regulation in the level of expression of LFA-3 molecules, while little or no significant increase of LFA-3 molecule expression was observed for smooth muscle cells infected with the low passage strain of CMV C1F. In a recent study, Fletcher et al. (1998) studied the effects of CMV infection on the level of expression of LFA-3 molecules on fibroblasts infected with a number of high and low passage strains of CMV. It was reported that the virus strains Towne, Toledo, Davis, and a low passage clinical isolate adapted for growth initially in fibroblasts and subsequently in endothelial cells (C1FE), down-regulated LFA-3 expression. On the other hand, infection of fibroblasts with CMV strain AD169 and the low passage clinical isolate C1F up-regulated the level of expression of LFA-3 molecules. The up-regulation and down-regulation of LFA-3 in the latter study was demonstrated to be mediated by CMV IE or early CMV genes, since neither of these effects was inhibited by ganciclovir treatment, which blocks viral DNA synthesis and late viral gene expression. The up-regulation of LFA-3 expression in C1F-infected fibroblasts was in contrast to the results of the present study, where infection of smooth muscle cells with C1F had no significant effect on the level of expression of LFA-3 molecules. This suggests that the cell type, as well as the CMV strain, affects the effect of CMV infection on LFA-3 expression. Thus it would be of great importance to study the effect of infection with a range of clinical isolates of CMV on LFA-3 expression in a variety of cell types, and ultimately identify the viral and cellular genes that affect LFA-3 expression.

The increased expression of adhesion molecules induced by viral infection may have functional significance. According to the response-to-injury hypothesis postulated for the pathogenesis of atherosclerosis, it can be envisaged that the denudation of the endothelium would expose the underlining intimal smooth muscle cells to the arterial lumen (Ross, 1993). This implies that the increased expression of adhesion molecules on smooth muscle cells during the initiation and progression of atherosclerotic lesion development could be important. In addition, there is a suggestion that smooth muscle

cells expressing certain adhesion molecules may facilitate the retardation and permanent residence of migrating monocytes and T-cells in the atherosclerotic plaque (Libby & Li, 1993; Braun et al., 1999). In recent studies, the expression of ICAM-1 has been detected in intimal smooth muscle cells in the atherosclerotic vascular wall (Jang et al., 1994). Whereas ICAM-1 expression could not be detected on smooth muscle cells in the normal aorta, the expression of this adhesion molecule has been observed on smooth muscle cells in the intima of atherosclerotic lesions (Printseva et al., 1992; Davies et al., 1993). Recently, Ballantyne et al. (1996) demonstrated an increase in the expression of ICAM-1 in endothelial and smooth muscle cells of the coronary artery in patients undergoing transplant-associated atherosclerosis after heart transplantation. Hence, it could be implied that the increased expression of ICAM-1 on endothelial cells and smooth muscle cells could play a contributory role in recruiting leukocytes to the vessel wall. In addition, this increased ICAM-1 expression has been shown to facilitate the adhesion of monocytes, neutrophils and lymphocytes via the β2-integrin (CD11/CD18) family of adhesion molecules (Meerschaert and Furie, 1995). Monocytes express all three of these β2-integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18). CD11a/CD18 and CD11b/CD18 are known to interact with ICAM-1. Several investigations have demonstrated a correlation between ICAM-1 expression and mononuclear cell infiltration, suggesting that mediators derived from these mononuclear cells may contribute to the induction of adhesion molecules in smooth muscle cells *in vivo* (O'Brien et al., 1996). Poston et al. (1992) performed immunohistochemistry for ICAM-1 expression on human atherosclerotic arteries, and demonstrated the expression of this molecule on endothelial cells, macrophages and smooth muscle cells in the plaque. Normal arterial endothelial cells and intimal smooth muscle cells outside plaques gave weaker or negative reactions. Thus, the increase in the level of expression of ICAM-1 on the surface of smooth muscle cells following infection with CMV may contribute to the development and progression of atherosclerotic lesions.

It has also been demonstrated that there is a significant increase in the adherence of $CD2^+$, but not $CD2^-$, lymphocytes to CMV-infected fibroblasts (Grundy et al., 1993). Such increased adherence occurred at days 2-4 post-infection, correlating with the period of maximum increase in adhesion molecule expression. The authors concluded that the increased expression of LFA-3, the ligand for CD2, was responsible for the increased adherence of $CD2^+$ lymphocytes to CMV-infected cells. Thus, increased expression of LFA-3 on CMV-infected cells could lead to an increase in the binding and localization of $CD2^+$ T lymphocytes to smooth muscle cells of the arterial wall, contributing to atherosclerotic plaque development. As discussed above, this property of CMV appears to

be strain and cell type dependent. Thus further studies on the infection of smooth muscle cells with clinical isolates of CMV from transplant patients would be required in order to assess whether the CMV-induced up-regulation of LFA-3 could play an important role *in vivo*.

In the present study, smooth muscle cells were shown not to express VCAM-1 molecules, and CMV infection with either a high or low passage strain of CMV did not induce its expression. Similar observations have been reported in human umbilical vein endothelial cells, where it has been shown that VCAM-1 molecules were not constitutively expressed in this cell type, and that infection with an endothelial cell propagated strain of CMV did not induce its expression (Sedmark et al., 1994). Couffinhal et al. (1994) demonstrated that the constitutive expression of VCAM-1 in smooth muscle cells was low, even though detectable levels of expression of VCAM-1 were found at both the mRNA and protein level by polymerase chain reaction and flow cytometry. VCAM-1 mRNA was enhanced between 1 and 24 hours following treatment with tumour necrosis factor, and then slowly declined over the subsequent 24 hours. Modulation of the expression of VCAM-1 by cytokines could thus play an important role in the patho-physiology of inflammatory and immune processes in atherosclerosis. In human atheroma, smooth muscle cells have been demonstrated to express VCAM-1, and its expression could be induced in intimal smooth muscle cells in rabbits fed on an atherogenic diet (Li et al., 1993). It was suggested that smooth muscle cell VCAM-1 serves to retard the migration of monocytes and T cells in the blood vessel wall, encouraging their permanent residence within the atherosclerotic plaque (Libby & Li, 1993). The present study suggests that CMV infection does not induce VCAM-1 expression in smooth muscle cells, and indirectly implies that the intracellular pathway for the induction of ICAM-1 is different from that of VCAM-1.

In the present study, smooth muscle cells were shown not to express the vitronectin receptor, and CMV infection with either a high or low passage strain of CMV did not induce its expression. There are no other reports investigating the effects of CMV infection on the expression of the vitronectin receptor in smooth muscle cells, endothelial cells or fibroblasts. However, several recent studies have reported that the vitronectin receptor is present in the atherosclerotic lesion, and is localized in sites of smooth muscle cell accumulation and angiogenesis in atherosclerotic plaques. These localization studies were performed on atherosclerotic plaque tissue isolated from the carotid artery (Dufourcq et al., 1997), and on the coronary artery from heart transplant recipients with diffuse

intimal thickening (Hoshiga et al., 1995). Regardless of its functional consequences, the finding of the vitronectin receptor in advanced human atherosclerotic plaques suggest the involvement of this molecule in mediating critical processes involved in smooth muscle cell migration and accumulation in atherogenesis. Further studies would be required to investigate whether CMV infection either directly or indirectly modulates the expression of the vitronectin receptor, facilitating smooth muscle migration and subsequent development of atherosclerotic lesions.

In contrast to the observed effects of CMV infection on the cell surface expression of ICAM-1 and LFA-3, the levels of expression of class I MHC antigens on smooth muscle cells were observed to fall progressively from 24 hours post-infection, to levels approximately 15% of that on uninfected cells by day 5 post-infection. These effects were not strain dependent, as CMV strains AD169 and C1F both produced essentially the same effect. In contrast, no expression of class II MHC antigens was observed on smooth muscle cells, and CMV infection failed to induce the expression of class II MHC antigens in these cells. Furthermore, it was observed in uninfected cells, that treatment with IFN-γ significantly increased the cell surface expression of class I MHC antigens, but not class II MHC antigens. CMV-infected cells treated with IFN-γ were observed to show the same reduction in the level of expression of class I MHC antigens as untreated infected cells.

A number of studies on the effects of CMV infection on the cell surface expression of class I on various cell types have reported conflicting data, and the results are not unequivocal depending on the experimental conditions. For example, enhanced class I MHC antigen expression has been reported on the surface of endothelial cells infected with CMV (Tuder et al., 1994). In the latter study, the percentage of cells expressing IE antigen was not reported, but no cytopathic effect was observed in infected cells for up to 4 weeks post-infection, suggesting a very low level of initial infection. Proximal tubular epithelial cells have also been reported to display increased levels of class I MHC expression following the infection of these cells with CMV strain AD169 at a multiplicity of infection of 10 (van Dorp et al., 1993). In the latter study, only approximately 5% of inoculated epithelial cells stained positive for CMV by indirect immunofluorescence with the monoclonal antibody E13, and the expression of class I MHC on the surface of proximal tubular epithelial cells was determined by radioimmunoassay. Grundy et al. (1988) reported that CMV infection resulted in the enhanced expression of class I MHC antigens either in the cytoplasm or on the cell surface in human fibroblasts. The latter study probably did not reflect the direct effect of CMV infection on the host cell, because a low MOI of 1PFU/cell was used, and the authors suggested that interferon produced by the infected cells was responsible for the up-regulation of the expression of class I MHC antigen on bystander

uninfected cells. However, it has been reported that when virtually 100% of cells are infected, CMV infection of fibroblasts results in a decrease in the cell surface expression of class I antigens (Barnes & Grundy, 1992; Warren et al., 1994; Yamashita et al., 1993). An explanation for these discrepancies in the expression of class I MHC antigens following infection with CMV has been demonstrated. Steinmassl & Hamprecht (1994) reported that in mixed cultures of uninfected and infected fibroblasts, the CMV-infected population of cells exhibited down-regulated class I MHC expression, while the uninfected cell population displayed elevated levels of expression of class I MHC antigens.

In contrast to the results presented here, and the proposed explanations for the discrepancies in the literature on the down-regulation of class I MHC antigens following CMV infection, Hosenpud et al. (1991) reported an increase in the expression of class I MHC antigens despite the fact that virtually 100% of inoculated smooth muscle cells were infected using a 10pfu/cell MOI. The authors infected human aortic smooth muscle cells with CMV strain AD169, and at 96 hours post-infection, the level of expression of class I MHC was measured by flow cytometry using the W6/32 anti-HLA-ABC monomorphic determinant. It was shown at this time point that there was an increase in the level of expression of class I MHC in a variable portion of CMV-infected smooth muscle cells. A second population of cells exhibited a decrease in class I MHC antigen expression. Hence, the present study is the first report to demonstrate the down-regulation of class I MHC antigens in smooth muscle cells following the infection of these cells with CMV. The down-regulation of class I MHC antigens by CMV might prevent the recognition of viral infected cells by cytotoxic T-cells, and thus reduce the susceptibility of infected cells to cytotoxic T lymphocytes-mediated lysis. This could result in the persistence of the virus within the smooth muscle cells of the vessel wall.

Several mechanisms have been postulated to explain the observed down regulation of class I MHC antigen on the surface of cells permissive to CMV infection. The finding that CMV encodes a protein, the UL18 gene product, that binds β_2-microglobulin, led to the suggestion that such binding resulted in the down-regulation of class I MHC antigen expression (Beck &Barrel, 1988). It is possible that such binding could prevent the assembly of class I MHC complexes in the endoplasmic reticulum. It was subsequently shown in transfected cells that the binding of the UL18 MHC heavy chain homologue to β_2-microglobulin was associated with decreased transport and reduced expression of the class I MHC light chain at the cell surface (Browne et al., 1990). However, a later study

has discounted the role of UL18 in the down-regulation of class I MHC antigen expression. It was observed in this study that infection of human fibroblasts with a CMV mutant with a non-functional UL18 gene decreases class I MHC expression to the same extent, as did the wild-type CMV (Browne et al., 1992). Presently, it is generally accepted that the CMV-induced down-regulation of class I antigens at the surface of host cells is the result of the concerted action of at least three separate genes in the unique region of the CMV genome. The US region of the CMV genome encodes most, if not all, glycoproteins known to play a role in the down-regulation of class I MHC expression. One of these, the US3 gene product, is expressed at immediate-early times of infection, and has been shown to retain class I MHC molecules in the endoplasmic reticulum (Ahn et al., 1996; Jones et al., 1996). At early times of infection, the US2 and US11 gene products have been shown to destroy and degrade newly synthesized class I MHC molecules (Jones et al., 1995; Wiertz et al., 1997). In addition, the US6 gene product has been demonstrated to bind to the transporter associated with antigen processing complex of the MHC antigen presentation pathway within the endoplasmic reticulum, inhibiting its peptide transporting function (Hengel et al., 1996).

A number of other viruses have been shown to reduce the class I MHC expression of host cells (Maudsley & Pound, 1991). They include adenovirus type 12 (Schrier et al., 1983; Bernards et al., 1983), adenovirus type 2 (Burgert & Kvist, 1985), HSV types 1 and 2 (Jennings et al., 1985), HIV-1 (Scheppler et al., 1989), pseudorabies virus (Mellencamp et al., 1991), myxoma virus, and malignant rabbit fibroma virus (Boshkov et al., 1992). The 13S transcript of the E1a gene of adenovirus type 12 down-regulates class I MHC in transformed cells at the transcriptional or posttranscriptional level (Schrier et al., 1983). In the case of adenovirus type 2, the E3 gene product E19K, a glycoprotein of 19kDa expressed at early times of infection, mediates the down-regulation of the cell-surface expression of class I antigens (Burgert & Kvist, 1985). Herpes simplex virus type 1 and type 2 inhibit the proteolysis of cytosolic proteins, the first step in class I MHC antigen presentation. Similarly, the Epstein-Barr virus nuclear antigen-1 protein contains a Gly-Ala repeat domain that inhibits antigen processing by the proteasome (Levitskaya et al., 1997). These findings suggest that viruses exhibit a range of mechanisms to down-regulate class I MHC expression, thereby potentially facilitating their persistence in the host by evading cytotoxic T cell-mediated destruction of infected cells.

In the present study, IFN-γ stimulation of uninfected smooth muscle cells was shown to up-regulate the cell surface expression of class I MHC antigens. Similar observations have been reported by others in smooth muscle cells (Warner et al., 1989; Hosenpud et al., 1991), and in human umbilical vein endothelial cells (Scholz et al., 1992).

The observed capability of IFN-γ to up-regulate the expression of class I MHC antigens in uninfected cells may be important for the pathogenesis of CMV *in vivo*. It could be suggested, since only very few numbers of cells are infected with CMV *in vivo*, that the production of IFN-γ by lymphocytes secondary to alloreactivity might play a role in the up-regulation of class I MHC antigens in the transplanted organ. Augmentation of MHC antigen expression has been associated with graft rejection and autoimmune reactivity (Bluestone et al., 1993; Lehmann et al., 1993). The up-regulation of MHC antigen expression as a result of the IFN-β release from infected cells may result in the activation of alloreactive cytotoxic T lymphocytes which could cause several different types of allograft damage (Grundy et al., 1988).

In the present study, there was no detectable expression of class II MHC antigens on the surface of smooth muscle cells, and CMV infection failed to induce such expression. The absence of class II MHC antigens under basal conditions has also been reported by others in smooth muscle cells (Warner et al., 1989; Hosenpud et al., 1991), as well as in human proximal tubular endothelial cells (van Dorp et al., 1993), human umbilical vein endothelial cells (Sedmak et al., 1990) and human aortic endothelial cells (Hosenpud et al., 1991). Hosenpud et al. (1991) reported that CMV infection failed to induce class II MHC antigens on the surface of human aortic smooth muscle and endothelial cells. However, the stimulation of human aortic smooth muscle and endothelial cells with IFN-γ was demonstrated to lead to an up-regulation in the expression of class II MHC molecules in both cell types. This observation is in contrast to the results presented here, which showed that IFN-γ failed to induce the up-regulation of class II MHC antigens on uninfected smooth muscle cells. There was no difference in the experimental design, dose of IFN-γ used, or in the staining protocol between the Hosenpud study and the present one that could suggest possible reasons for the discrepancy in the results obtained. Others have also reported that IFN-γ significantly increases the cell surface expression of class II MHC antigens in uninfected endothelial cells, and furthermore showed that CMV infection inhibited the IFN-γ induced up-regulation of class II MHC antigens in this cell type (Sedmak et al., 1990). However, since IFN-γ did not induce the expression of class II MHC antigens in uninfected smooth muscle cells in the present study, it was not possible to investigate any effect of CMV infection in this regard.

In conclusion, the findings presented here on the up-regulation of adhesion molecules in smooth muscle cells following CMV infection, could play a contributory role in the pathogenesis of atherosclerosis, by mediating the recruitment of leukocytes to the vessel wall. The data also suggest that the down-regulation of class I MHC antigens on

the surface of smooth muscle cells by CMV could prevent the recognition of viral infected cells by cytotoxic T-cells, thus resulting in persistence of the virus within the smooth muscle cell component of the vessel wall. The up-regulation of class I MHC antigens in uninfected cells by IFN-γ might play a role in facilitating the rejection of transplanted organs by enhancing alloreactivity in these tissues.

CHAPTER 6

The use of an organ culture of the saphenous vein to study CMV infection of the vessel wall.

6.1 INTRODUCTION

Most *in vitro* studies investigating the pathogenic role of viruses in the development of vascular pathology have been carried out using monolayer cultures of endothelial cells or smooth muscle cells. In the previous chapters, a potential pathogenic role for CMV infection in the development of atherosclerosis was sought by investigating the susceptibility of vascular smooth muscle cells to CMV infection. These studies revealed that vascular smooth muscle cells are preferential sites for CMV replication, as determined by the expression of CMV viral antigens and the production of infectious CMV virions in infected smooth muscle cells. However, these and other studies using single cell monolayer cultures of smooth muscle cells, suffer from the disadvantage that the cultures are not fully representative of the *in vivo* situation, where smooth muscle cells interact with the endothelium and other cell types.

It has been demonstrated that organ culture systems consisting of whole segments of human blood vessels can be maintained *in vitro* for extended periods of time, if careful attention is paid to the media composition, substrate selection and atmosphere (Resau et al., 1991). For example, Soyombo et al. (1990) demonstrated that human saphenous vein segments cultured in medium supplemented with 30% foetal calf serum were viable for at least 14 days. The cultured vein segments mimicked the effects of arterial implantation of saphenous veins by developing characteristic intimal hyperplasia features. Intimal hyperplasia essentially involves the migration of smooth muscle cells of medial origin to the luminal aspect of the graft where they continue to proliferate and secrete extracellular matrix proteins, forming a neointima which leads to narrowing, atherogenesis and eventually thrombotic occlusion of the graft. The organ culture system of the saphenous vein has been extensively used to study the early stages of intimal hyperplasia and neointima formation, which is seen in the blood vessel wall of transplanted organs. Similarly, an organ culture system in which whole segments of human foetal aorta displayed maximal periods of viability of up to 12 weeks has been reported (Blackwell et al., 1975). For the investigation of virus-blood vessel wall interactions that may have relevance to the pathogenesis of human vascular disease, it would be desirable to use an organ culture system of human blood vessels. Such organ culture systems offer several advantages over isolated monolayer cultures of single cells for the study of virus-blood vessel interactions. One important advantage is that the integrity and architecture of the vessel wall is maintained, and this allows the study of cell-cell and cell-substratum interactions, including the structure and function of the vessel wall matrix. In the present study, the organ culture system of the saphenous vein was used to study the susceptibility

of cells of the blood vessel wall to CMV infection, and its associated effects on the structure of the vein wall.

6.2 RESULTS

6.2.1 The establishment of an organ culture of the saphenous vein.

Human saphenous veins were obtained from patients undergoing coronary artery or lower limb bypass grafting and used as a source for organ culture. The saphenous vein organ culture system is based on the use of intact vein segments. It was therefore important to assess whether the saphenous vein specimen used possessed an intact structure, and was not damaged or stripped of the endothelium during its retrieval from the patient. On arrival in the laboratory, saphenous vein segments were cut into several segments. Prior to culture, one segment was examined by the trypan blue exclusion technique to investigate the integrity of the endothelium. This vein segment was incubated for 2 minutes in phosphate-buffered saline containing 0.01% trypan blue. Exclusion of the trypan blue stain from the endothelial surface served as a preliminary method to assess the presence of an intact endothelial layer in the vein sample. Vein samples showing signs of endothelial denudation retained the trypan blue stain, and were excluded from the study. To confirm the presence of an intact endothelial cell lining, another vein segment was immediately fixed in formalin and processed for histology and immunohistochemistry. Immunohistochemical staining using a monoclonal antibody specific for von-Willebrand factor demonstrated the presence of an intact endothelial cell lining in this vein segment, which was continuous throughout the vein segment, whilst the underlying media remained unstained. Figure 6.1 shows a photomicrograph of the reactivity of a pre-culture vein segment with a monoclonal antibody specific for von-Willebrand factor. A section from the vein of an umbilical cord was processed in parallel, and served as a positive control for the detection of von-Willebrand factor in endothelial cells (Figure 6.2).

Immunohistochemical staining using a monoclonal antibody specific for smooth muscle α-actin was used to assess the localization of smooth muscle cells in the pre-culture vein segment. As illustrated in figure 6.3, cells positive for smooth muscle α-actin were detected in the medial layer of the saphenous vein. An appendix tissue section processed in parallel, served as a positive control for smooth muscle α-actin staining (Figure 6.4). Saphenous veins with an intact endothelial cell lining and non-distorted vascular integrity were subsequently used for organ culture. The vessels were cut along their length into 0.5cm ring segments, and then immobilized on a layer of preformed sylgard resin in the bottom of a glass petri dish. The segments were then cultured in medium supplemented with 30% foetal calf serum in a humified chamber for up to 21

days, with a change of medium every 2-3 days. After culture periods of 7, 14 and 21 days, histological examination revealed that saphenous vein segments demonstrated good viability, as evidenced by the retention of morphological integrity. Photomicrographs of the structure of vein segments cultured for 7, 14 and 21 days are shown in figure 6.5. In addition to the preservation of all the three coats of the vein wall in all cultured vein segments, it could be observed in vein segments cultured for 14 and 21 days that a new cellular layer had developed on the luminal side of the vein wall. This new cellular layer is referred to as the neointima.

6.2.2 The infection of saphenous vein segments using a standard virus adsorption technique.

To investigate the susceptibility of saphenous vein segments to CMV infection, attempts were made to infect them with the AD169 CMV strain, and the low passage CMV strain C1F. Initially, the infection strategy of saphenous vein segments was carried out by submerging them in 1ml of virus stock at 1 x 10^6 pfu/ml for one hour at 37°C. Mock-infected vein segments were placed in organ culture medium. Following the adsorption period, vein segments were transferred to glass petri dishes and were cultured for 7 or 14 days. At each time point, segments were removed, fixed in formalin, and processed for histology and immunohistochemistry. In order to detect CMV-infected cells in the vein segments, the streptavidin-biotin-peroxidase technique using the monoclonal antibody, E13, which is directed against CMV-specific IE antigens, was used (see section 2.5.5). Histological examination of the vein segments exposed to both CMV strains revealed that this infection procedure was not effective at infecting cells of the vein segment. This was demonstrated by the fact that no IE-antigens could be detected in the vein segments, but were detected in a section of ulcerative colon tissue from a patient who died from disseminated CMV disease processed in parallel (Figure 6.6). In the latter section, large, cytomegalic cells, typical of cells infected with CMV were observed. Sections from this tissue served as a positive control for the detection of CMV antigens in all further studies.

6.2.3 The infection of saphenous vein segments using centrifugal inoculation with CMV strain AD169.

Based on experience in the laboratory in gaining permissive infection of endothelial cells in monolayer cultures (MacCormac et al., 1999), a centrifugal step was added to the above infection protocol. Hence, saphenous vein segments were individually placed in 1ml of CMV strain AD169 at 1 x 10^6 pfu/ml and centrifuged at 1500g for one hour. Following centrifugation, vein segments were incubated in the same viral inoculum for a further hour in an atmosphere of 5% CO_2 at 37°C. Mock-infected vein segments were centrifuged in culture medium. Mock-infected and infected vein segments were then transferred to glass petri dishes and cultured for 7, 14 or 21 days. At each time point, vein segments were removed, fixed in formalin and processed for histology and immunohistochemistry. To detect CMV antigens, immunohistochemical analysis using the monoclonal antibody E13 was performed. It revealed strong staining for CMV IE antigens in the nuclei of many cells in the intimal and adventitial layers of the saphenous vein wall, as early as day 7 post-culture (Figure 6.7). This observation represented the first demonstration in which the initiation of CMV infection in an intact human blood vessel wall had been achieved *in vitro*. There were no CMV antigens observed in the medial layer of infected vein segments at 7 days post-culture. At day 14 post-culture, immunohistochemical staining for CMV antigens showed that there was an increase in the number of cells expressing IE antigen in both the intimal and adventitial layers of infected vein segments, compared to those seen at day 7 post-culture (Figure 6.8). This implied a progression or spread of CMV infection within the vessel wall, and thus suggested that CMV was inducing a productive infection in the cells of the saphenous vein. As observed in vein segments examined at day 7 post-culture, there was no staining for CMV IE antigen in the medial layer of infected vein segments at day 14 post-culture. The type of intimal staining for CMV IE antigen at day 14 post-culture as determined in Figure 6.8 is shown at a higher magnification for a representative section in figure 6.9. The IE positive cells can be seen to line the luminal surface of the vein wall, strongly suggesting that these cells are endothelial cells, and that this cell type is a primary target for CMV replication. In addition, it was observed in some infected vein segments, that a number of IE antigen positive cytomegalic cells on the luminal side of the vein wall had detached from the underlying basement membrane.

Figures 6.10 and 6.11 show photomicrographs of a transverse section of an infected vein segment at day 21 post-culture stained for IE antigen using the streptavidin-biotin peroxidase technique. At this time point, it was observed that cells expressing IE-

antigens were localized in the intimal and adventitial layers of the infected vein segment. In particular, the adventitial layer of infected vein segments was observed to contain vast numbers of cells expressing IE antigen. As observed at earlier times post-infection, IE antigens were not detected in the medial layer of infected vein segments at 21 days post-culture (Figure 6.11). At this time point, a prominent neointima cell layer had developed in both infected and mock-infected cultures. CMV infection resulted in the severe disruption of neointimal cell morphology, with prominent cytomegalic cells observed in the neointima (Figure 6.12).

These observations suggested that CMV infection of an intact vessel wall could be achieved using the organ culture system, and that the cells comprising the intima, neointima and the adventitial layers of the vein wall were the prominent targets for CMV infection.

6.2.4 The expression of CMV early antigens in saphenous vein segments infected with CMV strain AD169.

To investigate whether CMV infection progressed beyond the expression of IE antigens, further analysis of infected saphenous vein segments was carried out using the monoclonal antibody CCH2. This antibody is specific for the DNA-binding protein p52 of CMV, which is expressed early, and more abundantly late, after CMV infection of a permissive cell. The vein segments used in the previous section were used in this study. Figure 6.13 shows the detection of CMV-specific early antigen in infected vein segments at day 21 post-culture by immunohistochemical staining. The streptavidin-biotin-peroxidase technique with the monoclonal antibody CCH2 revealed strong staining of the CMV early antigen in the nuclei of many cells in the neointimal, intimal and adventitial layers of the saphenous vein at 21 days post-culture. There was no staining observed for the CMV early antigen in the medial layer of infected vein segments at this time point. The observations from this study thus suggested that infection of the vein wall with CMV strain AD169 progressed beyond the expression of IE antigens to the expression of an early antigen. This indicated a progression of CMV replication, at least to the expression of p52 early antigens, in the cells comprising the neointimal, intimal and adventitial layers of the vein wall. The expression of CMV-specific late antigens in the CMV-infected vein segments was not carried out because of the unavailability of suitable monoclonal antibodies specific for late antigens that were reactive on formalin-fixed, paraffin-embedded tissue sections.

6.2.5 The ability of CMV strain C1F to infect saphenous vein segments.

In order to investigate the susceptibility of saphenous vein segments to CMV infection with the low passage CMV strain C1F, saphenous veins were placed in 1ml of this virus strain at 1 x 10^6 pfu/ml, or mock-infected, and centrifuged at 1500g for 1 hour as previously described for CMV strain AD169. The infected vein segments were cultured for 21 days. Figure 6.14 shows the detection of CMV early antigens in vein segments infected with the low passage CMV strain C1F, as detected with the monoclonal antibody CCH2. Immunohistochemical staining revealed that early antigens were detected in the neointimal, intimal and adventitial layers of the vein wall, as originally observed in vein segments infected with CMV strain AD169. There were no CMV antigens detected in the medial layer of the vein wall. A neointimal cell layer was also observed to develop in these vein segments, and the cells in this layer were highly susceptible to CMV infection. It was evident from a series of similar studies that both CMV strains were capable of permissively infecting the cells in the neointimal, intimal and adventitial layers of the vessel wall, as demonstrated by the localization of cytomegalic cells in these regions of the vein. Infection of cells in the medial layer of vein segments was not observed with either virus strain.

In conclusion, immunohistochemical staining using CMV-specific monoclonal antibodies E13 and CCH2, demonstrated that the infection of saphenous vein segments by centrifugal inoculation was effective in infecting cells of the saphenous vein. CMV IE and early antigens were detected in the intimal and adventitial cells of the saphenous vein, but not in the media, the smooth muscle component, of the saphenous vein. A neointima developed in the cultured vein segments, and the cells in this layer were demonstrated to be highly susceptible to CMV infection.

6.2.6 The quantitation of the number of infected cells in vein segments infected with CMV.

It was of interest to quantitate the number of cells susceptible to CMV infection in infected vein segments. This served as an indicator of the progression of CMV infection in the neointimal and adventitial layers of the vein wall with time post-culture. Quantitation was performed by counting the number of cells displaying IE antigen immunoreactivity over a defined area of an infected vein segment. It was also of interest

to compare the susceptibility of cells of the vein wall to CMV infection with the high passage AD169 strain and the low passage C1F strain.

As described in the previous sections, vein segments were centrifugally inoculated with CMV strain AD169 at 1 x 10^6 pfu/ml, or mock-infected, and subsequently cultured for 14 days or 21 days. At these time points, the vein segments were removed from culture, fixed in formalin, and stained by immunohistochemistry for the expression of CMV IE antigen with the monoclonal antibody E13. The quantification of cells expressing IE antigen was performed using a SeeScan image analysis software programme. This allowed distinct parts around the neointimal and adventitial layers of infected vein segments displaying IE-antigen immunoreactivity to be randomly constructed, using the drawing tools of the image analysis software. A total of 5 frames of area were outlined in each of the neointimal and adventitial layers of the vein wall, and their dimensions calculated by the computer. Within each defined area in the neointima or adventitia of the infected vein segment, the number of cells expressing IE antigen was counted. From the data obtained, the mean ± standard deviation of the number of cells expressing IE antigen per unit area in the infected vein segments at the various times post-culture was calculated. Table 6.1 shows the number of cells expressing IE antigen per unit area of the neointimal and adventitial layers of infected vein segments at 14 and 21 days post-culture. The data illustrates that for vein segments infected with CMV strain AD169, there was an increase in the number of cells expressing IE antigen in the adventitial layer of the vein wall between day 14 and day 21 post-culture. It was calculated using the Mann-Whitney t-test from the data obtained from six-infected vein segments, that the increase in the number of cells expressing IE antigen between day 14 and 21 post-culture was statistically significant ($P<0.05$). Similarly, there was an increase in the number of cells expressing IE antigen in the neointimal layer of the vein wall between the two time points. This was calculated using the Mann-Whitney t-test to be statistically significant ($P<0.05$).

Veins segments were also infected with the low passage CMV strain C1F, and the resultant number of cells expressing IE antigen were determined as described above. The results revealed that there was an increase in the number of cells expressing CMV IE antigen in the neointimal and adventitial layers of the vein wall between day 14 and day 21 post-culture. It was calculated using the Mann-Whitney t-test from the data obtained from six-infected vein segments, that the increase in the number of cells expressing IE antigen between day 14 and 21 post-culture was statistically significant ($P<0.05$). It was observed that there was no significant difference in the number of cells expressing IE antigen in

either the neointimal or adventitial layers between vein segments infected with CMV strain AD169 and strain C1F at either time points ($P>0.05$).

In conclusion, CMV infection of vein segments led to the expression of IE antigen in a number of cells in the neointimal and adventitial layers of the vein wall. Quantitation of the number of cells expressing IE antigen revealed that the AD169 strain and C1F low passage CMV strain equivalently infected cells comprising the neointimal and adventitial layers of the vein wall, and that such infection progressed from day 14 to day 21 post-culture.

6.2.7 The identification of cell types in areas of saphenous vein segments susceptible to CMV infection.

The streptavidin-biotin-peroxidase technique was used to identify the cells present in the areas of the vein segment that had been shown to be susceptible to CMV infection. Cells present in susceptible areas included endothelial cells located at the luminal surface of the vessel wall, smooth muscle cells in the inner longitudinal or outer circular layers of the media, and fibroblasts in the adventitia. It was also possible that monocytes and other leukocytes could have become resident in the vessel wall, following their transmigration through the endothelium *in vivo*. Hence, in order to identify the phenotype of cells in the areas of the saphenous vein susceptible to CMV infection, monoclonal antibodies specific for the above mentioned cell types were used. CMV infection of endothelial cells has been reported to lead to a down-regulation in the expression of certain endothelial cell specific markers *in vitro* (Bruggeman et al., 1988). It was therefore important to test a range of endothelial cell-specific monoclonal antibodies on infected vein segments. The panel of endothelial cell specific monoclonal antibodies used in this study included anti–von-Willebrand factor, anti-CD34, anti-PECAM-1 and the reagent, Ulex Europeaus Lectin. In order to identify smooth muscle cells, the monoclonal antibody, anti-α-smooth muscle-1 specific for smooth muscle α-actin was used. Furthermore, monoclonal antibodies specific for the CD68 and CD45 antigens were used to detect monocytes and other leukocytes, respectively in the vein wall. Monoclonal antibodies routinely used in immunohistochemical staining procedures to detect fibroblasts such as 5B5, which recognizes prolyl-4-hydroxylase and anti-vimentin are also reactive on smooth muscle cells (Dako). The fibroblast-specific antibody designated AS02, which was used earlier in chapter 3, is non-reactive on formalin-fixed, paraffin-embedded tissue sections. Hence, no suitable monoclonal antibodies were available to detect fibroblasts in the vein segments.

The vein segments used to demonstrate the infection of vein segments with CMV strain AD169 in section 6.2.3 were used in this study.

In Figure 6.1, in a pre-culture vein segment, endothelial cells were shown to line the lumen of the vein wall as detected using an anti-von-Willebrand factor monoclonal antibody by the streptavidin-biotin-peroxidase technique. This served to demonstrate the presence of an intact endothelium in the vein specimens retrieved from the patient. At day 14 post-culture, during which a neointima cell layer had developed in both infected and mock-infected vein segments, immunohistochemistry using monoclonal antibodies specific for von-Willebrand factor and anti-CD34 revealed that endothelial cells were observed in the neointima of infected and mock-infected vein segments (Figures 6.15 and 6.16, respectively). Endothelial cells could not be detected using Ulex European lectin (results not shown). It has been hypothesized that lectin molecules in endothelial cells are down-regulated under culture conditions (Dr Kwee Yong, University of London). Furthermore, endothelial cells could not be detected with an anti-PECAM-1 monoclonal antibody in either infected or mock-infected cultured vein segments (results not shown). At day 21 post-culture, the immunohistochemical detection of endothelial cells with monoclonal antibodies anti-von-Willebrand factor and CD34 revealed that endothelial cells were located in the intima and/or neointima of the vein wall. Figures 6.17 and 6.18 illustrate the location of cells reactive with anti-von-Willebrand factor and CD34, respectively in infected and mock-infected vein segments.

In order to identify smooth muscle cells in the vein wall, the streptavidin-biotin-peroxidase technique using a monoclonal antibody specific for smooth muscle α-actin was used. In pre-culture vein segments, smooth muscle cells in the media of the vein wall were strongly positive for the antigen as previously illustrated in Figure 6.3. After the culture of vein segments, the staining of smooth muscle α-actin became less intense in the smooth muscle cells of the media, in both infected and mock-infected vein segments (Figure 6.19). Smooth muscle cells in infected vein segments were observed in the neointima, and in the inner longitudinal smooth muscle cell area of the venous media. The upper layer of the neointima stained less for smooth muscle α-actin than the base of the neointima, while the outer circular smooth muscle cells of the media were negative for smooth muscle α-actin staining (Figure 6.19). Mock-infected vein segments had similar regions of the vein wall staining positively for smooth muscle α-actin as that observed for infected vein segments.

Immunohistochemical staining using monoclonal antibodies specific for CD45 and CD68 cellular antigens was performed. CD45 and CD68-reactive cells could not be detected in cultured vein segments (results not shown), but were detected in human tonsil

sections treated in parallel as positive controls, as illustrated in Figures 6.20 and 6.21, respectively.

Thus, immunohistochemical staining of infected saphenous vein segments using monoclonal antibodies specific for endothelial cells and smooth muscle cells suggested that the newly formed neointimal cell layer consisted of a mixture of endothelial cells and smooth muscle cells, and that these cell types were possible targets for CMV infection in the vein segments. The possibility of resident leukocytes in the vein wall being susceptible to CMV infection was ruled out by the demonstration that no CD45 and CD68 antigens were detected in the vein wall. The adventitial layer of the vein wall was non reactive with any of the monoclonal antibodies tested here, indicating that the cells of the adventitia were likely to be of a fibroblast phenotype. Hence, the data suggested that endothelial cells of the intima and neointima, smooth muscle cells of the neointima, and fibroblasts of the adventitia were probably the cell types in the areas of the vein wall susceptible to CMV infection.

6.2.8 The identification of CMV-infected cells in infected saphenous vein segments by immunohistochemical double-staining.

To further identify the target cells for CMV infection in infected saphenous vein segments, an immunohistochemical double-staining technique was used. This potentially allowed cell types displaying CMV immunostaining to be identified. Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 as described above, and then cultured for 14 and 21 days. At each time point, vein segments were removed, fixed in formalin and processed for histology and immunohistochemical double-staining for the simultaneous demonstration of CMV antigens and cell specific markers. There were limitations in performing this analysis, because most of the monoclonal antibodies previously used in this study were of mouse origin and of an IgG1 isotype. For example, in order to detect smooth muscle cells displaying CMV immunostaining, the monoclonal antibodies, anti-α-smooth muscle-1 and CCH2 or E13 would have to be used, which are all of an IgG1 isotype and of murine origin. With the reagents available, it was only possible to perform double-staining immunohistochemistry using the CMV early antigen specific monoclonal antibody, CCH2, and a rabbit polyclonal antibody specific for von-Willebrand factor. This served to investigate the possibility that endothelial cells were targets for CMV infection in the vein wall. The CMV-infected colon tissue used previously (section 6.2.2) served as a positive control for the simultaneous detection of

CMV early antigen and cells expressing von-Willebrand factor in the same tissue (Figure 6.22). In all cases, the prominent brown nuclear staining of CMV early antigens with CCH2 could easily be distinguished from the red cytoplasmic staining of von-Willebrand factor in endothelial cells. It was observed that a number of cytomegalic endothelial cells in the colon tissue section were doubly positive for the CMV early antigen and von-Willebrand factor. An essential control used in this analysis involved the substitution of the second primary antibody with an irrelevant isotype-matched antibody in the same concentration range. This would help to determine if any staining observed was due to binding of the second primary antibody by the first secondary antibody. Immunohistochemical double-staining on vein segments cultured for 14 days, revealed that brown-staining cells expressing CMV early antigen, and red staining cells positive for von-Willebrand factor were located at the intimal layer of the vein segments. It could be observed that certain cells in the intimal layer of the vein segment were doubly positive for CMV early antigen and von-Willebrand factor. This suggested that endothelial cells of the intimal layer of the vein wall were targets for CMV infection (Figure 6.23). There was no staining of cells with these antibodies in the medial component of the vein wall, but it was observed that the adventitial layer of the vein wall was singly positive for CMV-specific early antigen (results not shown). Control stained vein sections were singly positive for the CMV early antigen, and no staining for the irrelevant isotype matched antibody was observed (results not shown).

Immunohistochemical double-staining of vein segments cultured for 21 days with monoclonal antibodies, CCH2 and the rabbit polyclonal antibody against von-Willebrand factor showed that the majority of the cells expressing CMV early antigen were located in the neointima, and in the adventitial layer of the vein wall. von-Willebrand factor was also detected in the neointima. The cells expressing CMV early antigen were not doubly positive for CMV early antigen and von-Willebrand factor (Figure 6.24). This is in contrast to the double staining results observed at day 14 post-culture, where a few cells were observed to be doubly positive for CMV and von-Willebrand factor staining. The fact that endothelial cells were not doubly positive for CMV early antigen and von-Willebrand factor at 21 days post-culture, could be due to the possibility that at much later times post-infection of endothelial cells in the organ culture, the von-Willebrand factor in infected endothelial cells could have been down-regulated, as observed in monolayer cultures of infected endothelial cells (Bruggman et al., 1988).

In conclusion, the data presented here and in the previous section suggest that the cells in the vein wall that are susceptible to CMV infection include endothelial cells in the intima, endothelial cells and/or smooth muscle cells in the neointima, and fibroblasts in the

adventitia. There are no cells in the medial layer of the vein wall that are susceptible to CMV infection in this system.

6.2.9 The effect of CMV infection on neointimal thickness.

One of the prominent features of transplant-associated atherosclerosis is the development of a new cellular layer on the luminal side of the blood vessels of transplanted organs. This new cellular layer called the neointima is believed to result from the migration of smooth muscle cells of medial origin to the luminal aspect of the vein wall, where they proliferate, synthesize extracellular matrix, contributing to the narrowing of the luminal diameter. As CMV infection of the blood vessel wall is hypothesized to facilitate the development of atherosclerosis in transplant recipients, it was of interest to use the organ culture system to investigate whether the neointima formed in CMV-infected vein segments was significantly different from that formed in mock-infected vein segments. Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169, or mock-infected, and subsequently cultured for 7, 14 or 21 days post-infection. At each time point, they were removed, fixed in formalin, and subsequently analysed for the development of a neointima, using the Millers elastin/van Gieson staining protocol. Millers elastin stain was used to localize the internal elastic lamina, as it stains this, and all other elastic fibres present in the vein wall, black. The van Gieson stain stains smooth muscle cells and collagen components of the vein wall yellow and red, respectively. Measurements taken from the top of the internal elastic lamina to cells lining the luminal surface, using a computerised image analysis system, give an indication of the thickness of the neointima in cultured vein segments. There was no neointima in vein segments prior to infection and subsequent culture (Figure 6.25).

Histological examination of cultured infected and mock-infected saphenous vein segments showed the development of a neointima, which was clearly distinguishable from the original intima. The entire length of the saphenous vein section was examined in consecutive fields of view, and 25 measurements of neointimal thickness were obtained per field of view. The mean of these measurements denoted the thickness of the neointima across the length of the vein. Table 6.2 shows the mean ± standard deviation of measurements of the thickness of the neointima for infected and mock-infected cultures at 7, 14 and 21 days post-culture. At day 7 post-culture, a neointima was discernable, and its thickness could be measured. It was deduced using the Mann-Whitney t-test on the data obtained from three vein segments obtained from the same vein, that there was no

significant difference in the thickness of the neointima between infected and mock-infected vein segments at this time point ($P > 0.05$). At day 14 post-culture, the thickness of the neointima was found to have increased in thickness above that observed on day 7 post-culture. There was no significant difference in the thickness of the formed neointima between infected and mock-infected vein segments at this time point ($P > 0.05$). Prominent neointima formation continued in both cultures. Figure 6.26 illustrates the thickness of the neointima in infected and mock-infected vein segments at day 21 post-culture. At this time point, a mean thickness of 41μm (range 34-52) was found for mock-infected cultures, while a mean thickness of 46μm (range 40-60) was calculated for infected vein cultures (Table 6.2). These measurements were not statistically significantly different by the Mann-Whitney t-test ($P > 0.05$). Thus the measurements showed that there was no significant difference in neointimal thickness between infected and mock-infected vein segments at all time points examined. However, it was deduced using the Mann-Whitney t-test that there was a significant increase in neointimal thickness in both infected and mock-infected vein segments between day 7 and day 21 post-culture ($P < 0.05$) in both cases.

In conclusion, CMV infection had no effect on the development and thickening of the neointima formed in vein segments cultured for various times post-infection.

6.2.10 The effect of CMV infection on the structure of the saphenous vein in culture.

In addition to staining the internal elastic lamina and all other elastic fibres in the vein wall, the Millers elastin/van Gieson staining procedure also gives an indication of the content of collagen and smooth muscle cells in the vein wall. The van Gieson stain stains collagen fibres red, while smooth muscle cells are stained yellow. The vein segments used to demonstrate the infection of vein segments with CMV strain AD169 in section 6.2.3 were used to study the effect of CMV infection on the structure of the saphenous vein. Observations of saphenous vein segments cultured for 21 days showed that CMV infection resulted in an altered organization of the medial layer of the vein wall (Figure 6.26). The Millers elastin/van Gieson stain demonstrated that there was an apparent increase in collagen synthesis in the longitudinal smooth muscle cell region of the media, as compared with mock-infected saphenous vein segments. Consistent with the observations from smooth muscle α-actin staining by immunohistochemistry, the Millers elastin/van Gieson stain identified smooth muscle cells to be located in the neointima and

in the longitudinal smooth muscle layer of the media in both mock-infected and infected saphenous vein cultures.

Hence, observations from saphenous vein segments stained with the Millers elastin/van Gieson stain suggested that CMV infection of the saphenous vein led to an altered organisation of the medial layer of the vein wall, and also apparently increased collagen synthesis in this region of the vein wall.

Table 6.1. The number of cells expressing CMV-specific IE antigen per unit area of neointimal and adventitial tissue in infected saphenous vein segments[1].

	Number of cells expressing IE antigen per unit area ($10^4\mu m^2$). (Number ± standard deviation), n=6			
CMV Strain	AD169		C1F	
Vein wall layer	Neointima	Adventitia	Neointima	Adventitia
Days in Culture				
14	9 ± 3	11 ± 3	12 ± 1	10 ± 2
21	22 ± 1	19 ± 3	24 ± 2	21 ± 3

[1]Saphenous vein segments were infected with CMV strain AD169 or C1F by centrifugal inoculation. They were then cultured for 14 or 21 days post-infection, and at each time point were removed, fixed in formalin, and stained for the expression of CMV IE antigen using the monoclonal antibody E13. The stained sections were subsequently analysed using an image analysis software in order to quantitate the number of cells expressing IE antigen per unit area in the neointimal and adventitial layers of the infected vein segment. From a total of six infected saphenous vein segments, a total of five frames of area were outlined in each of the neointimal and adventitial layers of the vein wall with IE antigen immunoreactivity, and the defined areas were calculated by the computer. A count to deduce the number of cells expressing IE antigen in the defined areas was then performed. The number of cells expressing IE antigen per unit area for each area of the neointima and adventitial layer of the vein was calculated, from which the mean ± standard deviation of number of cells expressing IE antigen per unit area was deduced. The results shown are the mean ± standard deviation of the number of cells expressing IE antigen per $10^4\mu m^2$ unit area of six infected saphenous vein segments.

Table 6.2. A comparison of the thickness of neointima formation in infected and mock-infected vein segments at 7, 14 and 21 days post-culture[1].

	Thickness of neointimal formation (μm ± error) Range (min-max)		
Days in culture	7	14	21
Infected	8.16 ± 3.94 (3-14)	15.97 ± 4.27 (7-22)	46.99 ± 3.57 (40-60)
Mock-infected	6.27 ± 2.10 (3-10)	14.47 ± 3.06 (9-24)	40.75 ± 5.52 (34-52)

[1]Saphenous vein segments were either mock-infected or infected with CMV strain AD169 by centrifugal inoculation. They were then cultured for 7, 14 or 21 days, and at each time point were removed, fixed in formalin, and subsequently analysed for the development of a neointima. Measurements of the thickness of the neointima formed were made using a computerised image analysis system (Leica Q500MC). A total of 25 measurements were taken along the length of the vein section, from which the mean of these readings was calculated and denoted as the neointimal thickness of the section or vein segment. The data shown is the mean ± standard error of the data obtained from three separate experiments.

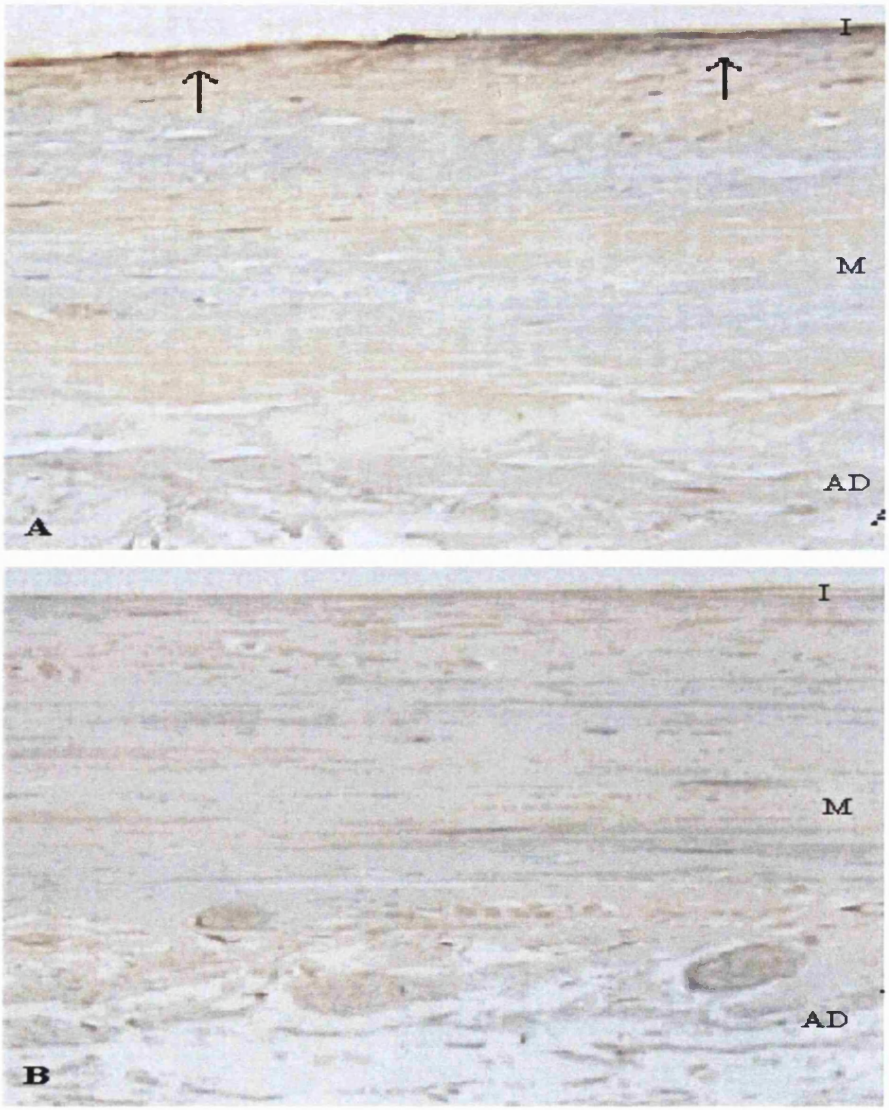


Figure 6.1. The demonstration of an intact endothelial cell layer in a pre-culture saphenous vein segment.

A pre-culture vein segment was fixed at day 0 and stained for endothelial cells using a monoclonal antibody specific for von-Willebrand factor (Part A), or an irrelevant isotype-matched control antibody (Part B). Immunohistochemical staining revealed the presence of an intact endothelial layer (arrowed) on the luminal surface of the vein segment (Part A), as visualized using diaminobenzidine. Mayers haematoxylin was used as a counterstain. (I-Intima, M-Media, AD-Adventitia). x360 magnification

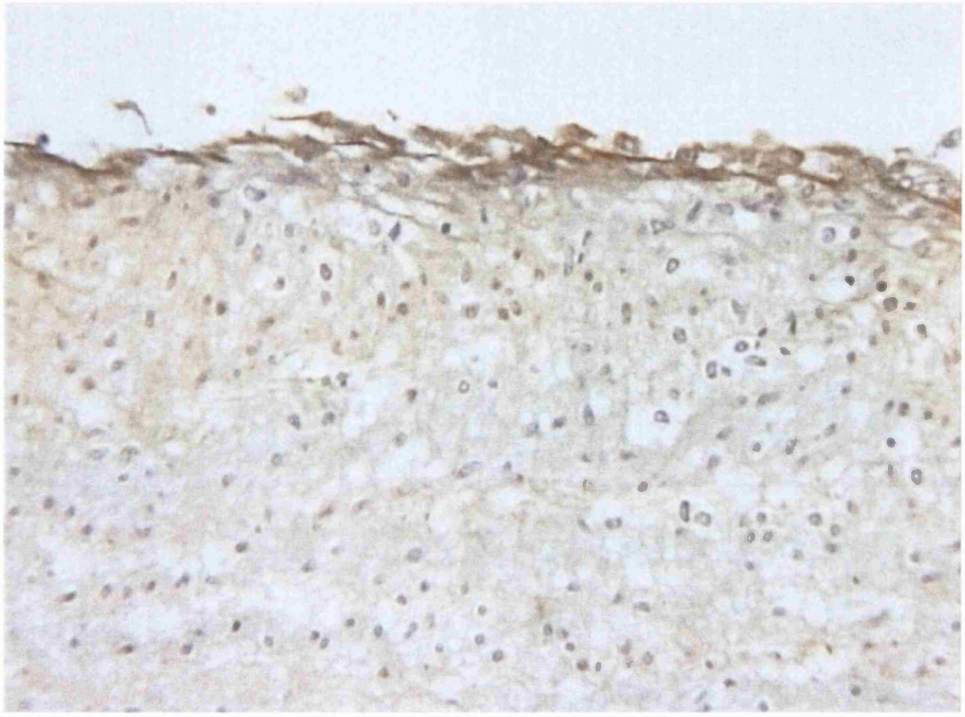


Figure 6.2. The demonstration of endothelial cells in a section of an umbilical cord.

Immunohistochemical staining of endothelial cells in a section of an umbilical cord using a monoclonal antibody specific for von-Willebrand factor. The streptavidin-biotin-peroxidase technique was used to detect the antigen, which was visualised using diaminobenzidine. Endothelial cells (brown-staining) are shown to line the luminal surface of the vessel of the umbilical cord. Mayers haematoxylin was used as a counterstain. x700 magnification.

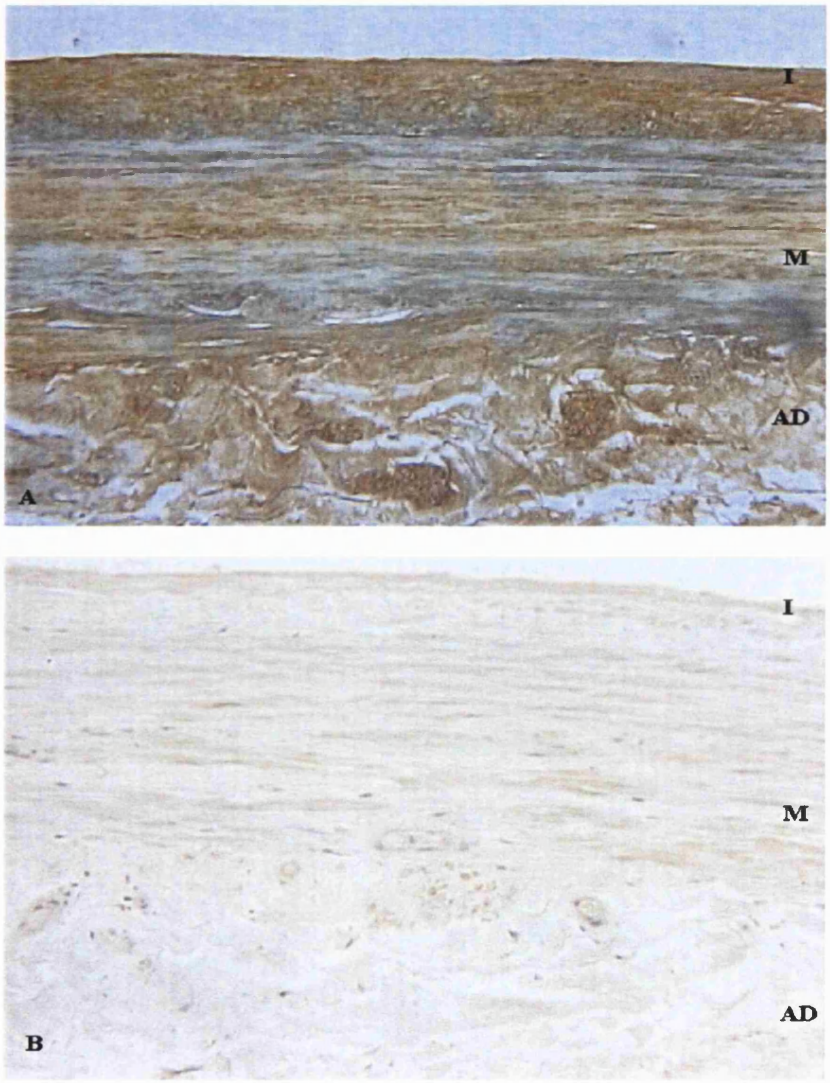


Figure 6.3. The demonstration of the localization of smooth muscle cells in a pre-culture saphenous vein segment.

Photomicrographs of a pre-culture vein segment fixed at day 0 and stained for smooth muscle cells using a monoclonal antibody specific for smooth muscle α-actin (Part A), or an irrelevant isotype-matched control antibody (Part B). Immunohistochemical staining revealed that smooth muscle cells were detected in the media of the vein segment (Part A), as visualized using diaminobenzidine. Mayers haematoxylin was used as a counterstain. (I-Intima, M-Media, AD-Adventitia). x350 magnification

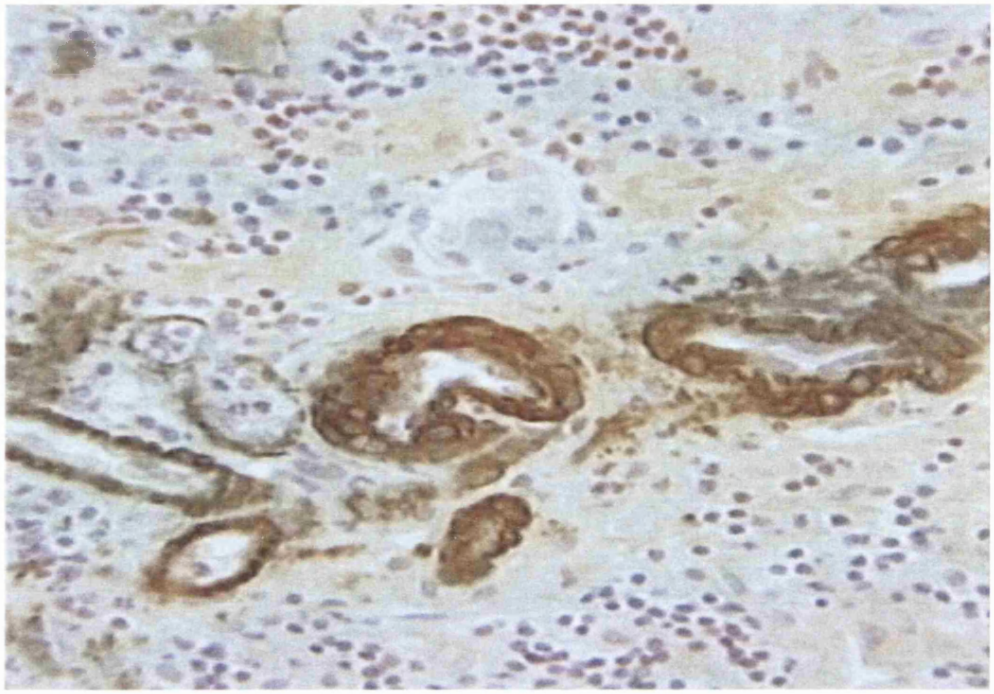


Figure 6.4. The demonstration of smooth muscle cells in a section of human appendix tissue.

Immunohistochemical staining of a section of human appendix tissue using a monoclonal antibody specific for smooth muscle α-actin. The streptavidin-biotin-peroxidase technique was used to detect the antigen, which was visualised using diaminobenzidine. Mayers haematoxylin was used as a counterstain. x700 magnification.

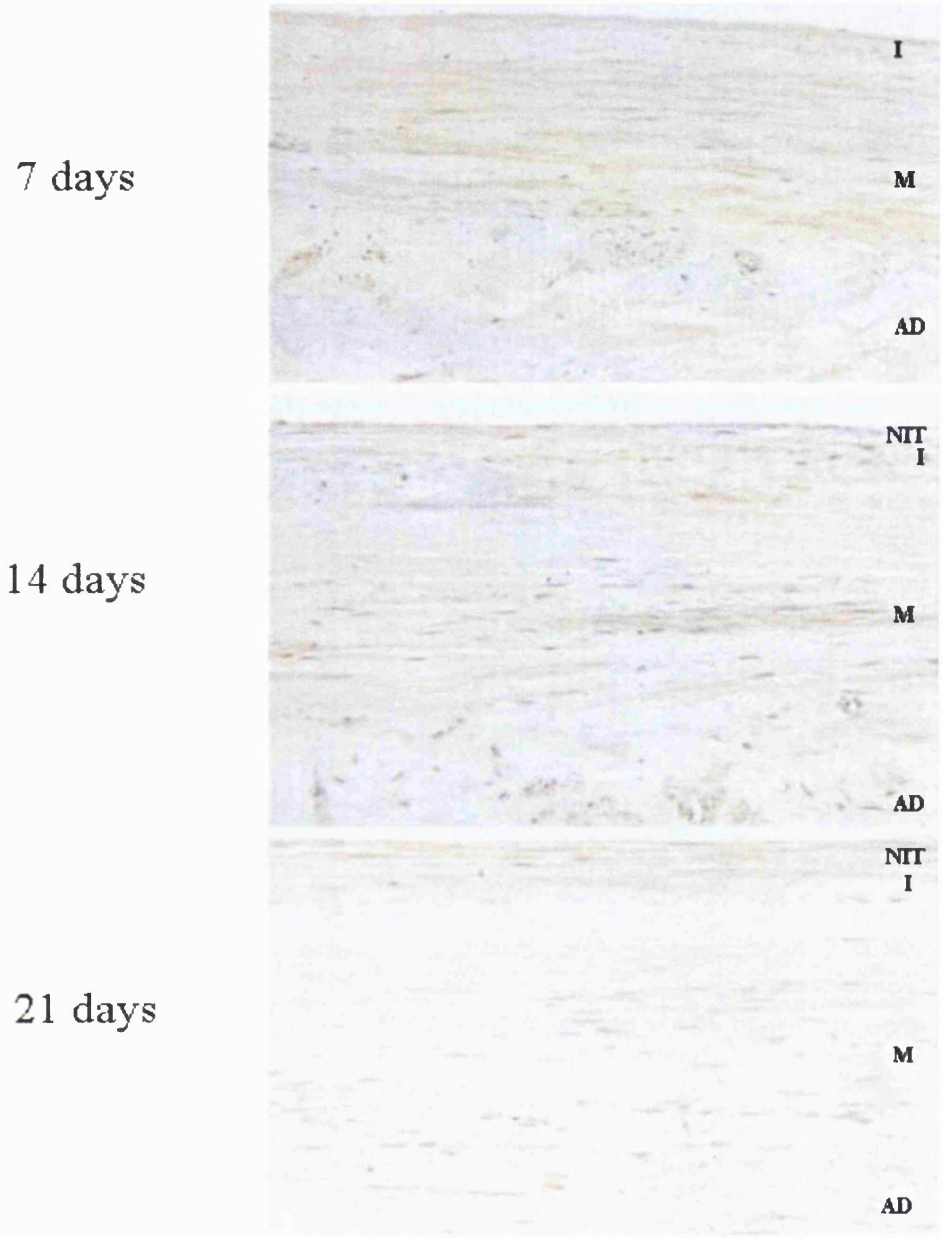


Figure 6.5. The demonstration of the retention of morphological integrity of the vein wall in vein segments cultured for various times.

Saphenous vein segments were harvested from patients undergoing coronary artery or lower limb bypass grafting, and were cultured in medium supplemented with 30% foetal calf serum. The photomicrographs shows Mayers haematoxylin staining of the transverse sections of vein segments cultured for 7, 14 and 21 days, and illustrates that the integrity of the vein wall was maintained over the various times post-culture. At day 21 post-culture, a segment is shown to have developed a prominent new cellular layer, which is referred to as the neointima. (NIT-Neointima, I-Intima, M-Media, AD-Adventitia). x270 magnification

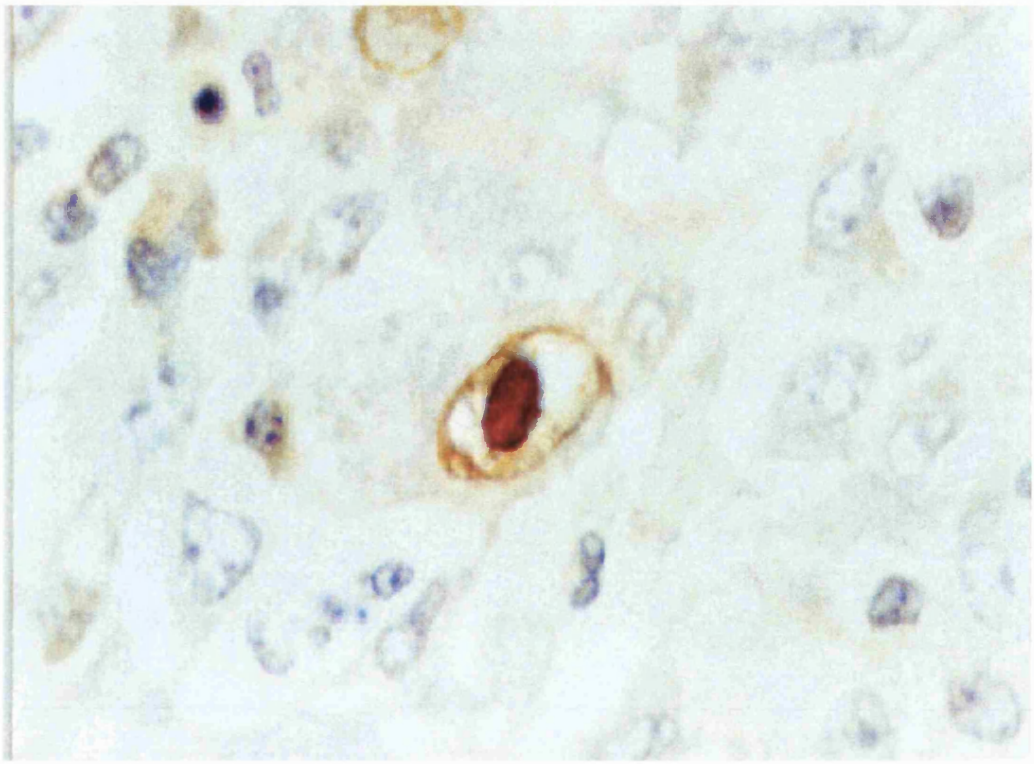


Figure 6.6. The demonstration of CMV-specific IE antigen in a section of CMV-infected colon tissue.

Immunohistochemical staining of a section from the colon of a patient with ulcerative colitis who died from disseminated CMV infection. The section shows a brown-staining cytomegalic cell in the centre of the field, typical of cells infected with CMV. The CMV-infected cell was detected using the streptavidin-biotin-peroxidase technique with the IE antigen specific monoclonal antibody E13, which was visualised using diaminobenzidine. Mayers haematoxylin was used as a counterstain. x1670 magnification

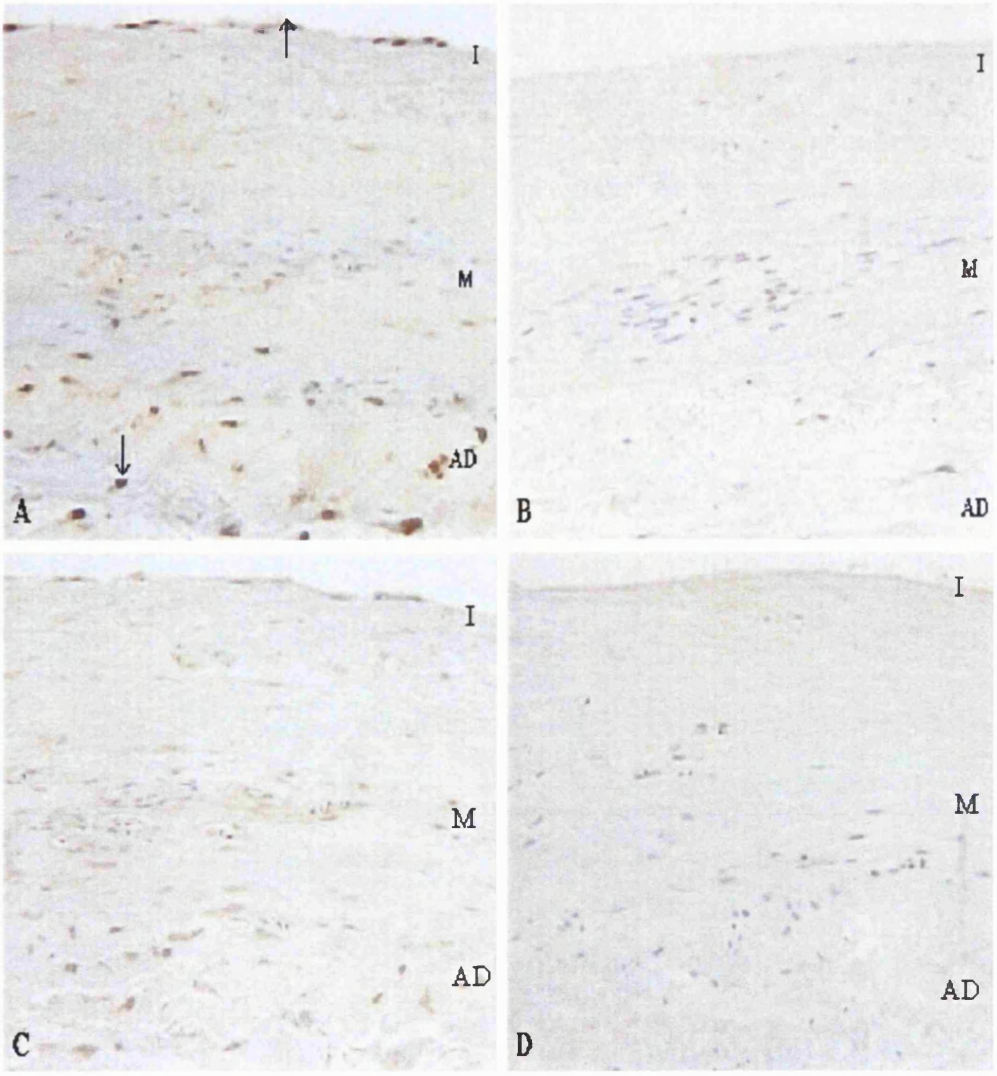


Figure 6.7. The demonstration of CMV-specific IE antigen in infected saphenous vein segments at day 7 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 7 days. At this time point they were removed, and stained for CMV IE antigen with the monoclonal antibody E13 using the streptavidin-biotin-peroxidase technique. Part A shows that brown-staining cells expressing IE antigen were detected in the intimal and adventitial layers of the vein wall (arrowed). Part B shows that no CMV IE antigens were detected in mock-infected vein segments. Irrelevant isotype-matched controls for infected and mock-infected vein segments are shown in (C) and (D) respectively. CMV antigens were visualized with diaminobenzidine, and the sections were counterstained with Mayers haematoxylin. (I-Intima, M-Media, AD-Adventitia). x220 magnification.

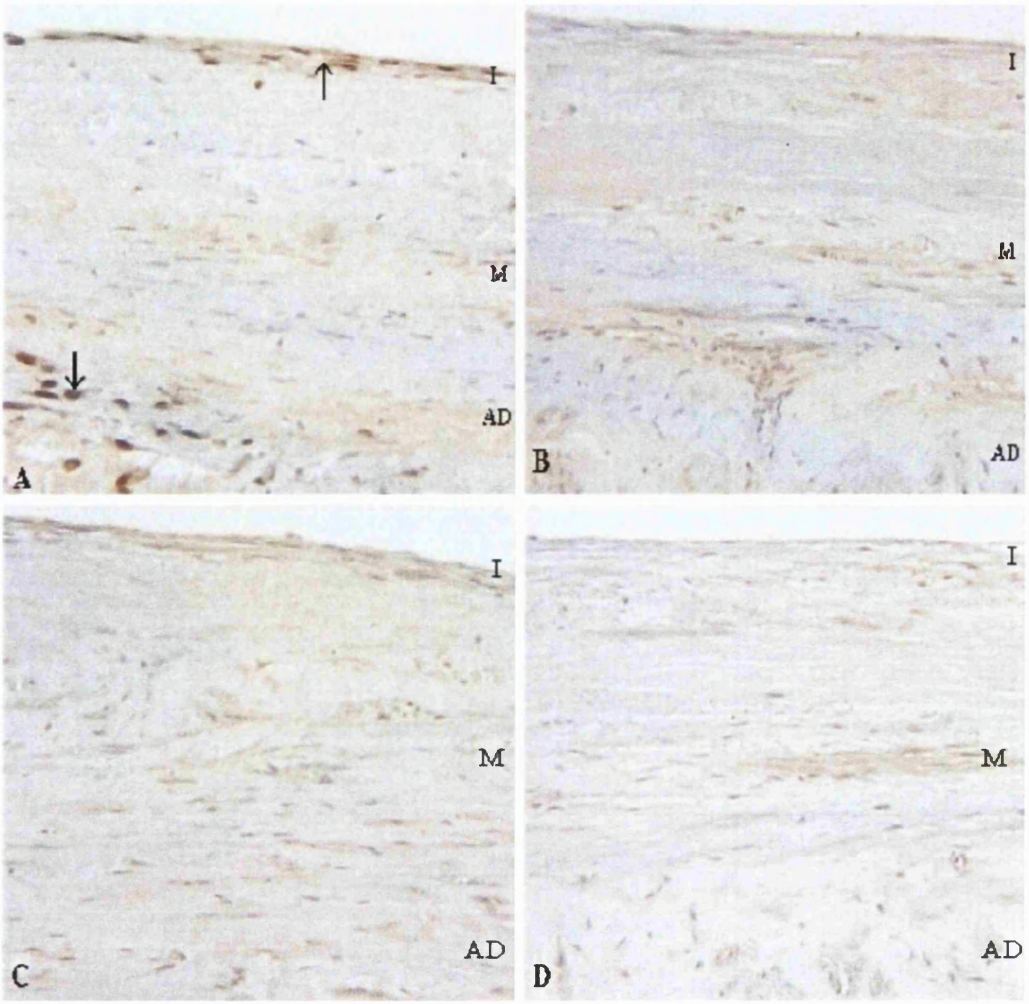


Figure 6.8. The demonstration of CMV-specific IE antigen in infected saphenous vein segments at day 14 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 14 days. At this time point they were removed, and stained for CMV IE antigen with the monoclonal antibody E13 using the streptavidin-biotin-peroxidase technique. Part A shows that brown-staining cells expressing IE antigens were detected in the intimal and adventitial layers of the vein wall (arrowed). Part B shows that no CMV IE antigens were detected in mock-infected vein segments. Irrelevant isotype-matched controls for infected and mock-infected vein segments are shown in (C) and (D) respectively. CMV IE antigens were visualized with diaminobenzidine, and the sections were counterstained with Mayers haematoxylin. (I-Intima, M-Media, AD-Adventitia). x220 magnification.

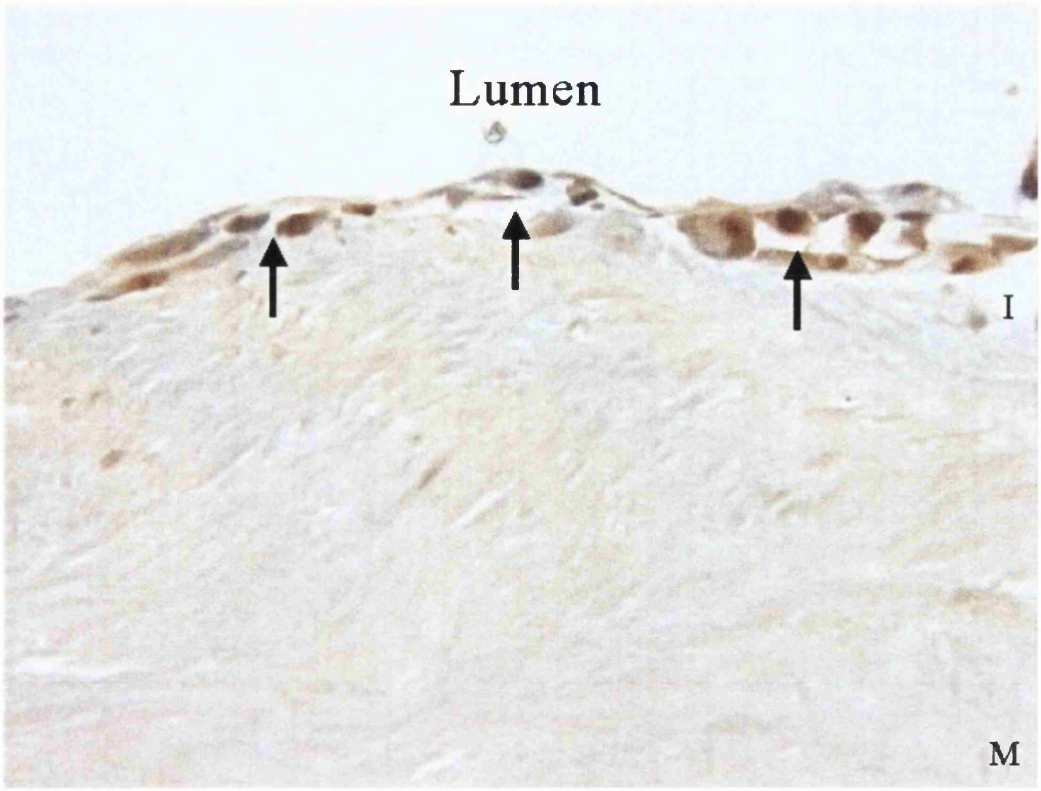


Figure 6.9. The demonstration of CMV-specific IE antigen at the luminal surface of an infected saphenous vein segment at day 14 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 and cultured for 14 days. At this time point, they were removed and stained for CMV IE antigen with the monoclonal antibody E13 using the streptavidin-biotin peroxidase technique. The photomicrograph shows brown-staining cells expressing IE antigens on the luminal surface of the vein wall (arrowed). Some of these cells appeared to have detached from the underlying basement membrane. CMV IE antigens were visualized using diaminobenzidine and the section was counterstained with Mayers haematoxylin. (I-Intima, M-Media). x700 magnification.

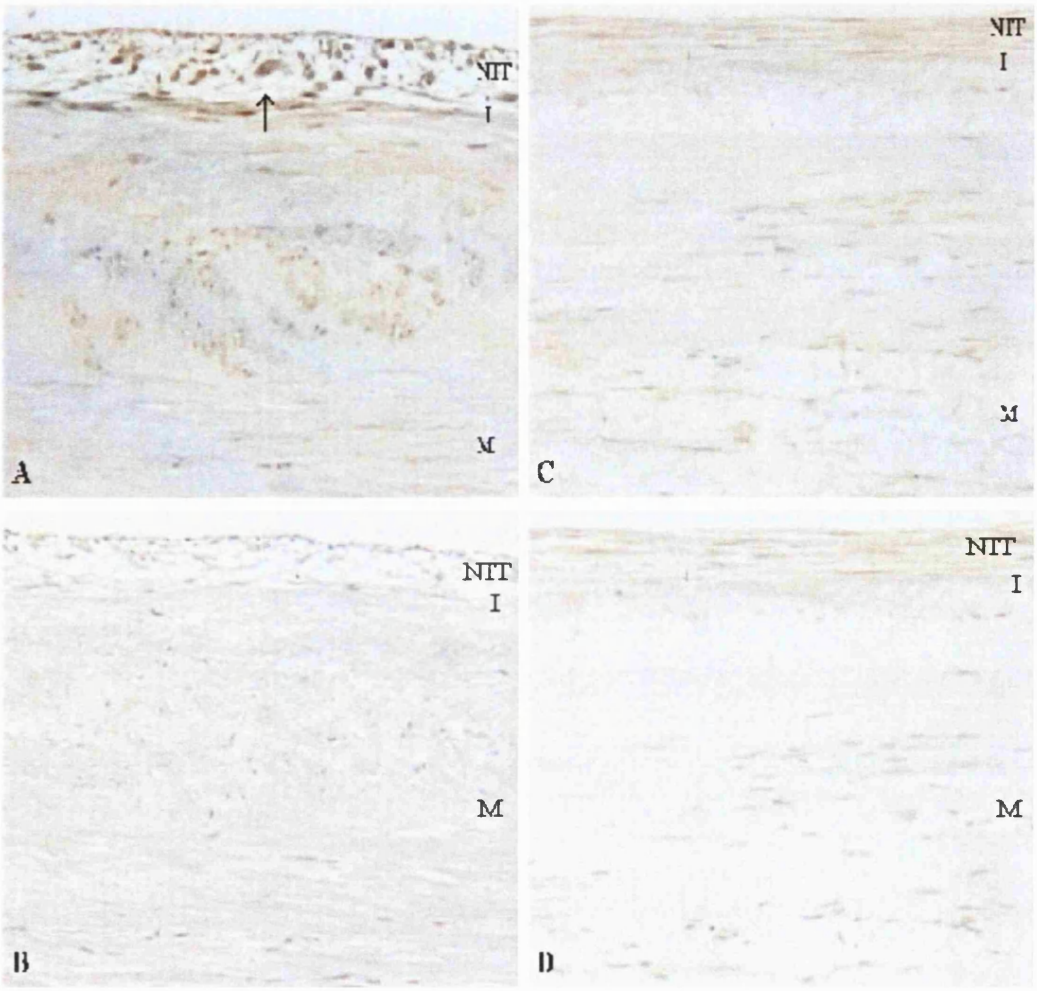


Figure 6.10. The demonstration of CMV-specific IE antigen in infected saphenous vein segments at day 21 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 21 days. At this time point they were removed, and stained for CMV IE antigen with the monoclonal antibody E13 using the streptavidin-biotin-peroxidase technique. Part A shows that brown-staining cells expressing IE antigen were detected in the neointimal and intimal layers of the vein wall (arrowed). Part B shows that no CMV antigens were detected in mock-infected vein segments. Irrelevant isotype-matched controls for infected and mock-infected vein segments are shown in (C) and (D) respectively. CMV IE antigens were visualized with diaminobenzidine, and the sections were counterstained with Mayers haematoxylin. (NIT-Neointima, I-Intima, M-Media). x220 magnification.

Figure 6.11. The localization of CMV-specific IE antigen in an infected saphenous vein segment at day 21 post-culture.

Saphenous vein segments were infected and processed for immunohistochemical staining for IE antigens as described in figure 6.10. The brown-staining cells expressing IE antigens were detected in the neointimal, intimal and adventitial layers of the vein wall. The IE antigen was visualized with diaminobenzidine, and the section was counterstained with Mayers haematoxylin. (NIT-neointima, M-Media, AD-Adventitia). x180 magnification.

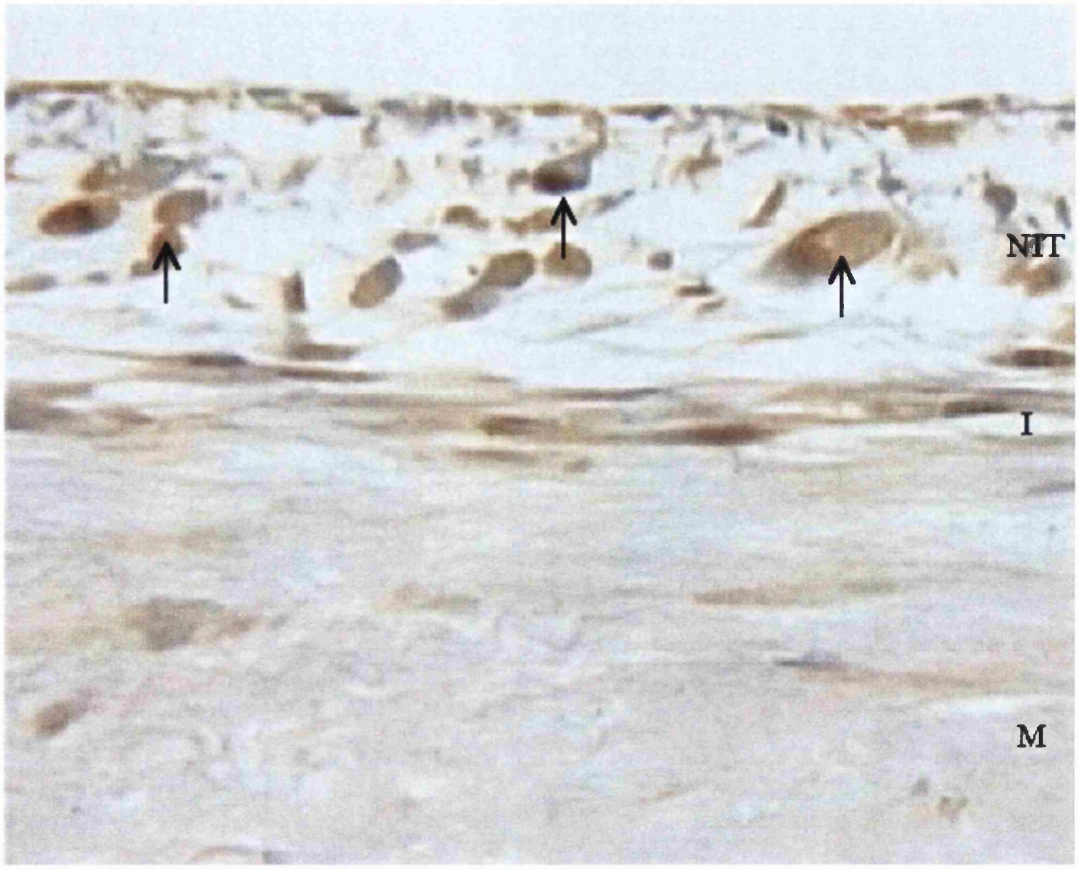


Figure 6.12. The demonstration of CMV IE antigen in the neointimal layer of an infected saphenous vein segment at day 21 post-culture.

The photomicrograph shows a higher magnification of Part A of Figure 6.10. CMV IE antigen positive cytomegalic cells, typical for cells infected with CMV, are shown in the neointimal layer of the vein wall (arrowed). CMV IE antigens were visualized with diaminobenzidine and the section was counterstained with Mayers haematoxylin. (NIT-neointima, I-Intima, M-Media). x1800 magnification..

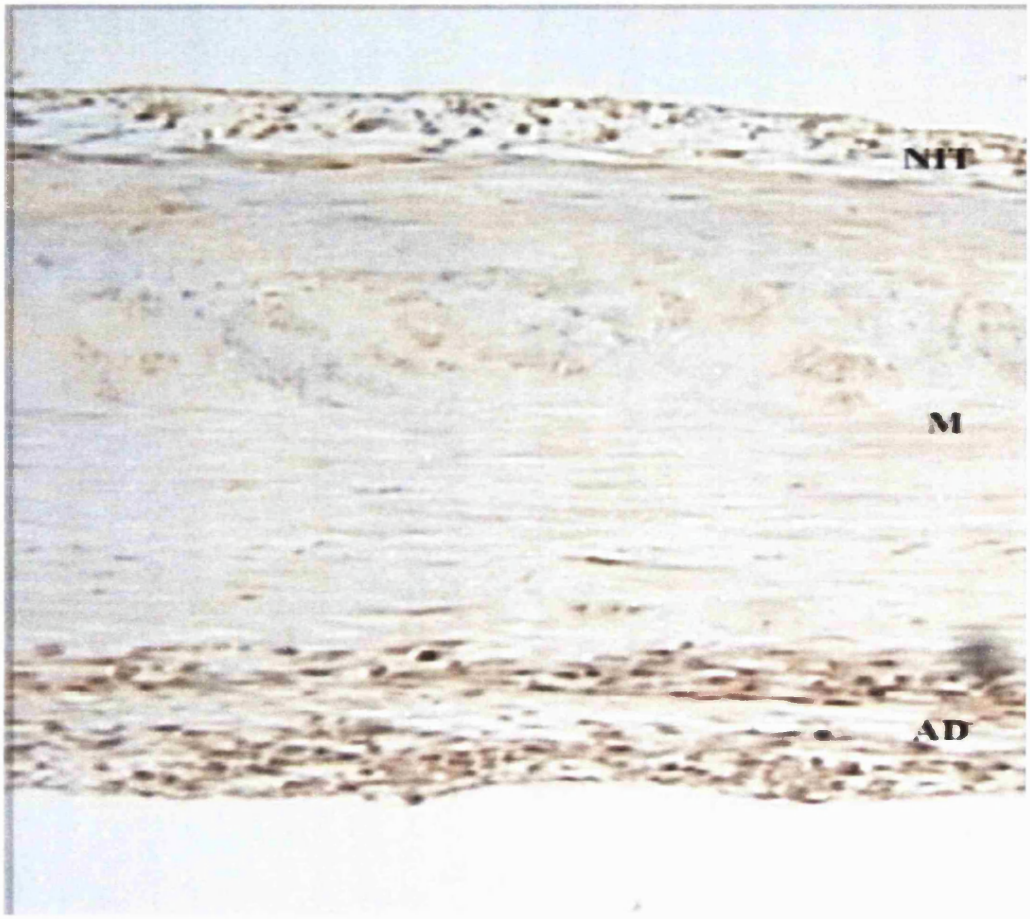


Figure 6.13. The demonstration of a CMV early antigen in a saphenous vein segment infected with CMV strain AD169 at day 21 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 and cultured for 21 days. At this time point, they were removed and stained for the CMV early antigen p52 with the monoclonal antibody CCH2 using the streptavidin-biotin-peroxidase technique. CMV-infected cells expressing early antigen (brown staining) were detected in the neointimal, intimal and adventitial layers of the vein wall. CMV early antigens were visualized using diaminobenzidine and the section was counterstained with Mayers haematoxylin. The irrelevant isotype matched control antibody staining is shown in Figure 6.10 Part C. (NIT-neointima, M-Media, AD-Adventitia). x400 magnification.

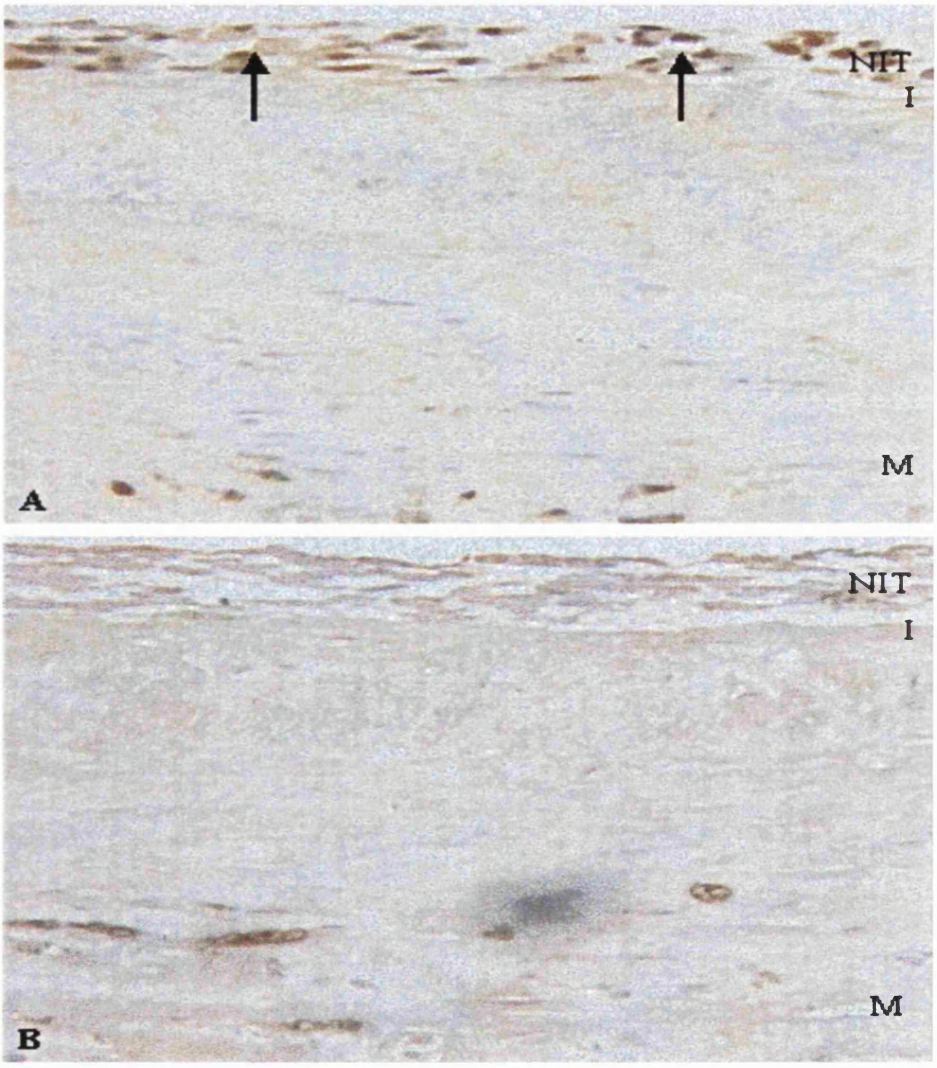


Figure 6.14. The demonstration of a CMV early antigen in a saphenous vein segment infected with CMV strain C1F at day 21 post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain C1F and cultured for 21 days. At this time point, they were removed and stained for the CMV early antigen p52 with the monoclonal antibody CCH2 (Part A), or an irrelevant isotype-matched control antibody (Part B) using the streptavidin-biotin-peroxidase technique. Part A shows that brown-staining CMV-infected cells expressing the early antigen in the neointima (arrowed). CMV early antigens were visualized using diaminobenzidine and the section was counterstained with Mayers haematoxylin. (NIT-neointima, I-Intima, M-Media). x640 magnification

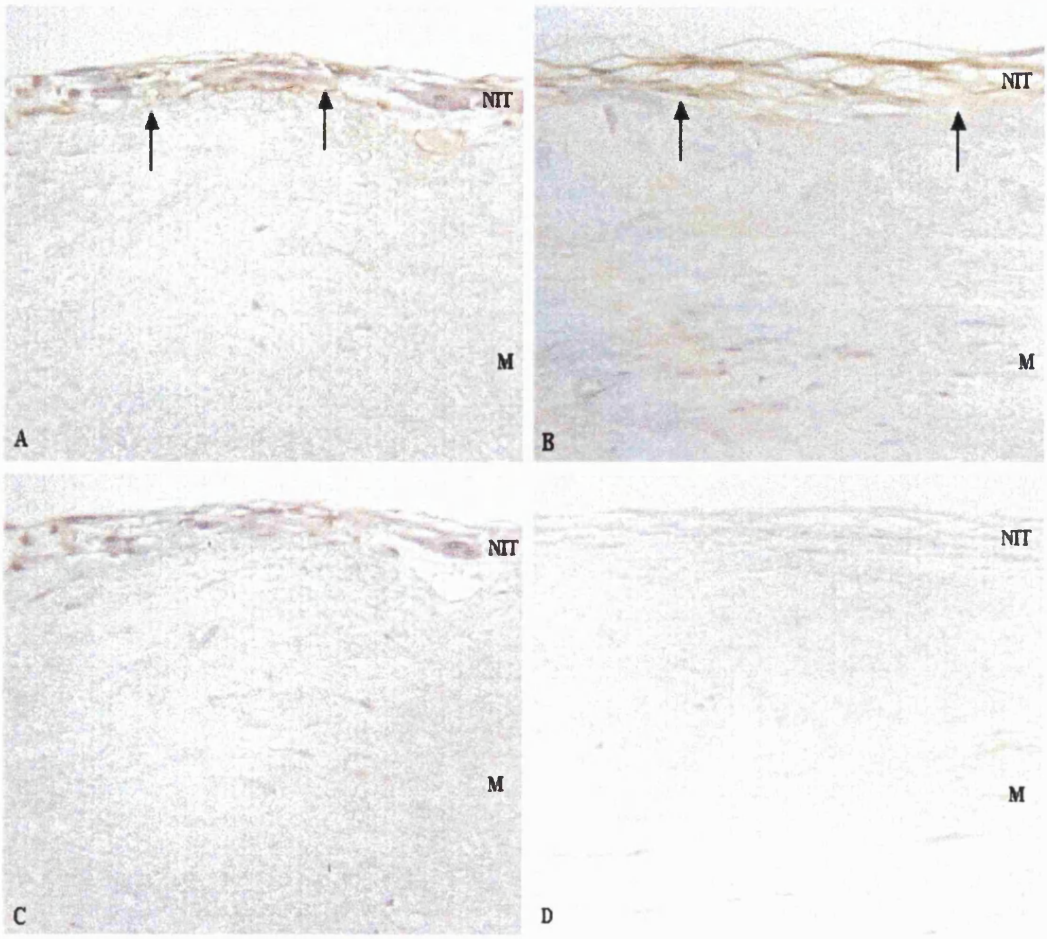


Figure 6.15. The demonstration of the localization of endothelial cells in cultured saphenous vein segments using an anti–von-Willebrand factor antibody at day 14 post-culture.

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 14 days. At this time point, they were removed, and stained for endothelial cells using a monoclonal antibody specific for von-Willebrand factor. Parts A and B show photomicrographs of endothelial cells (arrowed) in infected and mock-infected vein segments, respectively, as detected using the streptavidin-biotin peroxidase technique. Irrelevant isotype-matched control antibody staining for infected and mock-infected vein segments is shown in (C) and (D), respectively. Mayers haematoxylin was used as a counterstain. (NIT-Neointima, M-Media). x350 magnification.

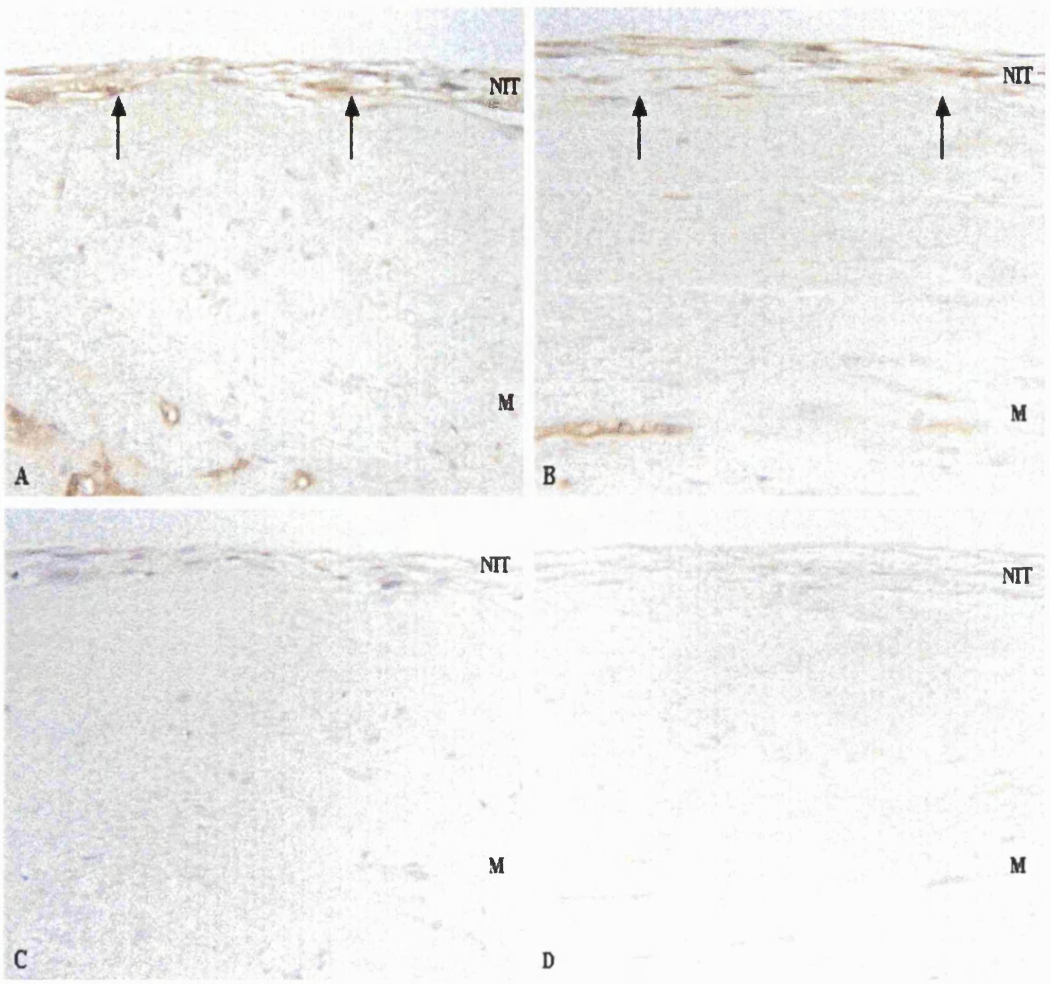


Figure 6.16. The demonstration of the localization of endothelial cells in cultured saphenous vein segments using a CD34 antibody at day 14 post-culture.

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 14 days. At this time point, they were removed and stained for endothelial cells using a CD34 monoclonal antibody. Parts A and B show photomicrographs of endothelial cells (arrowed) in infected and mock-infected vein segments, respectively, as detected using the streptavidin-biotin peroxidase technique. Irrelevant isotype-matched control antibody staining for infected and mock-infected cultures is shown in (C) and (D), respectively. Mayers haematoxylin was used as a counterstain. (NIT-Neointima, M-Media). x350 magnification.

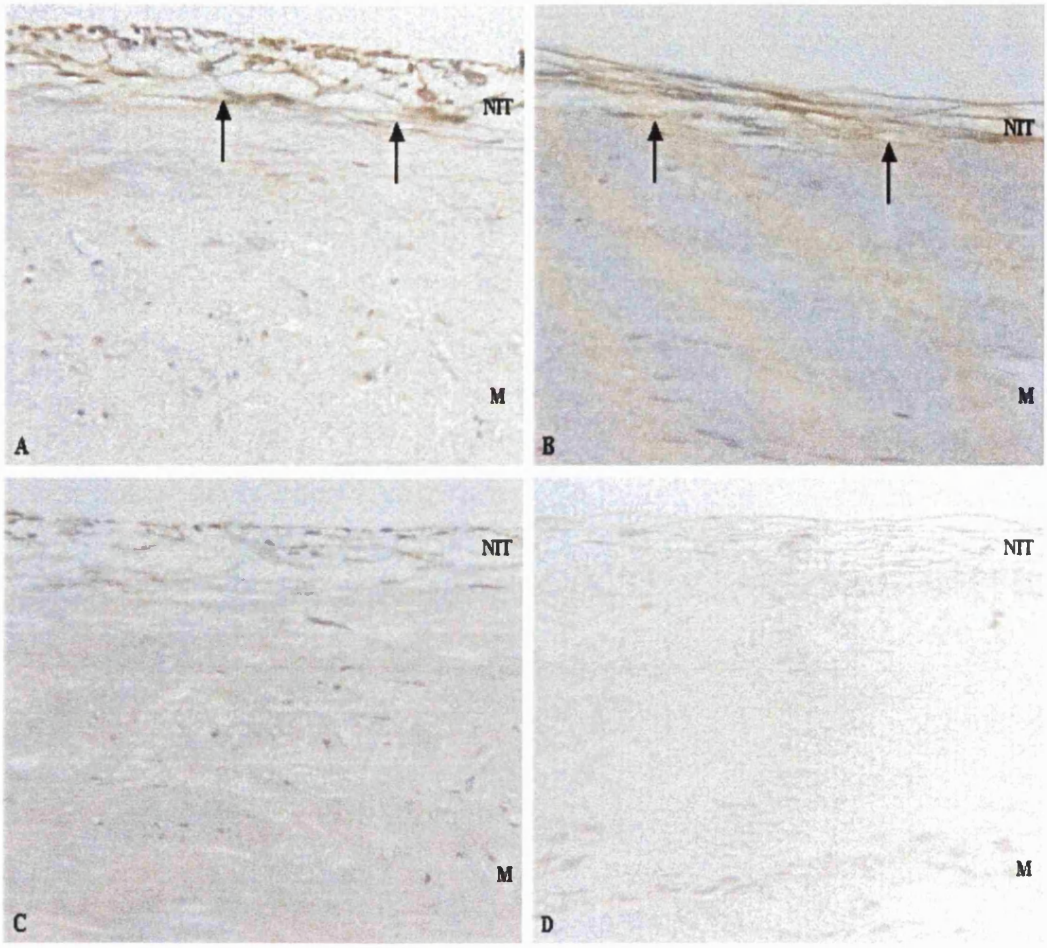


Figure 6.17. The demonstration of the localization of endothelial cells in cultured saphenous vein segments using an anti–von-Willebrand factor antibody at 21 days post-culture.

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 21 days. At this time point, they were removed and stained for endothelial cells using a monoclonal antibody specific for von-Willebrand factor. Parts A and B show photomicrographs of endothelial cells (arrowed) in infected and mock-infected vein segments, respectively, as detected using the streptavidin-biotin peroxidase technique. Irrelevant isotype-matched control antibody staining for infected and mock-infected cultures is shown in (C) and (D), respectively. Mayers haematoxylin was used as a counterstain. (NIT-Neointima, M-Media). x350 magnification.

Figure 6.18. The demonstration of the localization of endothelial cells in cultured saphenous vein segments using a CD34 monoclonal antibody at 21 days post-culture.

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 21 days. At this time point, they were removed and stained for endothelial cells using a CD34 monoclonal antibody. Parts A and B show photomicrographs of endothelial cells (arrowed) in infected and mock-infected vein segments, respectively, as detected using the streptavidin-biotin peroxidase technique. Irrelevant isotype-matched control antibody staining for infected and mock-infected cultures is shown in (C) and (D), respectively. Mayers haematoxylin was used as a counterstain. (NIT-Neointima, M-Media). x350 magnification.

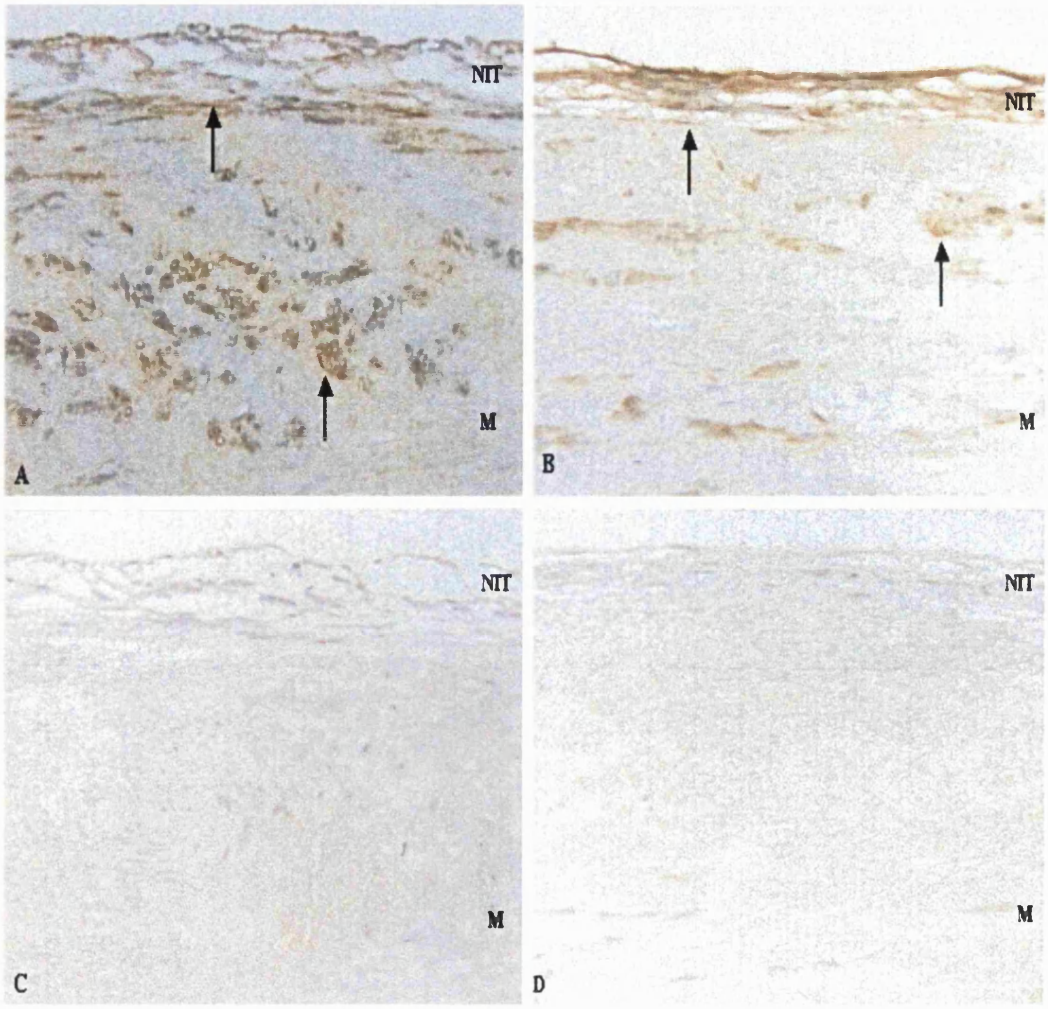


Figure 6.19. The demonstration of the localization of smooth muscle cells in cultured saphenous vein segments at 21 days post-culture..

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and subsequently cultured for 21 days. At this time point, they were removed and stained for smooth muscle cells using a monoclonal antibody specific for smooth muscle α-actin. Parts A and B show photomicrographs of smooth muscle cells (arrowed) in infected and mock-infected vein segments, respectively as detected using the streptavidin-biotin peroxidase technique. Irrelevant isotype-matched control antibody staining for infected and mock-infected cultures is shown in (C) and (D), respectively. Mayers haematoxylin was used as a counterstain. (NIT-Neointima, M-Media). x450 magnification.

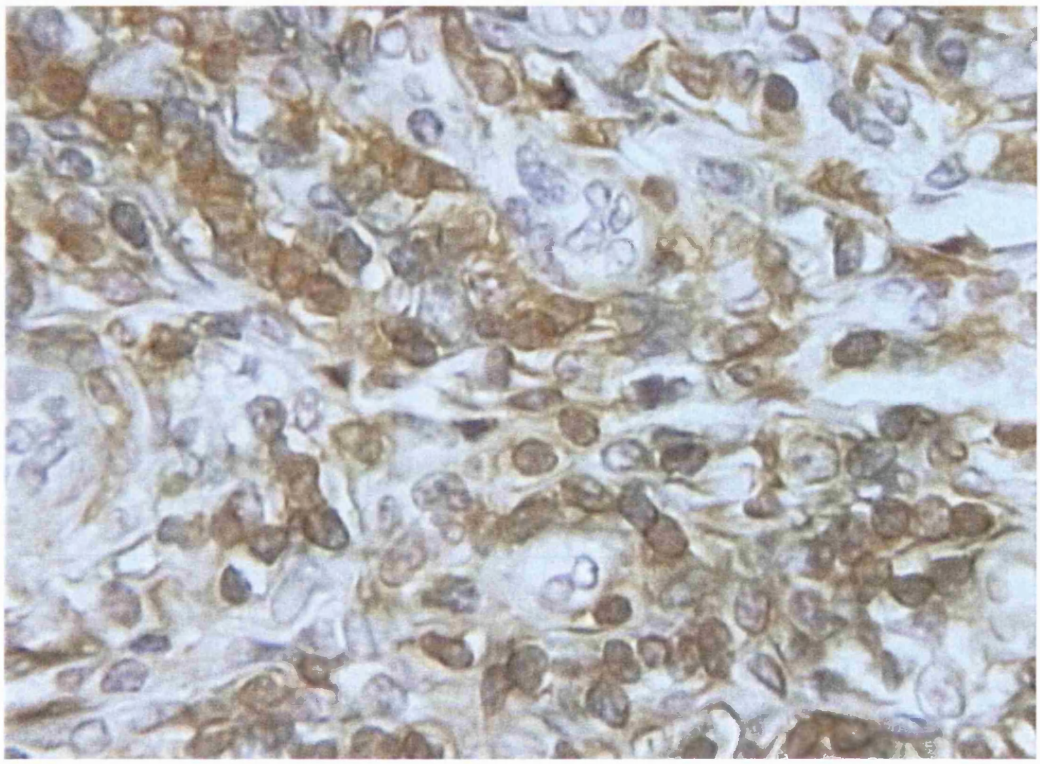


Figure 6.20. The detection of the CD45 lymphocyte common antigen in a human tonsil section.

Immunohistochemical staining of a human tonsil section with a monoclonal antibody specific for the CD45 leukocyte common antigen. The CD45 lymphocyte common antigen (brown-staining) was detected using a streptavidin-biotin-peroxidase technique and visualised with diaminobenzidine. Mayers haematoxylin was used as a counterstain. x700 magnification.

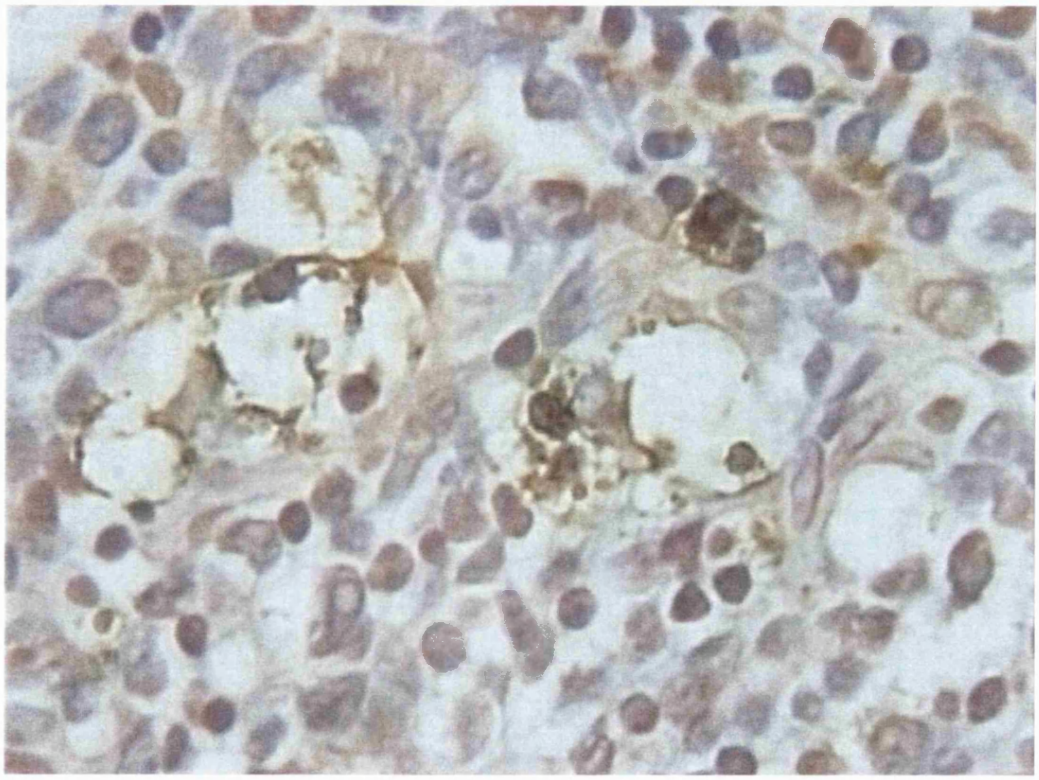


Figure 6.21. The detection of the CD68 monocyte/macrophage cell marker in a human tonsil section.

Immunohistochemical staining of a human tonsil section with a monoclonal antibody specific for the CD68 monocyte/macrophage cell lineage. The CD68 monocyte/macrophage positive cells (brown staining) were detected using a streptavidin-biotin-peroxidase technique and were visualised with diaminobenzidine. Mayers haematoxylin was used as a counterstain. x700 magnification.

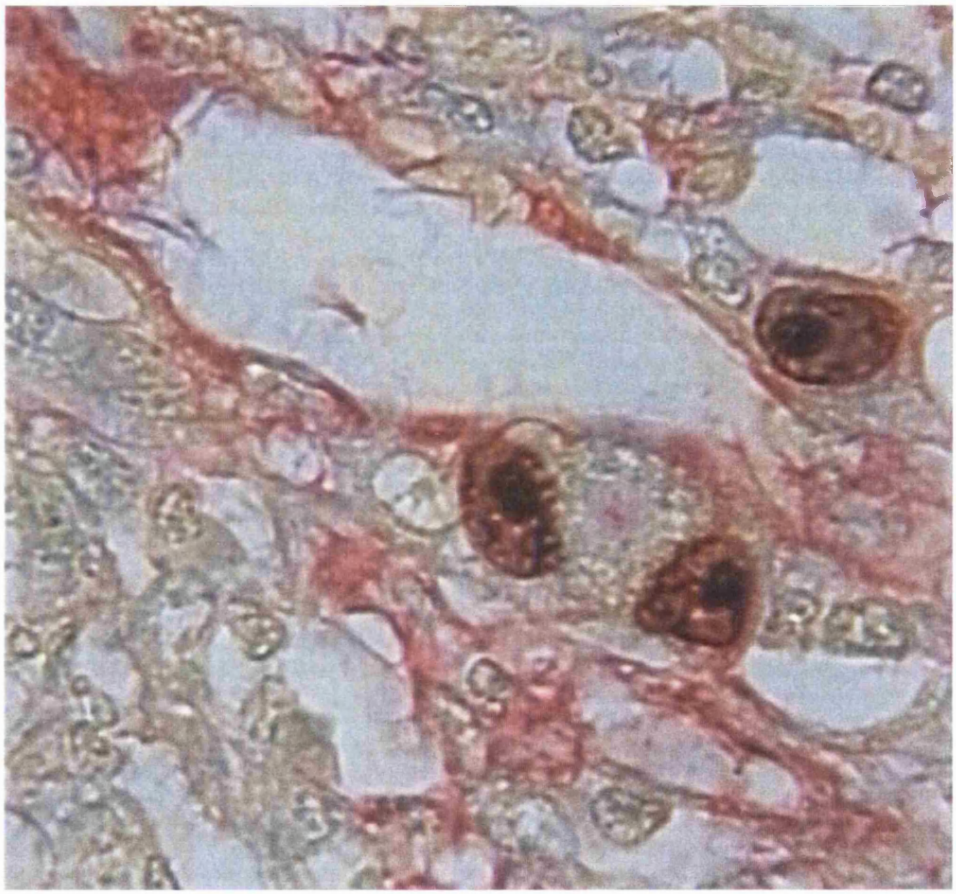


Figure 6.22. Double-immunohistochemical staining for CMV antigens and endothelial cell markers in a section of colon tissue from a CMV infected patient.

Double-immunohistochemical staining of a section from the colon of a patient with ulcerative colitis who died from disseminated CMV infection, using the CMV-specific monoclonal antibody, CCH2, and a rabbit antibody specific for von-Willebrand factor, respectively. CMV-specific early antigen (brown-staining) was detected by a streptavidin-biotin-peroxidase technique, while von-Willebrand factor (red-staining) was detected by a streptavidin-biotin-alkaline phosphatase technique. No counterstain was required. X1600 magnification.

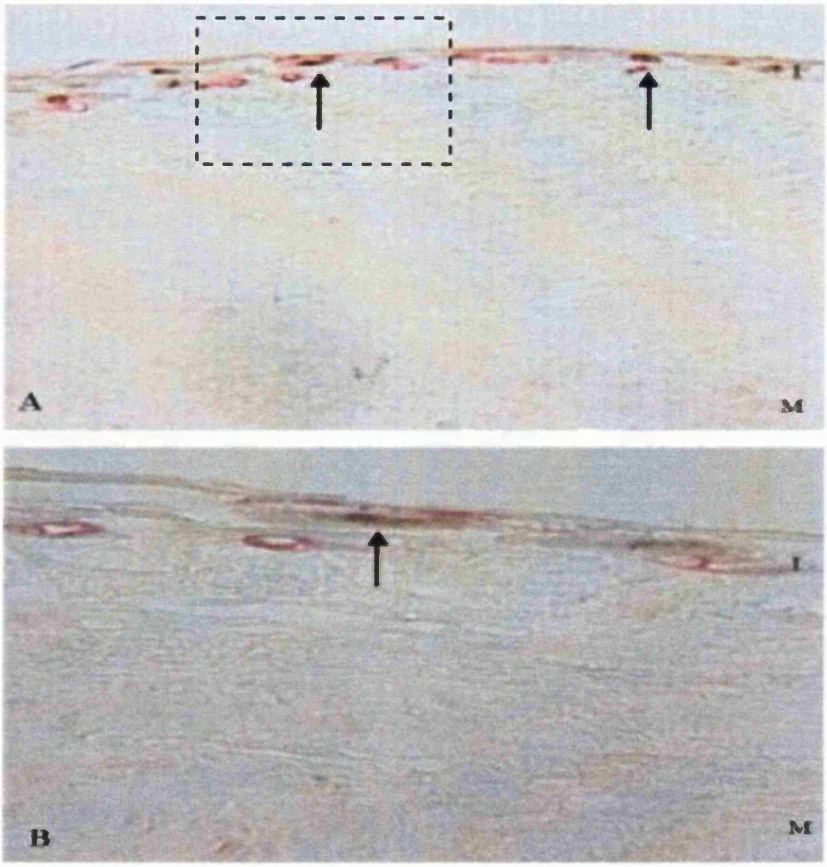


Figure 6.23 Double immunohistochemical staining for CMV antigens and an endothelial cell marker in a infected saphenous vein segment at 14 days post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 and cultured for 14 days. At this time point, they were removed and analysed by double-immunohistochemical staining for the simultaneous detection of CMV early antigens and endothelial cell markers using the monoclonal antibody CCH2 and a rabbit antibody specific for von-Willebrand factor, respectively. CMV early antigen as detected by an immunoperoxidase technique yielded brown-staining, while von-Willebrand factor as detected by an immunoalkaline phosphatase technique resulted in red cytoplasmic staining. Part A shows the presence of CMV early antigen and von-Willebrand factor positive cells lining the luminal surface of the vein wall (arrowed). Part B represents a higher magnification of the outlined section from part A. (I-Intima, M-Media). x280/x1150 magnification.

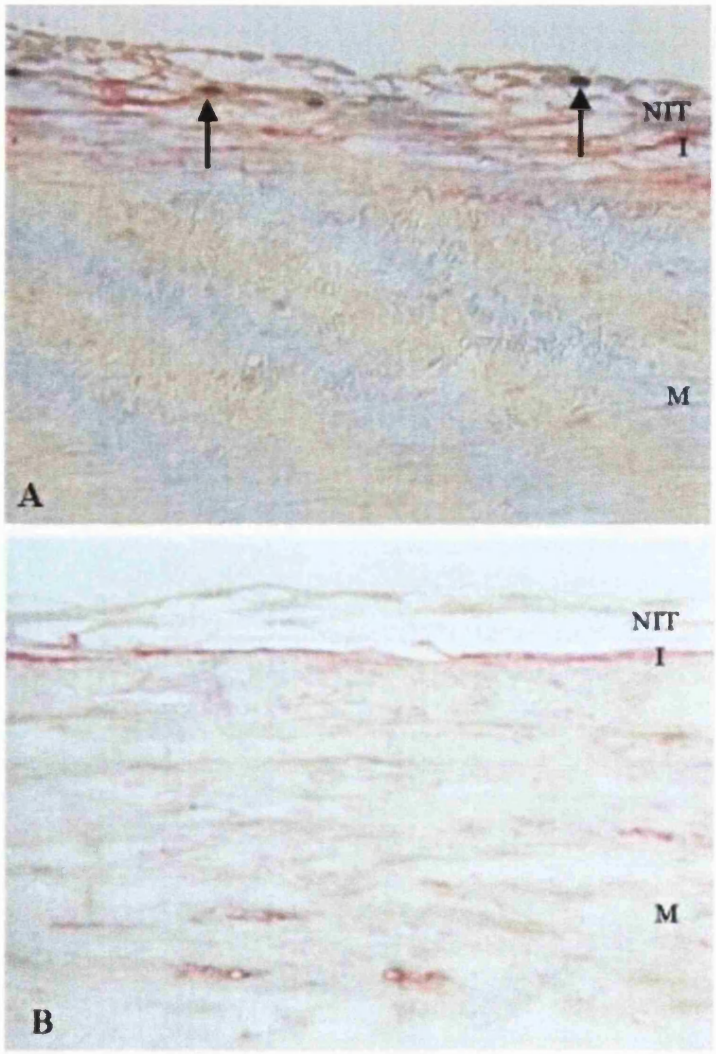


Figure 6.24. Double immunohistochemical staining for CMV antigens and an endothelial cell marker in a cultured saphenous vein segment at 21 days post-culture.

Saphenous vein segments were infected by centrifugal inoculation with CMV strain AD169 or mock-infected, and cultured for 21 days. At this time point, they were removed and analysed by immunohistochemical double staining for the simultaneous detection of the CMV early antigen p52 and von-Willebrand factor. A; an infected saphenous vein at 21 days post-culture showing the presence of CMV early antigen in neointimal cells lining the luminal surface of the vein wall. B: Mock-infected saphenous vein segments. The detection of the CMV early antigen by an immunoperoxidase technique yielded brown-staining (arrowed) while endothelial cells as detected with a rabbit antibody specific for von-Willebrand factor by an immunoalkaline phosphatase technique resulted in red cytoplasmic staining (NIT-Neointima, I-Intima, M-Media). x280 magnification.

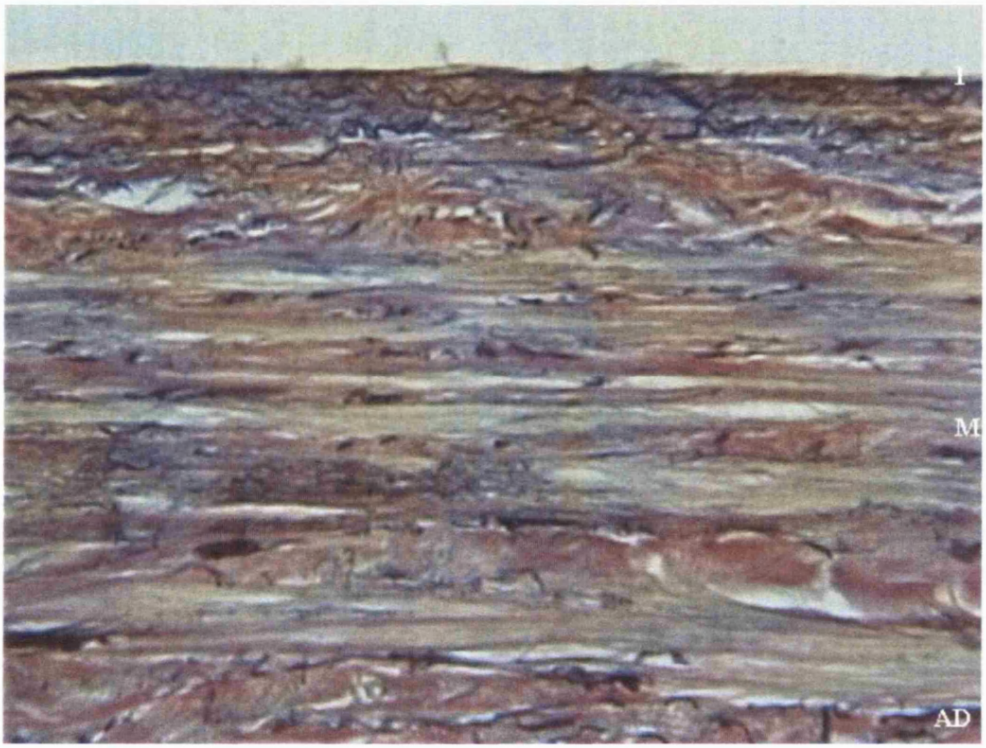


Figure 6.25. The demonstration of elastin fibres in a pre-culture saphenous vein segment.

Photomicrograph of a vein segment fixed at day 0 and stained with Millers elastin/van Gieson stain which stains all elastic fibres present in the vein wall black, muscle fibres – yellow and collagen-red. The photomicrograph illustrates that prior to vein culture, there was no neointimal layer formed in vein segments (I-Intima, M-Media, AD-Adventitia). x400 magnification

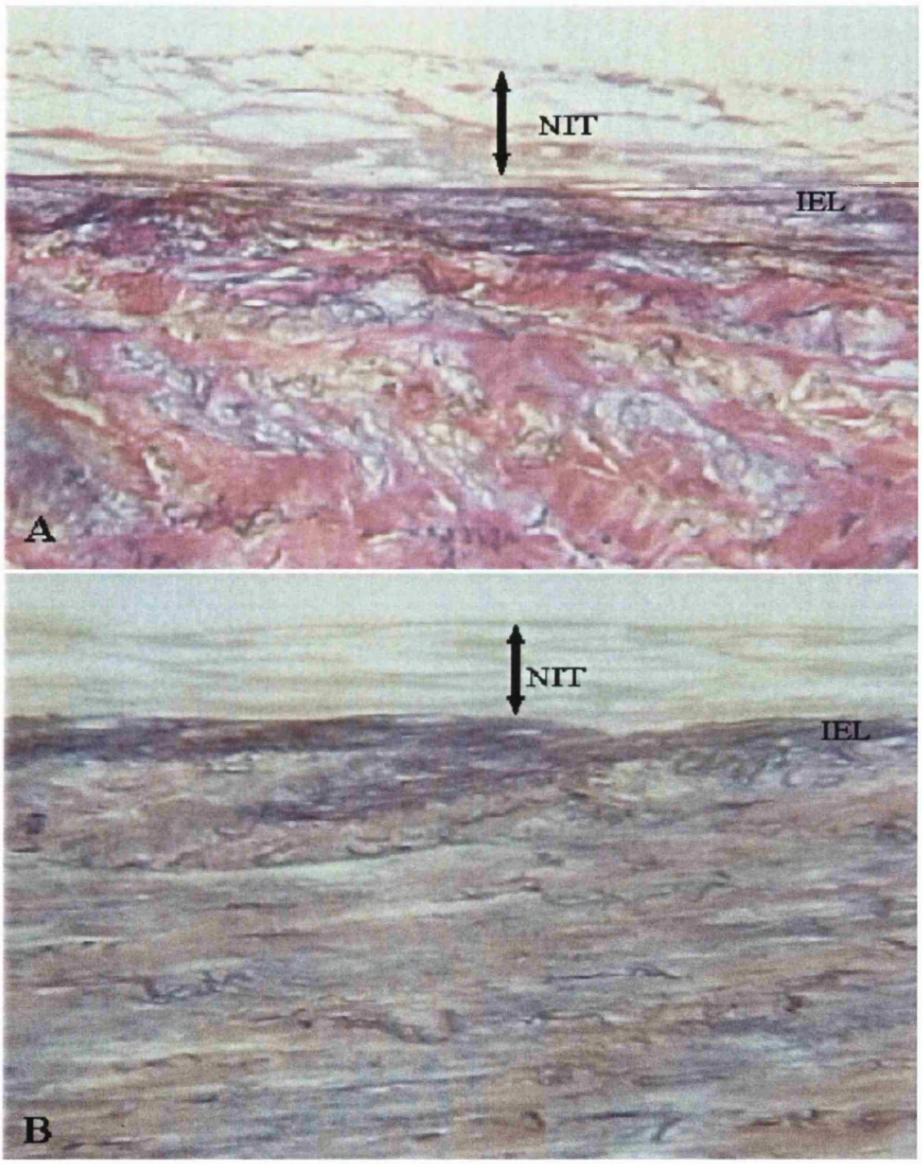


Figure 6.26. The demonstration of neointimal formation in cultured saphenous vein segments at 21 days post-culture.

Saphenous vein segments were either infected by centrifugal inoculation with CMV strain AD169, or mock-infected, and subsequently cultured for 21 days. At this time point, they were removed and stained with Millers elastin/van Gieson stain. Parts A and B illustrate representative transverse sections of infected and mock-infected vein segments, respectively. They show comparable neointima formation between infected and mock-infected vein segments (arrowed). In addition, infected vein segments show an altered organization of extracellular matrix, as judged by the differences in collagen matrix composition (red staining) between infected and mock-infected cultures in the medial layer of the vein wall. (IEL-Internal elastic lamina). x700 magnification.

6.3: DISCUSSION

In the present study, the organ culture of the human saphenous vein was used to study the susceptibility of cells of the vein wall to CMV infection. The organ culture of the saphenous vein is based on the use of intact vein segments. Hence, it was important to determine whether the intimal and medial cells of the vein segments remained viable during preparation and tissue culture. The examination of the intimal surface morphology of saphenous vein segments revealed that an intact endothelium was always present in these vessels as demonstrated by their ability to exclude trypan blue, and their reactivity with monoclonal antibodies specific for endothelial cell markers. Others have studied the viability of the harvested vein segments by quantitating the total tissue adenosine triphosphate (ATP) and adenosine diphosphate (ADP) concentrations of the predominant cell type of the vessel wall, the smooth muscle cell. The concentration of ATP and the ATP/ADP ratio normally observed in saphenous vein segments immediately after surgical removal, and rinsing in physiological saline indicated that the preparative procedure caused little additional injury to the vein wall (Angelini et al., 1987).

It has been demonstrated that the viability of cells of the vein wall is maintained when vein segments are cultured in medium supplemented with 30% foetal calf serum (Soyombo et al., 1990; Porter et al., 1996a; Jeremy et al., 1997). In the present study, cultures of saphenous vein segments appeared to be viable for up to 21 days, as suggested by their retention of morphologic integrity, and the preservation of the three coats of the vein wall as shown by histological examination. Thus, under these culture conditions, saphenous vein segments maintained their morphological integrity and closely resembled blood vessels *in vivo*. They were therefore possibly more suitable for studies of virus-blood vessel interactions than monolayer cell cultures derived from vascular tissues. In view of recent studies implicating CMV infection in the pathogenesis of human vascular diseases, this organ culture system was used to study CMV infection of cells of the vein wall.

In previous chapters, CMV infection of monolayer cultures of smooth muscle cells was achieved using the standard virus adsorption technique. This virus inoculation procedure was demonstrated to be inadequate in infecting cells of the saphenous vein in organ culture. On the other hand, centrifugal inoculation of the saphenous vein segment at 1500g for one hour, followed by a further hour of virus adsorption at 37°C proved to be a more effective procedure for achieving permissive CMV infection of cells of the saphenous vein. The effects of low-speed centrifugation enhanced the efficiency of CMV

infection of cells of the vein wall. In primary cultures of monocytes/macrophages, and monolayer cultures of endothelial cells, it has been demonstrated that centrifugal inoculation improves the efficiency of CMV infection *in vitro* (Ho et al., 1993; MacCormac & Grundy, 1999). The mechanism underlining the enhancement of CMV infectivity by centrifugal inoculation is unknown, but it is believed that the interaction between the CMV virion and the target cell is greatly increased during centrifugation (Hughes, 1993; Plachter et al., 1996).

Immunohistochemical analysis of infected saphenous vein segments with a monoclonal antibody specific for CMV IE antigen, revealed that IE antigens were observed in infected saphenous vein segments as early as day 7 post-culture. CMV-specific IE antigens were detected in the intimal and adventitial cells of the vein wall. There were no IE antigens observed in the medial layer of the saphenous vein segment. The infection apparently progressed, with increasing numbers of cells expressing IE antigen observed beyond day 7 post-culture in the intimal and adventitial cells of the saphenous vein. At day 21 post-culture, a prominent neointima cell layer had developed in infected and mock-infected vein segments, and this was shown to be highly susceptible to CMV infection, as indicated by the presence of numerous cells expressing IE antigen and the presence of distinct cytomegalic cells. Further immunohistochemical analysis of infected saphenous vein segments with a monoclonal antibody specific for an early CMV antigen was carried out, in order to assess whether the observed infection proceeded beyond IE times. It was observed that the infection of saphenous vein segments had progressed with the CMV early antigen expressed in the neointimal, intimal and adventitial layers of the vein wall. Thus the data obtained in the present study suggested that CMV infection was initiated in cells of the saphenous vein, as determined by the expression of IE antigens, and that this progressed to the expression of an early CMV antigen. In addition, the fact that the number of cells expressing IE antigen significantly increased between days 14 and 21 post-culture in vein segments infected with either strain of CMV, suggested that such infection became fully productive with the resultant spread of virus within the vessel wall. These results represent the first demonstration of CMV infection of an intact human blood vessel segment *in vitro*.

A full understanding of the interaction of CMV with cells of the vein wall requires the identification of the cells that allow permissive infection as opposed to those cell populations which are either abortively infected or not infected at all. As described above, CMV-specific IE and early antigens were localized in the intimal, neointimal and adventitial layers of the vein wall in infected saphenous vein segments.

Immunohistochemical analysis using monoclonal antibodies specific for the predominant cell types in the vein wall, notably, endothelial cells and smooth muscle cells, was performed to deduce the phenotype of cells that were susceptible to CMV infection. The streptavidin-biotin-peroxidase staining technique using the monoclonal antibodies, anti-von-Willebrand factor and CD34 revealed that endothelial cells were localized in the neointimal and intimal, but not in the medial and adventitial layers of the vein wall. Smooth muscle α-actin staining was observed to be localized in the neointimal, intimal and medial layers of the vein wall. There was no smooth muscle α-actin staining present in the adventitial layer of the vein wall. The characterization of the phenotype of cells using monoclonal antibodies specific for fibroblast markers was not carried out. This was because antibodies specific for fibroblast markers such as 5B5, which recognize prolyl-4-hydroxylase, and anti-vimentin are also reactive with smooth muscle cells (Esterre et al., 1992, Lazarides, 1980). The fibroblast-specific marker designated Fib AS02, which was described in Chapter 3 was also not suitable for the studies presented here because of its non-reactivity with fibroblasts in formalin-fixed paraffin-embedded tissue sections (Saalbach et al., 1996). There was no staining for CD45 and CD68 antigens in CMV-infected saphenous vein segments. Hence, immunohistochemical analysis using the streptavidin-biotin-peroxidase staining technique revealed that cells permissive for CMV infection in infected saphenous vein segments included endothelial cells in the intima, and a mixture of endothelial and smooth muscle cells in the neointima. The media was composed of smooth muscle cells, and from its non-reactivity with endothelial and smooth muscle cell markers, the adventitia was most likely to be composed of fibroblasts. There were no leukocytes present in the vein wall, and thus monocytes and lymphocytes that could have transmigrated through the endothelium and become resident in the vein wall *in vivo* were not the cellular targets of CMV infection observed in the vein wall. It is of interest that the smooth muscle α-actin monoclonal antibody, anti-α-smooth muscle-1, was specific in its reactivity to smooth muscle cells in the organ culture model, and was not reactive with the adventitial layer of the vein wall, which is largely composed of fibroblasts. It was shown in chapter 3 that this monoclonal antibody was highly reactive with fibroblasts in monolayer cultures of this cell type. This suggests that the reactivity of anti-α-smooth muscle-1 with fibroblasts in formalin-fixed paraffin embedded tissue sections is different from that in monolayer cultures of this cell type.

In an attempt to conclusively identify the target cells for CMV infection in the vein wall, double-staining immunohistochemistry was used. It was observed with this

procedure, using a monoclonal antibody specific for the CMV early antigen and a rabbit polyclonal antibody specific for von Willebrand factor, that endothelial cells and CMV infected cells were present in the intima and/or neointima. In some cases at earlier times post-culture, endothelial cells appeared to be doubly positive for the endothelial cell marker and the CMV early antigen, suggesting that endothelial cells in the vein wall were primary targets for CMV infection. However, at later times post-culture, the vast majority of infected cells were not clearly positive for both antigens. Hence, conclusive identification of endothelial cells as targets for CMV infection was hindered. It has been reported that CMV infection of endothelial cells leads to the down-regulation in expression of several known endothelial cell markers such as von-Willebrand factor (Bruggeman et al., 1988). Bruggeman et al. 1988 demonstrated in cultured human endothelial cells isolated either from umbilical cord veins or arteries, or adult veins, that CMV infection with strain AD169 led to the disappearance of von-Willebrand factor from infected cells. Thus, there are restrictions in identifying CMV-infected endothelial cells in tissue or monolayer cultures of this cell type because the expression of the principal endothelial cell marker, von Willebrand factor is down-regulated as a result of CMV infection. In renal, bone-marrow and heart-lung transplant recipients, the use of a monoclonal antibody designated PAL-E, which specifically recognizes an undefined endothelial cell antigen has been reported to be capable of identifying circulating cytomegalic endothelial cells which are productively infected with CMV (Percivalle et al., 1993; Grefte et al., 1995; Salzberger et al., 1997). It was not possible to use this antibody in the present study because of its non-reactivity with endothelial cells in formalin-fixed, paraffin-embedded tissue sections. Hence, with the use of the reagents available, it was not possible to conclusively identify endothelial cells as one of the phenotype of cells infected in the intimal layer of the vein wall. However, at early times post-infection of the saphenous vein segments, it could be postulated, based on the anatomical location of endothelial cells in the vein wall, that the infected cells lining the luminal surface of infected vein segments were probably endothelial cells. Further studies should be carried out in which infected and mock-infected vein segments are frozen for immunohistochemical analysis, as opposed to being paraffin-embedded. This would enable the use of monoclonal antibodies specific for several cell markers that are non-reactive on formalin-fixed paraffin-embedded sections, but reactive on frozen tissue sections. This procedure would assist in conclusively identifying the cell types susceptible to CMV infection in the vein wall.

In a few infected vein segments, the CMV-infected luminal surface cells were observed to lose their adherence, resulting in the detachment of these cells from the basement membrane of the vessel wall. These cells resembled late-stage CMV-infected cells, with prominent cytomegalic alterations. These infected luminal surface cells, possibly endothelial cells, may have some implication in the dissemination of CMV during acute infection. In heart or heart-lung transplant recipients, marrow transplant recipients and AIDS patients with active CMV infection, it has been observed that giant cells fully permissive for CMV infection circulate in the peripheral blood (Grefte et al., 1993; Percivalle et al., 1993; Salzberger et al., 1997). These giant cells have been identified to be of endothelial origin, and have been shown to contain viral antigens from all three stages of the viral replication cycle, indicative of a productive CMV infection (Percivalle et al., 1993; Grefte et al., 1995; Salzberger et al., 1997). It is believed that CMV-infected endothelial cells progressively enlarge till they detach from the vessel wall and enter the blood stream. The detachment of actively infected endothelial cells from the basement membrane into the bloodstream may provide a means by which circulating endothelial cells infected with CMV may be involved in the haematological spread of the virus to other cells and organs of the body (Grefte et al., 1993; Percivalle et al., 1993). These giant cells are carried by the bloodstream, and may get trapped in microvessels where they could distribute infectious virus to adjacent cells. Thus the data provided here provides support for the hypothesis that CMV infection of the endothelium leads to the detachment of infected endothelial cells, which could then circulate in the blood stream and play a role in virus dissemination. Furthermore, the organ culture system provides a model to study such endothelial cell detachment in more detail. Overall, the observed infection, enlargement and subsequent detachment of luminal cells of the saphenous vein in the organ culture system is consistent with observations obtained from immunocytochemical and histological studies in heart transplant recipients and AIDS patients acutely infected with CMV (Percivalle et al., 1993; Grefte et al., 1995; Salzberger et al., 1997). In these systems CMV infection has been shown to lead to the enlargement and subsequent detachment of endothelial cells from the infected blood vessel wall.

Intimal surface cells in saphenous vein cultures, presumably endothelial cells, were equivalently infected with the high passage CMV strain AD169 and the low passage clinical CMV strain C1F. This is of interest, since little or no infection is achieved in single cell type monolayer cultures of endothelial cells infected with the AD169 strain (Waldman et al., 1989; MacCormac & Grundy, 1999). This is true even when infection of monolayer cultures of endothelial cells with CMV strain AD169 is performed by

centrifugal inoculation (MacCormac & Grundy, 1999). In contrast, low passage strains of CMV such as C1F can be successfully propagated in endothelial cell cultures *in vitro* (MacCormac et al., 1996). This suggests that the physiological state and/or the environment of the endothelial cell is different between endothelial cell monolayer cultures and the endothelial cells in the organ culture of the saphenous vein segments, and that this difference affects the ability of high passage strains of CMV, such as strain AD169, to replicate in endothelial cells. It is possible that soluble factor(s) released from components of the vein wall, possibly smooth muscle cells, or the interaction of these vessel wall components with the endothelium in the intact vein wall, increases the susceptibility of endothelial cells to infection with high passage strains of CMV. The demonstration of the importance of such intercellular interactions in CMV replication illustrates an advantage of the organ culture system over monolayer cultures of single cell types in studying CMV-vascular cell interactions.

A neointima cell layer developed in cultured saphenous vein segments. With the use of monoclonal antibodies specific for endothelial cell markers and smooth muscle α-actin, it was demonstrated by immunohistochemical staining that the neointima was composed of a mixture of endothelial and smooth muscle cells. While neointimal smooth muscle cells appeared to be susceptible to CMV infection, medial smooth muscle cells were never found to be infected with CMV. Neointimal and medial smooth muscle cells are known to have distinct properties both *in vitro* and *in vivo* (Dartsch et al., 1990; Majesky et al., 1992), and such differences may underline their susceptibility to CMV infection. An alternative explanation for the lack of CMV infection of medial smooth muscle cells is that CMV virions could not penetrate through the intact endothelial cell layer into the vessel wall and infect the underlining medial smooth muscle cells. However, since in this *in vitro* system, penetration of CMV virions was possibly through the cut edges of the vein wall or through the adventitial side of the saphenous vein segment, the latter possibility seems unlikely. As CMV is normally permissive in actively proliferating cells (Plachter et al., 1996), the quiescent phenotype of medial smooth muscle cells could limit their susceptibility to CMV infection. In support of the conclusion that neointimal, and not medial smooth muscle cells are susceptible to CMV infection in the organ culture system presented here, are results obtained using the rat model of accelerated transplant atherosclerosis (Persoons et al., 1994; Persoons et al., 1997). Persoons et al. (1994) investigated the susceptibility of neointimal and medial smooth muscle cells to acute CMV infection in immunocompetent and immunosuppressed rats. In their experiments, the left carotid arteries of these rats were injured by balloon

catheterization while the right carotid artery was left untouched. On day 14 after injury, rats were either intravenously infected with rat CMV or mock-infected with a salivary gland homogenate derived from non-infected rats. It was observed after 2 weeks of rat CMV infection, that active CMV infection was shown in the neointima of the injured arteries of immunosuppressed rats, but that medial smooth muscle cells were never infected. No infection was seen in control right carotid arteries or in the injured arteries of immunocompetent rats. It was concluded that the phenotype of the neointimal smooth muscle cell is important in its susceptibility to rat CMV infection. Furthermore, since the change in the phenotype of neointimal smooth muscle cells is associated with enhanced proliferation of these cells, it was postulated that the change in phenotype and subsequent proliferation of these cells may be a prerequisite for rat CMV infection (Persoons et al., 1997). The data presented here suggests that the same requirements might also be necessary for the replication of human CMV in smooth muscle cells.

In the organ culture system described here, the thickness of the neointima formed was not significantly affected by CMV infection. This suggests that smooth muscle cell proliferation was not increased in the infected vein wall compared to mock-infected vein cultures. This could be due to the fact that neointima formation and/or smooth muscle cell proliferation *in vitro* were already maximal, and therefore could not be increased by CMV infection. The lack of effect of CMV infection on neointimal thickness described here is in contrast to observations obtained from studies of CMV infection in the rat model of accelerated allograft atherosclerosis. Using donor-recipient combinations of aortic allografts of inbred Darkagouti (AG-B4, $RT1^a$) and Wistar-Furth (AG-B2, $RT1^v$) rats, Lemstrom et al. (1993a) infected recipient rats with rat CMV and left control rats uninfected. The rats received 300μCi [^{3}H] thymidine by intravenous injection 3 hours before sacrifice, and the grafts were removed at various time points for histology, immunohistochemistry, and autoradiography. It was shown that rat CMV infection doubled the proliferation rate of smooth muscle cells, increased the thickness of the neointima, and significantly enhanced the generation of allograft arteriosclerosis. Thus the effects of CMV infection on neointimal thickness *in vivo* might be different from that observed in the organ culture system *in vitro*, where the effects of alloreactivity cannot be assessed.

In the present study, it was of interest to observe that there was an increase in the presence of collagen extracellular matrix in the medial component of the vein wall in infected vein cultures. Histological staining using the elastin/van Gieson stain, revealed that the increased content of collagen in the media of infected vein segments led to an

altered organization of the vein wall compared to mock-infected vein segments. This observation was of interest because in advanced lesions of atherosclerosis, the focal thickening of the intima is due in part to a large increase in the number of smooth muscle cells, and the formation and deposition of new connective tissue matrix by these smooth muscle cells. Such smooth muscle cells synthesize up to 45-fold more collagen, particularly type I collagen, than quiescent smooth muscle cells, and thereby contribute to the formation of a fibrous cap characteristic of atherosclerotic lesions. In the rat renal model of transplant-associated atherosclerosis, similar observations of increased collagen synthesis in the blood vessels of the transplanted organ have been described (Inkinen et al., 1997; Inkinen et al., 1999). In this study, renal transplantation was performed with a combination of inbred Darkagouti (AG-B4, $RT1^a$) and Wistar-Furth (AG-B2, $RT1^v$) rats under triple drug treatment. One group of rats was infected with rat CMV and the other mock-infected. One of the characteristic features of transplant-associated atherosclerosis of the renal allograft is interstitial fibrosis. It was demonstrated that rat CMV infection accelerated transplant-associated atherosclerosis as well as the development of fibrosis in renal allografts. This was shown by an increase in collagen synthesis, and a significant increase in the expression of collagen type III mRNA in the virus infected grafts as early as one week post-transplantation. The mechanism of this phenomenon is unknown, but it might be mediated by cytokines and growth factors produced in association with the immune response induced by the viral infection of the graft (Lemstrom et al., 1995; Arkonac et al., 1997). Hence, the data obtained from the organ culture system presented here, suggests that CMV infection may be involved in facilitating the synthesis and subsequent deposition of extracellular matrix characteristic of atherosclerotic lesions. In addition, the organ culture system provides an *in vitro* system in which the identity of possible growth factors and cytokines involved in the CMV-induced synthesis and deposition of extracellular matrix components could be studied.

In conclusion, the observations obtained from the organ culture system of the saphenous vein are fairly similar to findings in the rat model of transplant-associated atherosclerosis, where infection of rat allograft recipients with rat CMV has been demonstrated to be associated with the development of atherosclerosis. The present study suggests that CMV infection of endothelial and smooth muscle cells of the blood vessel wall leads to several morphological alterations in the blood vessel structure that could ultimately contribute to the pathogenesis of atherosclerosis. This suggests that the organ culture model of the saphenous vein with its capability to support CMV replication provides a useful model for the *in vitro* study of CMV-vessel wall interactions and their potential role in vascular pathology.

CHAPTER 7:

GENERAL DISCUSSION

It is at present generally accepted that infection of the blood vessel wall may play an important role in the development of atherosclerotic lesions. This concept is compatible with the commonly held belief that atherogenesis is mediated in a large part by inflammation occurring in the vessel wall (Ridker et al., 1997). Infection would fit this paradigm as being one of the several triggers of the inflammatory processes ultimately leading to the initiation of atherosclerotic plaque development. Evidence implicating infectious agents as a cause of atherosclerosis is based on seroepidemiologic, histopathologic, and mechanistic studies. For example, seroepidemiologic studies have shown an association of several pathogens, in particular CMV and *Chlamydia pneumoniae,* with atherosclerosis (Hendrix et al., 1990; Kuo et al., 1993). CMV has been found in atherosclerotic vessels (Melnick et al., 1994), and can infect cells residing within the vessel wall (Gyorkey et al., 1984), inducing changes in these cells that could be considered pro-atherogenic (Speir et al., 1994; Zhou et al., 1996). In addition, CMV has been found to augment the atherogeneic process in animal models of atherosclerosis (Zhou et al., 1999).

In the present study, an investigation into the potential role of CMV infection of smooth muscle cells in the pathogenesis of atherosclerosis was reported. This investigation proceeded with the isolation, culture and characterization of smooth muscle cells from human blood vessels. Smooth muscle cells were isolated and cultured from human saphenous veins and from the veins of the umbilical cord. Aortic smooth muscle cells were also obtained from a commercial source, and served as an arterial alternative to smooth muscle cells isolated from the blood vessel wall of the venous vasculature. The isolated smooth muscle cells in culture were observed to grow in a "hill and valley" morphology, typical of cultured smooth muscle cells, and were characterized using an α-actin smooth muscle cell marker to be of a smooth muscle cell phenotype. These observations paralleled the results obtained with aortic smooth muscle cells. Primary cultures of smooth muscle cells prepared by either enzyme dispersion or explantation rapidly induce de-differentiation under normal culture conditions (Chamley-Campbell et al., 1979). De-differentiation involves a change in the phenotype of smooth muscle cells from a quiescent, contractile state to a proliferative, synthetic phenotype, a phenomenon referred to as phenotypic modulation. Vascular smooth muscle cells in the synthetic state have a fibroblastic–like appearance, proliferate readily, and synthesize increased levels of various extracellular matrix components, particularly fibronectin, collagen type I and III, and tropoelastin (Chamley-Campbell et al., 1979). To support this phenotype, these cells contain large amounts of free ribosomes, rough endoplasmic reticulum, Golgi complexes,

and mitochondria. Contractile vascular smooth muscle cells, on the other hand, have a muscle-like or spindle-shaped appearance, and a well-developed contractile apparatus resulting from the expression and intracellular accumulation of thick and thin muscle filaments. Contractile cells can readily assume a synthetic phenotype within 1-2 passages after they are placed in culture *in vitro*, as well as when the vessel is injured either mechanically or immunologically *in vivo* (Chamley-Campbell et al., 1979). This modulation from a contractile to a synthetic phenotype appears to be an important early event in the pathogenesis of many vascular diseases, including atherosclerosis, and may be a prerequisite for the migration of the vascular smooth muscle cells that form the neointima following balloon catheter injury of the vessel wall (Pauly et al., 1992). Thus in trying to design studies to understand the abnormal growth of smooth muscle cells, and to identify the factors or agents that promote them, it is necessary to use methods of smooth muscle cell isolation in which the process of phenotypic modulation can be controlled. However a culture system which maintains a differentiated phenotype of smooth muscle cells, and controls the phenotypic conversion from a de-differentiated to a differentiated state has not been reported. There are no smooth muscle cell lines that maintain a fully differentiated phenotype (Chamley-Campbell et al., 1979). The present study using primary cultures of smooth muscle cells has therefore only characterized the de-differentiated cells with the synthetic phenotype.

Using a high passage CMV strain, AD169, and a low passage CMV strain, C1F, it was demonstrated that CMV was capable of undergoing a full cycle of replication in smooth muscle cells isolated either by explantation of the saphenous vein, or by enzyme dispersion of the veins of the umbilical cord. Human aortic smooth muscle cells were also demonstrated to be fully permissive to infection with either strain of CMV. Thus, there was no difference between smooth muscle cells isolated from the venous vasculature and arterial smooth muscle cells in their ability to support CMV replication. Viral replication was demonstrated by the immunofluorescent staining of CMV viral antigens, which are induced following CMV infection of a permissive cell. The replication of CMV in these smooth muscle cell cultures led to the production of infectious virus particles. These studies demonstrated that CMV infection of smooth muscle cells proceeded in a fashion comparable to CMV infection in fully permissive fibroblasts, and suggested that smooth muscle cells may be a host for the replication of the virus *in vivo*. This finding is likely to be significant in the pathogenesis of atherosclerosis and/or vascular disease. However, as mentioned above, it was only possible to study CMV infection in cultured de-differentiated smooth muscle cells with the synthetic phenotype. In order to try to extend

these studies to smooth muscle cells of the differentiated contractile phenotype, an organ culture system of the saphenous vein was developed, and used to study CMV replication of smooth muscle cells in the intact blood vessel wall, which might represent a more differentiated phenotype.

Whole segments of saphenous veins were cultured for extended periods of time *in vitro*, with the preservation of cell viability and vessel structure. Studies into the infection of these vein segments with CMV strain AD169 and strain C1F were undertaken. At various stages post-infection, CMV replication was observed in the intimal and adventitial layers of the vessel wall. This was evident by the detection of the expression of CMV-specific IE and early antigens by immunofluorescent staining in these layers of the vein segment. The medial portion of the vessel wall was apparently not infected with CMV. No CMV viral antigens were detected by immunofluorescent staining in this layer of the vein wall at any time point. These results represented the first *in vitro* demonstration of CMV replication in an intact blood vessel wall, and the localization of cells of the vessel wall that were susceptible to CMV infection. In addition, the results demonstrated the inability of human CMV to replicate in the medial smooth muscle cells of the blood vessel wall. A new cellular layer termed the neointima, which developed in culture, was shown to be highly permissive to CMV infection. Using monoclonal antibodies specific for certain cell surface markers that were likely to be present in the vasculature of the saphenous vein, it was deduced that the cell types susceptible to CMV infection were endothelial cells and/or smooth muscle cells in the neointima, and endothelial cells in the intima. However because monoclonal antibodies specific for fibroblasts were not available for use in this study, the possibility remains that cells of a fibroblast phenotype could have been present in the neointimal layer of the vein wall, and that these cells were permissive for CMV infection. However, this cell type has not been found in the neointima of such organ cultures by others (Soyombo et al., 1990; Holt et al., 1992; Slomp et al., 1996). Fibroblasts were the most likely cell type in the adventitia to be infected by CMV, due to the non-reactivity of cells in this layer with monoclonal antibodies specific for endothelial and smooth muscle cell markers. There was no detection of monocytes or other lymphocytes in the vein wall, which could have served as targets for CMV infection.

Definitive identification of the cells in the intima, neointima and adventitia which were permissive for CMV replication was not possible in the present study for two reasons. First, because of the unavailability of suitable monoclonal antibodies against cell specific markers reactive on formalin-fixed paraffin-embedded tissue which could be used for double-labelled immunofluorescence studies with CMV-specific antibodies. Second, because CMV infection can down-regulate the expression of certain cellular antigens. It is

possible that future studies using frozen sections instead of formalin-fixed paraffin-embedded tissue could provide evidence to definitively prove the cell types permissive for CMV in cultured vein segments. This is because a broader range of antibodies are available for studies using frozen sections, including an antibody against an endothelial cell marker, PAL-E, which is not believed to be down-regulated following CMV infection (Schlingemann et al., 1985; Grefte et al., 1993; Percivalle et al., 1993).

In the organ culture system, it was observed that the neointima, which was demonstrated to consist of endothelial and smooth muscle cells, was highly susceptible to CMV infection. Smooth muscle cells in the medial layer of the vein wall were however not susceptible to CMV infection. The difference between the latter smooth muscle cell population and neointimal smooth muscle cells is that medial smooth muscle cells are quiescent and contractile in nature, whilst neointimal smooth muscle cells are synthetic, proliferating cells. This suggests that the phenotype of the smooth muscle cell could affect its susceptibility to CMV. The phenotype of the smooth muscle cell in isolated smooth muscle cell cultures is similar to those of neointimal smooth muscle cells. Thus the findings that CMV replicates in monolayer cultures of smooth muscle cells supports the idea that neointimal smooth muscle cells are infected and are permissive to CMV infection in the organ culture system. Further support for this idea comes from *in vivo* studies in rats infected with rat CMV (Persoons et al., 1994). Persoons et al. (1994) demonstrated that neointimal smooth muscle cells, but not medial smooth muscle cells, were susceptible to rat CMV infection. In the latter study, there was no infection of smooth muscle cells in the normal vessel wall, but when the vessel wall was injured by balloon catheterization, rat CMV replication in the proliferating neointimal smooth muscle cells was found. This supports the idea that replicating cells are more susceptible to CMV infection than non-replicating cells. The apparent high permissiveness of synthetic smooth muscle cells to CMV infection has potential clinical relevance, because these cells are present in large numbers in atherosclerotic lesions and in the arteries of transplanted allografts.

The formation of a neointima is a feature of atherosclerotic lesions *in vivo*, and several reports have suggested that CMV infection may well provoke neointima formation *in vivo* (Lemstrom et al., 1993a, b). Using computerized morphometry, it was demonstrated in the present study that CMV infection of saphenous vein segments had no effect on the neointimal formation that arises in cultured vein segments relative to mock-infected vein segments. There are a few studies in rats infected with rat CMV that are consistent with this observation (Persoons et al., 1994; Persoons et al., 1997). In both of these studies, Persoons et al. (1994, 1997) reported that there was no difference in the size

of the neointimal area of the carotid artery in rats either infected with rat CMV or mock-infected two weeks after balloon catherization of the artery. In contrast, Lemstrom et al. (1993a) demonstrated, using the rat model of transplant-associated atherosclerosis, that CMV infection had a significant contributory effect on the neointimal thickening and smooth muscle cell proliferation in allogeneic transplant recipients, and that an allogeneic response was required for this effect. In this rat model, the first signs of transplant-associated atherosclerosis were an inflammatory influx of monocytes/macrophages and T lymphocytes into the adventitia of the vein wall. This suggests that the development of transplant-associated atherosclerosis in CMV-infected recipients is likely to be due to an interaction of CMV with the immune system. The finding that immunosuppression by triple drug therapy significantly reduced the development of transplant-associated atherosclerosis in rat CMV-infected aortic allografts, provides support for the concept that transplant-associated atherosclerosis is due to an immune reaction, and that rat CMV has a stimulatory effect on its development (Lemstrom et al., 1994a). This hypothesis is consistent with the lack of any effect of CMV infection on neointimal thickness in the present study, or in the rat studies of Persoons et al. (1994, 1997), since no allogeneic reaction was generated in either of these systems.

In addition to the development of the neointimal cell layer, it was observed that there was an increase in the presence of collagen extracellular matrix in the vein wall of infected vein segments. Histological staining using the elastin/van Gieson stain, revealed that there was an increased content of collagen in the media of infected vein segments, together with an altered organization of the vein wall, compared to mock-infected vein segments. These observations were of interest, because in advanced lesions of atherosclerosis, the focal thickening of the intima is due in part to a large increase in the number of smooth muscle cells, and to the formation and deposition of new connective tissue matrix by these smooth muscle cells. The data obtained from the organ culture system thus suggests that CMV infection may be involved in facilitating the synthesis and subsequent deposition of extracellular matrix components characteristic of atherosclerotic lesions. In the rat model of transplant-associated atherosclerosis, it has been demonstrated that CMV infection enhances the mRNA expression of platelet-derived growth factor-β and transforming growth factor β in rat aortic allografts. It would therefore be of interest to use the organ culture system in future studies to investigate the effect of CMV infection on the production of growth factors, which might contribute to the proliferation of smooth muscle cells, and on the synthesis of extracellular matrix components by cells of the vein wall.

The anatomical localization of the endothelial cell at the junction of the blood vessel and underlying tissues makes it a pivotal cell type during the generation of an inflammatory reaction. The cell surface of vascular endothelial cells is the first contact between the tissue and its host immune system, and this cell type has been implicated as a primary target of graft destruction during transplant rejection (Gohra et al., 1995). According to the response-to-injury hypothesis postulated for the pathogenesis of atherosclerosis, the injury or denudation of the endothelium is the initial event in the atherogenesis (Ross, 1999). Several clinical studies have shown that during acute CMV infection, endothelial cells are commonly infected with this virus regardless of the organ involved (Sinzger et al., 1993b: Sinzger et al., 1995). Although these *in vivo* results suggest that CMV replicates in the endothelial cell, studies of the *in vitro* infection of endothelial cells have yielded conflicting results (Friedman et al., 1981; Ho et al., 1984; Smiley et al., 1988; Bruggeman et al., 1988; Waldman et al., 1989; MacCormac & Grundy, 1999). In the organ culture system, it was observed that infected intimal surface cells, presumably endothelial cells, were permissive to CMV infection with either the highly passaged AD169 strain or the low passage clinical strain C1F. The luminal infected cells stained positively for IE and early CMV antigens, and exhibited cytomegalic features, characteristic of cells infected with CMV. *In vitro*, CMV infection of endothelial cells with CMV strain AD169 has been reported to be restrictive, with <5% of the inoculated cells expressing CMV-specific IE antigens (Friedman et al., 1981; Ho et al., 1984; MacCormac & Grundy, 1999). It is however possible to initiate CMV replication in a proportion of these cells by treatment of the inoculated cells with the short chain fatty acid, sodium butyrate (MacCormac & Grundy, 1999). The infectivity of endothelial cells with CMV strain AD169 could be further increased by low speed centrifugal inoculation of the virus (MacCormac & Grundy, 1999). The combination of low speed centrifugal inoculation plus sodium butyrate treatment resulted in the optimal permissivity of endothelial cells for CMV strain AD169, however the resultant CMV replication was restricted to only one cycle, as the virus produced could not infect endothelial cells in the absence of further such treatments (MacCormac & Grundy, 1999). In contrast, using low passage clinical CMV isolates propagated in endothelial cells, instead of the fibroblasts which are normally used to propagate CMV, to infect endothelial cell monolayers, resulted in the optimal permissivity of the endothelial cells and higher levels of virus production than did infection with CMV strain AD169 (Waldman et al., 1989; MacCormac & Grundy, 1999). These *in vitro* experiments suggested that the outcome of CMV infection of endothelial cells was dependent on the virus strain used, and on the activation state of the cell. However, in the organ culture system, it was observed that the luminal surface

cells, presumably endothelial cells, were equivalently infected with the high passage CMV strain AD169 and the low passage clinical CMV strain C1F. This observation is in contrast to the results of CMV infection of monolayer cultures of endothelial cells where, as mentioned above, little or no infection of endothelial cells with CMV strain AD169 was achieved. This suggests that the physiological state and/or the environment of the endothelial cell is different between endothelial cell monolayer cultures and the endothelial cells in the organ culture of the saphenous vein segments, and that this difference affects the ability of the high passage CMV strain AD169 to replicate in endothelial cells. It is possible that soluble factor(s) released from components of the vein wall, possibly smooth muscle cells, or the interaction of vessel wall components with the endothelium in the intact vein wall, increases the susceptibility of endothelial cells to infection with CMV strain AD169. The demonstration of the importance of such intercellular interactions in CMV replication illustrates an advantage of the organ culture system over monolayer cultures of single cell types in studying CMV-vascular cell interactions.

In addition to the observed permissiveness of luminal surface cells to either strain of CMV, it was observed that the infected luminal surface cells appeared to be detached from the basement membrane of the vessel wall. The observed detachment of these cells lining the luminal surface may be caused by an altered interaction of the cells with extracellular matrix proteins as reported for CMV infection of fibroblasts (Pande et al., 1990). In monolayer cultures of fibroblasts and endothelial cells, CMV infection leads to a progressive detachment of these cells from the substratum (Compton, 1993b; Waldman et al., 1989). The detachment of luminal surface cells in the organ culture system is consistent with the *in vivo* observation that immunocompromised patients with an active CMV infection have enlarged, cytomegalic endothelial cells in the peripheral blood which are believed to have detached from the basement membrane and entered the blood circulation (Grefte et al., 1993; Percivalle et al., 1993). Support for this belief has come from studies in which it was observed that there was the loosening of infected endothelial cells from the basement membrane of rats infected with rat CMV (Span et al., 1993). This was attributed to the fact that the infected endothelial cells could have detached from the basement membrane and became lost from the intimal surface of the vessel wall. It has been suggested that circulating infected endothelial cells may contribute to CMV dissemination throughout the body (Grefte et al., 1993; Percivalle et al., 1993). Thus the use of the organ culture system described here to study the effect of CMV infection on the attachment of endothelial cells to the basement membrane of the vessel wall could help in the understanding of the dissemination of CMV via the bloodstream.

It can be envisaged that the denudation of the endothelium following CMV infection of the vessel wall would expose the underlying neointimal smooth muscle cells to the arterial lumen. These smooth muscle cells arise from their migration from the media through the internal elastic lamina into the intima to form the neointima (Angelini et al., 1991), where they then undergo a change of phenotype from a contractile to a synthetic proliferative state. Thus the results from experiments investigating the effects of CMV infection on cultured smooth muscle cells, which are in the synthetic proliferative phenotype, could potentially have some relevance to the situation *in vivo* with regard to the interaction of the vessel wall with circulating leucocytes.

In the present study, the infection of cultured smooth muscle cells with CMV led to the modulation of a number of molecules involved in immune regulation and in leukocyte trafficking. It was shown that such infection with CMV strains AD169 and C1F altered the constitutive expression of ICAM-1. The increase in the expression of ICAM-1 molecules on smooth muscle cells could play a contributory role in recruiting leukocytes to the vessel wall. The increased expression of ICAM-1 has been shown to facilitate the adhesion of monocytes, neutrophils and lymphocytes via the β2-integrin (CD11/CD18) family of adhesion molecules (Meerscharet & Furie, 1995). Support for the proposal that ICAM-1 might play a role in allograft rejection comes from *in vivo* animal studies using monoclonal antibodies that block the function of this molecule. The 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 the rejection of xenogeneic islet cells in diabetic mice (Zeng et al., 1994a, b). In heart transplant recipients undergoing transplant-associated atherosclerosis, it has been demonstrated that there is an increase in the expression of ICAM-1 in endothelial and smooth muscle cells in the coronary artery of these patients (Ballantype et al., 1996). In the organ culture system, it was demonstrated that luminal surface cells, presumably endothelial cells, were infected with either strain of CMV. In human umbilical vein endothelial cells infected with an endothelial cell-propagated strain of CMV, it has been demonstrated that there is an enhanced cell surface expression of ICAM-1, but not of ELAM-1 and VCAM-1 (Sedmak et al., 1994). The increased expression of ICAM-1 was specific to infected cells, and was not found on adjacent uninfected cells. Infection with CMV actually inhibited the TNF-α-induced expression of ELAM-1 and VCAM-1, but further increased the ICAM-1 expression observed with TNF-α-stimulation. In human

liver allografts, CMV infection resulted in the induction of ICAM-1 on endothelial cells (Lautenschlager & Hockerstedt, 1993). Similarly, the expression of ICAM-1 on endothelial cells in the vessels of rat renal transplant recipients was reported to be markedly increased in rats who were infected with rat CMV as compared to non-rat CMV infected allograft recipients (Yilmaz et al., 1996). In addition, enhanced expression of ICAM-1 on lung endothelial cells was also demonstrated in lung transplant recipients infected with rat CMV as compared to mock-infected rat controls. The up-regulation of ICAM-1 in these studies was accompanied by an increase in the infiltration of inflammatory cells into the allografts, particularly LFA-1 and VLA-4 positive cells (Steinhoff et al., 1996). The increased expression of ICAM-1 during allograft rejection is generally believed to be a result of cytokine production by infiltrating leukocytes. In cultured fibroblasts, it has been shown that the ability of CMV to induce ICAM-1 is independent of cytokine secretion by this cell type (Craigen & Grundy, 1996). No cytokines, notably interleukin-1β, tumour necrosis factor-α, interferon-γ, or interleukin-4, all of which are known to up-regulate the expression of ICAM-1 were detected in the supernatant fluid of CMV-infected fibroblasts (Craigen & Grundy, 1996). In addition, virus-free supernatant or ultra-violet inactivated virus was shown to be incapable of up-regulating the expression of ICAM-1 in fibroblasts (Craigen & Grundy, 1996), suggesting that infectious virus and/or the initiation of a viral replicative cycle were required for this effect. Similar mechanisms might also be responsible for the increased expression of ICAM-1 on smooth muscle cells (reported here) and endothelial cells (Sedmak et al., 1994). Thus CMV infection might influence the initial infiltration of inflammatory cells, by directly promoting ICAM-1/LFA-1 interactions, and might thereby contribute to the development or to the progression of atherosclerotic lesions.

In the present study, it was also shown that there was an increase in the expression of LFA-3 molecules on smooth muscle cells following their inoculation with CMV strain AD169. However the expression of LFA-3 was not altered following the infection of smooth muscle cells with the low passage CMV strain C1F. This suggested that the ability of CMV to alter LFA-3 expression was strain-dependent. 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; Somersalo et al., 1996). This speculation is supported by reports from 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). Furthermore, a decrease in the ability of natural killer cells to lyse cells infected with certain low passage strains of CMV which did not induce LFA-3 expression, was also

demonstrated (Fletcher et al., 1998). However, these strains of CMV which do induce LFA-3 expression in particular cell types could contribute to the inflammatory process, by increasing the adhesion of CD2+ lymphocytes to infected cells, since LFA-3 is the ligand for CD2. Increased adhesion of CD2+ lymphocytes to CMV-infected fibroblasts has previously been demonstrated (Grundy et al., 1993). CD2+ cells include the T cell and natural killer cell subsets, thus the increased expression of LFA-3 on CMV-infected smooth muscle cells could result in increased retention of these cells in the blood vessel wall. Since T cells are believed to play an important role in the development of atherosclerotic plaques, the enhancement of LFA-3 expression by some strains of CMV might contribute to atherogenesis. The organ culture system described here could be used to study the effect of CMV infection of the blood vessel wall on the expression of a variety of adhesion molecules on endothelial cells and smooth muscle cells. This system would have the advantage over monolayer cultures of single cell types that the various cell types are in their normal relationship to each other, so that the effects of CMV infection of one cell type on other cell types could be studied. In addition, the organ culture system of the saphenous vein could potentially be used to study the effect of CMV infection on the adhesion of leukocytes to cells of the vessel wall. However, *in vivo* such adhesion occurs under conditions of blood flow, and a major drawback of the organ culture system used here is that it does not experience blood flow conditions, although it has been reported that vein segments can be cultured under predetermined conditions of venous and arterial shear stress in a closed blood flow circulatory system (Porter et al., 1996b). Future studies using this *in vitro* flow model of the organ culture system of human saphenous veins could therefore investigate the effects of CMV infection on leukocyte recruitment and adherence. Furthermore, the phenotype of inflammatory cells infiltrating the vessel wall, and the overall effects of the ensuing inflammatory response on the vessel wall could be studied. Such studies could provide valuable information which could help in understanding the role of CMV infection in the patho-physiology of the development and progression of atherosclerosis.

In the present study, it was demonstrated that CMV could potentially interfere with the adaptive immune response, by altering the expression of class I MHC, but not class II MHC, molecules on the surface of smooth muscle cells. The infection of smooth muscle cells with CMV strain AD169 and a low passage CMV strain, C1F, both resulted in the down-regulation of class I MHC antigens, suggesting that these cells might be able to evade recognition and killing by cytotoxic T cells and escape immune surveillance. Such immune evasion might help CMV to persist in the blood vessel wall. In contrast to the effect of CMV infection on class I MHC expression on infected cells, it has been

shown that CMV infection of fibroblasts increases the level of expression of class I antigens on bystander uninfected cells (Steinmassl & Hamprecht, 1994), presumably due to the release of IFN-β by CMV-infected cells (Grundy et al., 1988a). Given the similarity between the replication of CMV in fibroblasts and smooth muscle cells reported here, it is likely that similar effects of CMV infection on class I MHC expression on bystander cells would be found with smooth muscle cells. Increased class I MHC expression on bystander cells is likely to be an important factor *in vivo*, where uninfected cells outnumber infected cells. In immunocompromised individuals, such cytokine-induced up-regulation of MHC molecules on donor derived tissues has been associated with allograft rejection (Shoskes & Wood, 1994; 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* (von Willebrand et al., 1986; O'Grady et al., 1988; Manez et al., 1993).

The question arises as to how CMV might get into the vascular wall. In immunocompetent CMV-seropositive persons, the monocyte appears to be the major cell type harbouring the virus in the blood, presumably in a latent state (Taylor-Wiedeman et al., 1991). Circulating monocytes has been shown by polymerase chain reaction analysis to harbour CMV DNA (Taylor-Wiedeman et al., 1991). CMV does not easily infect monocytes, but can infect monocyte precursors present in the bone marrow, and the viral genome has been demonstrated to persist in these cells (Maciejewski et al., 1992; Minton et al., 1994; Kondo et al., 1994). These observations, when considered together with the fact that clinically significant CMV infection develops in bone marrow transplant recipients seronegative for CMV, but whose donors are CMV seropositive (Meyers et al., 1986), suggest that myelomonocytic precursor cells may act as a reservoir of CMV, and that circulating monocytes may act as a vector, delivering the virus to sites of vascular inflammation or injury. CMV could contribute to atherosclerosis if it undergoes periodic reactivation, during which time either it expresses its full complement of gene products and replicates, or it expresses only its IE genes producing an abortive infection. It has been demonstrated that even the limited viral gene products expressed during an abortive infection have the capacity to elicit cellular changes that could be atherogeneic (Speir et al., 1994; Zhou et al., 1996; Zhou et al., 1999). Circulating monocytes could deliver the virus to the site of injury or inflammation. Indeed, it has been demonstrated that the constituents of the vessel wall, namely endothelial cells, smooth muscle cells and oxidized low density lipoprotein, have the capacity to reactivate latent CMV within monocytes, as part of the inflammatory response to areas of vascular injury (Guetta et al., 1997). The

CMV major IE promoter controls IE gene expression and thereby virus replication. Guetta et al. (1997) demonstrated that the transfection of the pro-monocytic cell line, HL-60 with a chloramphenicol acetyl transferase (CAT) reporter gene construct driven by the CMV major IE promoter led to the reactivation of CMV present in monocytes. Major IE promoter activity increased 2-fold when transfected HL-60 cells were co-cultured with endothelial cells, 5-fold when co-cultured with smooth muscle cells, and 2-fold when exposed to oxidized low density lipoprotein. The combination of oxidized low density lipoprotein and endothelial cell co-culture increased major IE promoter activity by more than 7-fold. These results indicated that the monocyte could deliver latent CMV locally to the site of vascular injury, where constituents of the vessel wall could enhance CMV IE gene expression and thereby contribute to CMV reactivation and/or replication. Reactivated CMV could then stimulate immune and inflammatory responses that could further contribute to the atherogenic process taking place in the vessel wall.

Monocytes and neutrophils have both been shown to harbour infectious CMV in the peripheral blood of immunocompromised patients with CMV viremia. In these patients, CMV could potentially be delivered to the vessel wall via the contact of these cells with components of the blood vessel wall, or by their transmigration through the endothelium. Furthermore, it has been demonstrated that there is a bi-directional transfer of infectious virus between endothelial cells and monocytes (Waldman et al., 1995). In the latter study, it was demonstrated that the co-culture of monocytes with CMV-infected endothelial cell monolayers led to approximately 30% of monocytes adhering to infected endothelial cells. The infected monocytes were demonstrated to transmit infectious virus by their ability to generate cytopathic foci on monolayers of uninfected endothelial cells.

The neutrophil has been reported in a number of studies to be a major reservoir of infectious CMV in both immunocompetent and immunocompromised persons with viremia (Gerna et al., 1990; Gerna et al., 1991). In these patients, infectious virus was more often associated with the neutrophil fraction than the monocyte fraction of peripheral blood (Gadler et al., 1982; Turtinen et al., 1987; Saltzman et al., 1988; Gerna et al., 1991). In addition, the quantity of CMV DNA was significantly greater in polymorphonuclear cells than in mononuclear leukocytes (Saltzman et al., 1988). One important finding in a few of these studies was that the neutrophil was able to harbour the virus in an infectious state, leading to the conclusion that neutrophils play an important role in virus dissemination during acute infection (Turtinen et al., 1987; Saltzman et al., 1988; Gerna et al., 1991). The origin of CMV carried by neutrophils is not certain, since the neutrophils from viremic patients stain positively for the CMV structural virion phosphoproteins pp65 (ppUL83) and pp150 (ppUL32), but not for the CMV IE antigen p72 (Revello et al., 1992;

Grefte et al., 1994), which is not found in the virion but is induced in infected cells. Similarly, viral DNA, but not transcripts of the p72 gene have been found in neutrophils in viremic patients (Turtinen et al., 1987). These observations suggested that neutrophils acquire virion phosphoprotein or viral DNA from ingestion of the virus or from the debris from virus-infected cells (The et al., 1995). However, although recent studies indicate that the presence of mRNA transcripts for IE and late CMV genes suggests that limited replication of CMV in neutrophils might occur, the majority of CMV viral structural proteins and DNA found in neutrophils in the peripheral blood of viremic patients is believed to originate from another source. In a recent communication by members of this laboratory, it was reported that neutrophils can acquire the CMV tegument protein pp65 and infectious virus, following either their co-culture with endothelial cells infected with an endothelial cell tropic clinical isolate of CMV, or by their transmigration through infected endothelial cells (Grundy et al., 1998)

These findings suggest that the monocyte and the neutrophil can act as vectors for CMV transmission, targeting the virus to arterial lesions, and thereby contributing to CMV-induced atherogenic-related processes.

CMV infection has been implicated in the development of transplant-associated atherosclerosis, which results in neointima formation in all arteries and arterioles of the transplanted organ. There are several mechanisms by which CMV infection can occur in transplant recipients. These include the primary infection in a seronegative recipient as a result of receiving an organ or blood products from a seropositive donor. This situation carries the highest risk for the development of CMV disease in seronegative transplant recipients. CMV infection may also occur in these patients as a result of undergoing vigorous immunosuppressive therapy for allograft rejection. The cellular source of the virus is not known, but it is more likely to be in tissue cells than in contaminating leukocytes from the donor, which are syngeneic to the transplanted organ. CMV reactivates in the transplanted organ and disseminates. Another mechanism by which CMV can get to the transplanted organ is as a result of a seropositive individual receiving a transplant from a seronegative donor. In this situation, CMV could be present in any tissue cells and/or in the leukocytes of the recipient. The reactivation of latent virus in these patients could be due to immunosuppression, or to the reactivation of CMV from recipients monocytes stimulated by contact with the allograft, in a mechanism similar to that described above. Finally, the superinfection of a seropositive recipient with virus acquired from the donor organ, or blood transfusion from a seropositive donor, could lead to active infection, and virus replication at many sites in the patient.

A new concept of multiple infections is emerging in research into the pathogenesis of vascular disease, based on the fact that serological responses towards more than one pathogen seem to be associated with an increased prevalence of coronary artery disease. In a recent communication, Wanishsawad et al. (2000) tested the generic hypothesis that when a host cell harbouring one of the several pathogens implicated in atherogenesis in a latent state is newly infected with another such pathogen, the new infection would increase the capacity of the latent pathogen to express its gene products. *Chlamydia pneumoniae* is currently the infectious agent most often associated with the inflammation found in atherosclerosis (Saikku, 2000). Because activation of the major IE promoter is critical for CMV gene expression and thereby viral activity, their experiments focussed on determining whether *Chlamydia pneumoniae* infection of HeLa cells augmented CMV major IE promoter activity. Their studies used HeLa cells transfected with a plasmid construct containing a reporter gene controlled by the major IE promoter of CMV. The results showed that *Chlamydia pneumoniae* significantly increased the CMV major IE promoter activity in these transfected cells. This investigation provided proof of the concept that two pathogens commonly considered to contribute to atherogenesis have the capacity to interact, such that *Chlamydia pneumoniae* infection of a cell that harboured CMV could potentially transactivate the major IE promoter of the virus. This interaction could possibly reactivate latent CMV, or augment existing levels of expression of viral gene products, or both. The study showed how infection with one pathogen might exacerbate the injurious effects of the second pathogen in vascular cells. The ability of one pathogen to affect the ability of another pathogen to express its gene products is not unique to *Chlamydia pneumoniae* and CMV. For example, previous studies have established that CMV infection of cells harbouring latent HIV activates the latent HIV (Fiala et al., 1991). It is therefore possible that cells infected with latent pathogens, or with pathogens expressing genes at low and perhaps biologically insignificant levels, can be stimulated by infection with a second pathogen, such that it augments the activity of the resident pathogen. Such a paradigm could be one mechanism by which the reactivation of latent CMV could occur, resulting in the infection of the vessel wall.

In conclusion, monolayer cultures of vascular smooth muscle cells were demonstrated to be highly susceptible to CMV infection. This finding is of clinical relevance due to the fact that smooth muscle cells in monolayer cultures, and in the neointima of restenotic and atherosclerotic lesions, are both of the synthetic proliferative phenotype. The saphenous vein organ culture system provided a more realistic means over isolated cultures of single cells to study the potential role of CMV infection of cells

of the blood vessel wall. It was demonstrated that CMV infection of saphenous vein segments resulted in the replication of CMV in the cells of the neointimal, intimal, and adventitial layers of the vein wall. From these studies, it appeared that the smooth muscle cell phenotype was a crucial factor in their permissiveness to cytomegalovirus infection. This implied that the degree to which synthetic smooth muscle cells are present in the blood vessel wall determines whether or not CMV infection could occur. The enlargement of luminal surface cells, possibly endothelial cells, and the increase in the synthesis of extracellular matrix components, suggested that CMV infection provoked changes to the vessel wall which were similar to those observed in transplant recipients undergoing transplant-associated atherosclerosis. An advantage of the organ culture system over the isolated monolayer cultures of single cells was that the cells of the vessel wall were present in their normal relationship to each other, and thus the overall effects of CMV infection on the vessel wall could be assessed. In addition, the preservation of the vein wall architecture maintains components of the extracellular matrix, which are known to influence smooth muscle cell proliferation, and are lost in isolated monolayer cultures of single cells. The organ culture model of the saphenous vein described in the present study should thus provide a basis for the further study of the short- and possibly long-term effects of CMV infection on the blood vessel wall. In particular the effects of CMV infection on the proliferation of smooth muscle cells, the deposition of extracellular matrix, the production of growth factors and the expression of adhesion molecules could be studied. The results presented in this study provide further support for the premise that CMV infection of vascular smooth muscle cells plays an important pathogenic role in the development of atherosclerosis and/or vascular disease and has provided an insight into the possible mechanisms involved. Furthermore, this study has successfully set up important models which could be used in future studies to determine exactly how CMV could contribute to atherogenesis.

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