The Role of Herpesviruses in Atherosclerosis

Cairistine Naomi Ellinor Grahame-Clarke

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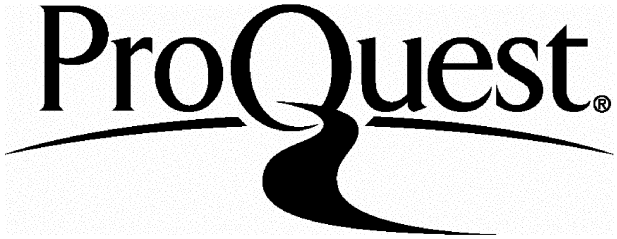

Abstract

Atherosclerosis is a series of inflammatory processes within the arterial wall that results in the formation of atherosclerotic plaques, or atheroma. It is the leading cause of death in the Western world and chronic infection is increasingly being cited as a hitherto unconsidered risk factor for the disease.

Herpesviruses are one group of potentially pro-atherogenic organisms, and members of the beta- and gamma-herpesvirus families exhibit certain biological properties which make them especially good candidates for being involved in the pathogenesis of atherosclerosis. However sero-epidemiological studies in man have been far from conclusive in demonstrating such a possible link. Animal models have therefore acquired a pivotal role in examining the involvement of herpesviruses in atherogenesis.

This thesis addresses the role of herpesvirus infection in atherosclerosis, firstly through the use of two novel animal models, and secondly through two human studies on patients potentially predisposed to atheroma formation - HIV infected and diabetic patients.

The Apolipoprotein E-deficient mouse and the C57Bl-diet-induced mouse are models of atherogenesis that have been further developed for this thesis by infection with murine gammaherpesvirus-68 (MHV-68). This has made possible the establishment of a direct aetiological link between herpesvirus infection and

accelerated atherogenesis. Further work also explored the underlying mechanisms involved in these models, with enhanced vasoconstriction being strongly implicated.

The results from these murine models influenced the approach in the human studies. One study looked at Human herpesvirus-8 (HHV-8), a human virus that is closely related to MHV-68 and that causes the distinctive clinical condition of Kaposi's sarcoma. In a retrospective survey of post-mortem reports on HIV positive individuals, Kaposi's sarcoma was found to be strongly associated with the presence of atheroma.

Finally, in a further study in Type 1 diabetic subjects, a potential atherogenic role of human cytomegalovirus (HCMV) was addressed. HCMV infection was found to be strongly and significantly associated with impaired vascular responses (an early marker of atherogenesis); there was also a weaker, non-significant association with coronary artery calcification (a means of quantifying atheroma).

This thesis thus summarizes a range of work, both in animal models and in the context of human disease, that has been undertaken in order to explore the role of herpesviruses in the genesis of atherosclerosis.

This thesis is dedicated to my parents and to Philip

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(and Hermione's) life, but also volunteered to read through this thesis when I could hardly bear to do so. Words cannot express my thanks to him.

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ACh	acetylcholine
AIDS	acquired immunodeficiency syndrome
ApoE	Apolipoprotein E
ApoE-/-	Apolipoprotein E deficient mouse
BHK-21	baby hamster kidney cell line
BK	bradykinin
C57Bl	C57black/6J mouse
CAC	coronary artery calcification
CCR2-/-	C-chemokine receptor-2 deficient mouse
CM	chylomicron
CMR	remnant particle
cpe	cytopathic effect
CRP	C-reactive protein
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
EBCT	electron beam computerised tomography
EBV	Epstein Barr Virus
EC	endothelial cell
EdtU	2'-deoxy-5-ethyl-β-4'-thiouridine
FBS	foetal bovine serum
GC	guanylate cyclase
GTN	glyceryl trinitrate
GMEM	Glasgow's modified Eagles' medium
HAART	highly active anti-retroviral therapy
HCMV	human cytomegalovirus
HDL-c	high density lipoprotein cholesterol
HHV-8	human herpesvirus-8 (KSV, Kaposi's sarcoma virus)
HIV	human immunodeficiency virus-1
HSP	heat shock protein
HSV-1	herpes simplex virus-1
HUVEC	human umblical vein endothelial cell line
HVS	herpesvirus saimiri
IDL-c	intermediate density lipoprotein cholesterol

IFNγ	interferon-gamma
IL-1	interleukin-1
IL-6	interleukin-6
in	intranasal route
ip	intraperitoneal route
IP_3	inositol trisphosphate
KS	Kaposi's sarcoma
LDL-c	low density lipoprotein cholesterol
LDLR-/-	LDL receptor deficient mouse
L-NAME	L^{ω}-nitro-L-arginine methyl ester
L-NMMA	N^{G}-monomethyl-L-arginine
LPS	lipopolysaccharide
MCMV	murine cytomegalovirus
MCP-1	monocyte chemoattractant protein-1
MCSF	macrophage colony stimulating factor
MDV	Marek's disease virus
MHV-68	murine gammaherpesvirus-68
MLCK	myosin light chain kinase
moi	multiplicity of infection
NBS	newborn bovine serum
NE	norepinephrine
NIH 3T3	National Institute of Health 3T3 cell line
NO	nitric oxide
OSM	oncostatin M
OxLDL	oxidised low density lipoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phenylephrine
PKG	cGMP-dependent protein kinase
PLC	phospholipase C
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEnd.1	(murine) skin endothelioma cell line
SMC	vascular smooth muscle cell
SNP	sodium nitroprusside
STZ	streptozotocin
TNFα	tumour necrosis factor alpha

U19	U46619, a thromboxane analogue
VLDL	very low density lipoprotein
VZV	varicella zoster virus

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Figure 1.1

'We can distinguish a stage of irritation preceding the fatty metamorphosis....which we have seen in other inflamed parts. I have therefore felt no hesitation in siding with the old view that an inflammation of the arterial coat to be the starting point of the so-called atheromatous degeneration...this is exactly the same as what is universally termed endocarditis, when it occurs in the parietes of the heart.'

From Lecture XVI: The atheromatous process

In Cellular Pathology. Rudolf Virchow 1859

With thanks to the Wellcome Library, London.

CHAPTER 1:INTRODUCTION

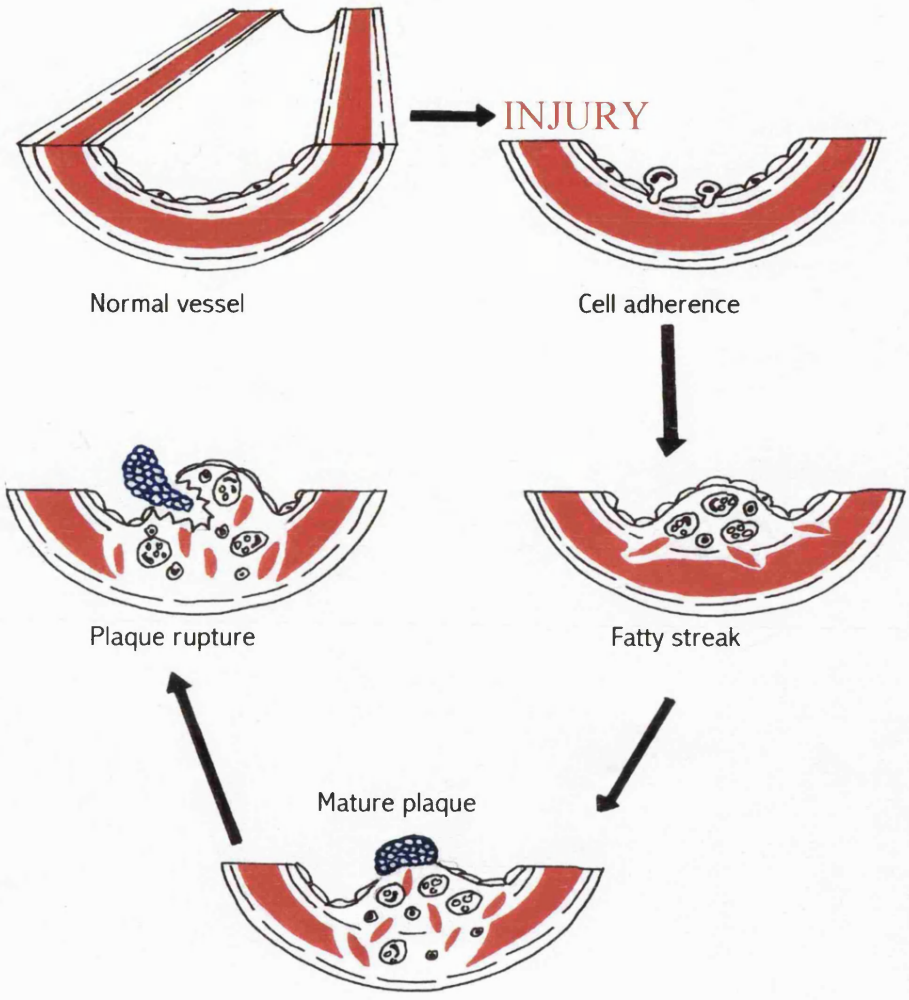
1.1 HISTORICAL PERSPECTIVE

The aetiology of atherosclerosis has been a matter of debate for over 150 years. In 1859 Rudolf Virchow (figure 1.1) emphasised the importance of *cholestearine* (cholesterol) in the formation of such lesions. He also stated that he was then of the **old** view that a process similar to endocarditis underlay the inflammation that preceded atheroma formation (Virchow, 1859). Klotz in 1906 described experiments where injections of typhoid or streptococcus organisms produced atheromatous lesions in rabbits (Klotz, 1906). By 1932, MacCallum in his Textbook of Pathology stated that the theory of infection influencing atherosclerosis in man 'lacked the stamp of conviction that would make [it] acceptable by everyone at once' (MacCallum, 1932). He was however of the view that a disturbance of cholesterol metabolism was the initial step in atherosclerosis. In the 1970s a seminal paper by Fabricant demonstrated that infection with Mareks' Disease virus, a herpesvirus caused atheroma in cholesterol fed chickens (Fabricant et al., 1978). Since then there has been an explosion in reports of various and diverse infections playing a role in atherogenesis (Cook and Lip, 1996; Ellis, 1997; Epstein et al., 1999). This thesis further explores the role of herpesviruses in the aetiology of atheroma.

1.2 PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Atherosclerosis is the leading cause of death in the Western World, and an increasing problem in developing countries (Pearson, 1999). It starts in childhood although it may only become apparent in old age (Sternby et al., 1999). Certain risk factors are known to predispose to atherosclerosis namely cigarette smoking, diabetes mellitus,

Figure 1.2: Schematic representation of the 'response to injury' theory. After R.Ross 1993



The normal arterial wall is composed of an inner endothelial monolayer, a surrounding media including smooth muscle cells (red) and an outer adventitia. These three layers are divided by inner and outer elastic laminae (black dashed lines). After an initial injury to the endothelium monocytes and T lymphocytes adhere to the endothelium and migrate beneath it. Monocytes become foam cells and together with lymphocytes and endothelial cells secrete chemokines which induced smooth muscle cell proliferation and matrix accumulation. The fatty streak so enlarges and develops into a mature plaque over which the covering endothelium can become discontinuous. The exposed pro-thrombotic core may cause a platelet plug (blue) to occlude the lumen or induce plaque rupture.

hypertension, hypercholesterolaemia and male sex. These risk factors however explain only 50% of the incidence of the disease (Crouse, 1984), suggesting that other as yet unidentified factors are also involved.

Evidence suggests that risk factors interact at the endothelium to induce a series of inflammatory changes which lead to atherosclerosis. This 'response to injury' theory of atherogenesis is outlined in figure 1.2 (Ross, 1993). Thus the endothelium becomes dysfunctional and a series of pathophysiological stages can be seen both in man and in experimental animal models of atherosclerosis (Mora et al., 1987; Ross et al., 1986; Tsukada et al., 1986). At areas of endothelial injury lipoproteins accumulate (Mora et al., 1987) and endothelial cells express adhesive glycoproteins known as adhesion molecules (Cybulsky and Gimbrone, 1991; Springer, 1990). Circulating monocytes and T lymphocytes adhere to these and migrate across the endothelium (Munro and Cotran, 1988). Once in the subendothelial space monocytes differentiate to form macrophages which accumulate cholesterol - specifically oxidised LDL cholesterol (oxLDL) - to become foam cells (Hajjar, 1991; Minick et al., 1979). Foam cells, lymphocytes and smooth muscle cells make the earliest visible sign of atherosclerosis, the fatty streak (Masuda and Ross, 1990). Cell proliferation and extracellular matrix production cause the plaque to enlarge and project into the lumen of the vessel. Macrophage accumulation and smooth muscle cell apoptosis occur at the shoulders of the plaque rendering it more unstable and liable to rupture. It is plaque rupture and/or thrombus formation that commonly leads to vessel occlusion and clinical sequelae (Davies and Thomas, 1984). The entire process is orchestrated by a series of growth factors and cytokines, which typically modify inflammatory processes, such as TNFα , IL-1, IL-6 , IFNγ (Ross,

1993). Systemic levels of IL-6 and TNFα induce the production of C-reactive protein (CRP) by the liver and are associated with the risk of myocardial infarction in man (Ridker et al,2000a;Ridker et al,2000b). Thus atherosclerosis can be viewed as an excessive if episodic wound healing response to noxious stimuli (Ross, 1993).

1.3 INFECTION AND ATHEROSCLEROSIS IN MAN

Moderately elevated levels of C-reactive protein (a non-specific measure of inflammation/infection), are associated with a higher risk of developing peripheral vascular disease or a myocardial infarction (Ridker et al,1998a;Ridker et al,1998b). A variety of evidence has implicated a wide range of infective organisms in this process (Cook and Lip, 1996; Danesh et al., 1997; Ellis, 1997; Epstein et al., 1999). The main contenders that have emerged are *Helicobactor pylori* (Danesh et al., 1999), *Chlamydia pneumoniae* (Gupta, 1999) and the herpesviruses such as Herpes Simplex Virus (HSV-1) & Human Cytomegalovirus (HCMV) (Nicholson and Hajjar, 1998) (table 1.1). These organisms are commonly encountered in childhood and have the ability to remain latent within the individual, periodically reactivating to cause further inflammation.

	HSV-1	*CMV*	*Chlamydia pneumoniae*	*Helicobactor pylori*
Sero-epidemiological evidence in...				
1) Atherosclerosis	**No.** (Adam et al., 1987; Ridker et al., 1998)	**Yes & No.** (Adam et al., 1987; Ridker et al., 1998; Sorlie et al., 1994)	**Yes.** (Gupta and Camm, 1997; Saikku, 1997)	**Yes.** (Danesh et al., 1999)
2) Restenosis	-	**Yes.** (Zhou et al., 1996)	-	**No.** (Danesh et al., 1999)
3) Transplant arteriosclerosis	-	**Yes.** (Grattan et al., 1989)	-	**No.** (Danesh et al., 1999)
Pathogen found in human plaques	**Yes.** (Melnick et al., 1994)	**Yes.** (Hendrix et al., 1991; Hendrix et al., 1990; Melnick et al., 1994)	**Yes.** (Saikku, 1997)	**No.** (Blasi et al., 1996)
Causes atheroma in animal models	-	**Yes.*** (Berencsi et al., 1998; Dangler et al., 1995; Lemstrom et al., 1993)	**Yes.** (Moazed et al., 1999)	-
Antimicrobials reduce atheroma in animal models	-	**Yes.** (Lemstrom et al., 1994)	**Yes.** (Muhlestein et al., 1998)	-

Table 1.1 Comparison of the evidence for each main infective agent possibly involved in the pathogenesis of atherosclerosis. Where (-) denotes that the relevant studies have not been performed. * signifies that as cytomegalovirus is species-specific the experimental studies in either the rat or the mouse have used the appropriate virus for that species not human cytomegalovirus.

1.4 THE HERPESVIRIDAE

There are eight human herpesviruses and over 100 characterised in other animal species. Herpesviruses are defined on the basis of their structure; namely an envelope surrounding an amorphous tegument, inside which is the capsid surrounding the DNA core. The trilaminar envelope extends numerous short glycoprotein spikes. The icosadeltahedral capsid is 100-110nm in diameter with 162 capsomeres and a 4nm channel down the long axis. The core is a linear double stranded DNA in the form of a torus and is large for a virus (100-235kbp long). Herpesviruses are divided into three families on the basis of their biological properties and genetic code; alpha, beta and gamma herpesviruses (Roizman, 1982). Each family having a prototype virus, usually the most studied in that group. These families are further subdivided into seven genera on the basis of their DNA sequence homology and degree of similarity of their viral proteins (Roizman, 1982).

1.4.1 The Alphaherpesviridae

The prototype virus for the alphaherpesviridae (α-herpesviridae) is HSV-1, which is the most widely studied of all the herpesviridae. Alphaherpesviruses have a broad host range with a short reproductive cycle so causing a destructive lytic infection in cell culture. Their site of latency is neural sensory ganglia. For example, HSV-1 reactivates from latency in the trigeminal ganglion to cause cold sores in the distribution of the trigeminal nerve. This family includes the simplex viruses (HSV-1&2) and the varicelloviruses (VZV). These viruses are not species-specific; so for example HSV-1 can infect mice but is not a natural infection. Marek's disease virus is an α-herpesvirus (see section 1.6).

1.4.2 The Betaherpesviridae

Human CMV is the prototype of the betaherpesviridae (β-herpesviridae). The β-herpesviridae have a far more restricted host range compared to the α-herpesviridae with a slow reproductive cycle and therefore spread more slowly in cell culture. Cells enlarge (cytomegalia) as cellular protein synthesis is stimulated, rather than undergo lysis. The nucleocapsids accumulate in the nucleus to be packaged with DNA resulting in the typical 'owls eye' appearance. Cytomegaloviruses generally become latent in ductal epithelial cells in secretory glands and kidneys. There is no animal model for HCMV as it is species-specific so murine cytomegalovirus (mCMV) is often used, although the viruses do differ in several aspects. HCMV emerges not only as infectious, but also as non-infectious particles - the so called 'dense bodies' or 'enveloped particles'; in contrast mCMV has no dense bodies. Murine CMV can grow in immortalised fibroblasts, whereas HCMV is preferentially grown in primary human cell cultures. In both cases the disease that is produced *in vivo* depends on the age and immune status of the host. Thus young or immunosuppressed individuals have a far more serious disease. Murine CMV, unlike HCMV, does not cross the placenta and therefore does not result in the devastating congenital infections that can be seen with human CMV. Murine CMV, whilst being a natural infection of mice, is difficult to grow and titrate and often requires immunosuppression of the host to achieve an active infection.

1.4.3 The Gammaherpesviridae

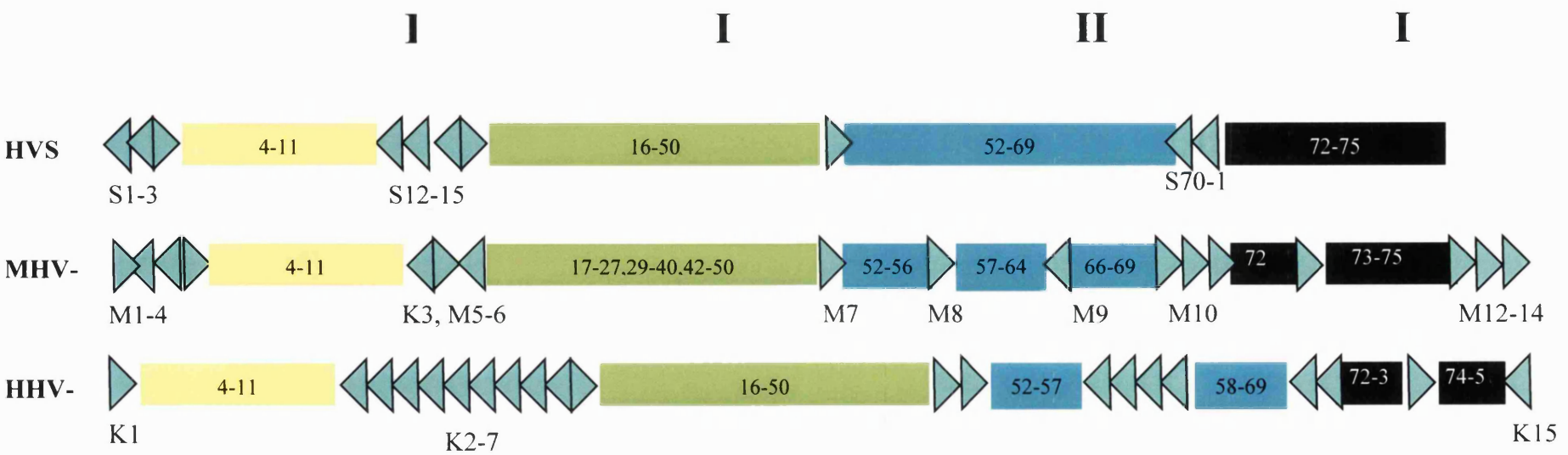
The gammaherpesviridae (γ-herpesviridae) have an intermediate host range between that of the α and β-herpesviruses, with the host range restricted to the family to

which the natural host belongs. They have a slow replication cycle primarily in lymphoblastoid cells, which may be lytic or latent, although latency is established in lymphoid tissue. The family is further subdivided into γ1– and γ2 –herpesviruses of which the prototypes are Epstein Barr Virus (EBV) (human) and Herpesvirus Saimiri (HVS) (Old World monkeys) respectively.

EBV commonly causes an asymptomatic childhood infection or infectious mononucleosis in young adults. Initial infection is in epithelial cells, but this is followed by an activation and lytic infection of B-lymphocytes, which depletes their number. Latent infection in lymphoid tissue can give rise to malignancy; EBV was first identified from cells derived from Burkitt Lymphoma specimens. It has also been associated with neoplasia in the form of Hodgkin's Lymphoma, Hairy Leucoplakia and nasopharyngeal carcinoma.

The γ2-herpesviruses include Human Herpesvirus-8 (HHV-8) and murine γ-herpesvirus-68 (MHV-68), as well as the prototype virus HVS. The genetic organisation of the γ2-herpesviruses is summarised in figure 1.3.

Figure 1.3. Comparison of genetic organisation of several members of the γ-herpesviruses after Simas et al, 1998



The conserved gene blocks I (yellow), II (green), III (blue) and IV (black) are shown; Open reading frame (ORF) designations within these boxes relate to the HVS numbering system. Between these are ORFs that are mainly unique to each virus and contain several homologues to cellular genes (blue triangles). Some of these genes are thought to regulate the immune response and viral latency. Genes with no recognisable homologues to HVS are numbered separately and given the letter M for MHV-68-specific and K for HHV-8-specific homologues. For example K2 encodes viral interleukin-6, ORF 74 encodes viral interleukin-8 receptor and K4 encodes viral macrophage inflammatory protein.

1.4.3.1 Human herpesvirus-8 / Kaposi's sarcoma virus

The DNA sequence of HHV-8, which is similar to HVS, was first identified in specimens from Kaposi's sarcoma (KS) in AIDS patients (Chang, 1994). Evidence now points to HHV-8 being causal in this disease, as well as in classic Kaposi's sarcoma of West Africa, primary effusion lymphoma and multicentric Castleman's disease (Brooks et al., 1997). However HHV-8 is not a common infection in the general population. Although precise figures are not known it would seem that the seroprevalence for HHV-8 lies between <1% and 20% (Schulz, 1998), with a greater prevalence in Africa and Italy and a lower prevalence in the USA and UK (Boshoff and Weiss, 1998). Nevertheless it is 20,000 times more common in HIV positive individuals, and 10 times more frequent again in bisexual or homosexual men with AIDS than in other HIV-infected groups (Whitby et al., 1998).

In man KS lesions are composed of endothelial cells , spindle cells with a leucocyte infiltration and marked angiogenesis (Jones, 1986). HHV-8 is found in both endothelial and spindle cells as well as monocytes and B-lymphocytes of many individuals at risk from KS, thus suggesting that these cells may act as viral reservoirs for HHV-8 infection and are the likely source that initially infects the endothelial and spindle cells (Offerman, 1999).

1.4.3.2 Murine gammaherpesvirus-68

Murine γ-herpesvirus–68 (MHV-68) was first isolated from the wood vole *Clethrionomys gariolus* (Blaskovic et al., 1980), and appears to be a common infection of European rodents (Svobodova et al., 1982). After initial intranasal infection, MHV-68 undergoes lytic replication in the lungs with some

haematogenous spread to the adrenals and heart. After 10-12 days this resolves but a lifelong latent infection of lymphoid tissue is established. This causes a marked splenomegaly peaking at 2-3 weeks after inoculation (Simas and Efstathiou, 1998). The primary cell target for latent infection is the B cell, with macrophages possibly being an additional reservoir after intraperitoneal infection (Sunil-Chandra et al., 1994; Weck et al., 1999). The germinal centres in the spleen regress as the B cell response to the virus matures and therefore the splenomegaly diminishes. Acute infection is accompanied by high levels of IFNγ and IL-6 production (Sarawar et al., 1996). Although neither cytokine appears essential for recovery from acute infection (Sarawar et al., 1998; Sarawar et al., 1997). Mice lacking IFNγ develop arteritis when infected with MHV-68, and the virus is found within the smooth muscle cells in the walls of the aorta (Weck et al., 1997). Ongoing viral replication appears to be necessary for such an arteritis, as antiviral therapy results in viral clearance and improvement in vascular lesions (Dal-Canto et al., 2000). Additionally, MHV-68 is associated with lymphoma after long-term infection in rodents (Sunil-Chandra et al., 1994), a feature reminiscent of the malignant potential of HHV-8 and EBV.

1.5 EVIDENCE FOR HERPESVIRUS INVOLVEMENT IN ATHEROSCLEROSIS IN MAN

1.5.1 Epidemiological evidence

Post mortem studies in children have shown an association between viral infection and coronary intimal thickening (Pesonen et al., 1999). Serological studies in adults have shown an association between herpesviridae serology and atherosclerosis. HCMV serology has been linked to atherosclerosis in surgical patients (Adam et al.,

1987), cardiac transplants (Grattan et al., 1989), carotid ultrasound studies (Sorlie et al., 1994) and restenosis after atherectomy (Zhou et al., 1996). However the largest serological study to date however did not show an association between HSV-1 or HCMV serology and the CRP concentration or the risk of having a myocardial infarction (Ridker et al., 1998). However, a single serological measurement gives no indication of the frequency of viral reactivation (which may be important in promoting atherosclerosis), and therefore seroepidemiological studies cannot prove or disprove an association between herpesvirus infection and atherosclerosis.

1.5.2 Evidence from *in vitro* studies in human tissue

1.5.2.1 Studies on post-mortem tissue

Herpesviruses have been found in human plaques. Post-mortem studies in man have shown HSV-1 & HCMV to be within plaques but not normal vessel wall (Melnick et al., 1994; Yamashiroya et al., 1988). This evidence is not conclusive, as with such ubiquitous infections, macrophages may simply engulf such infective organisms in the lung and migrate to areas of inflammation such as a plaque. This 'innocent bystander' theory needs to be overcome if a role for herpesviruses in atherosclerosis is to be proven (Capron, 1996; Muhlestein, 1998).

1.5.2.2 Studies on the cellular components of the vascular wall

Herpesviruses have a direct effect on cellular components of the vessel wall, namely endothelial cells (ECs), smooth muscle cells (SMCs) and macrophages which could promote atherosclerosis.

There is substantial evidence for the herpesviruses affecting ECs. HSV-1, HCMV and HHV-8 can all infect the cells (Boshoff, 1995; Flore et al., 1998; Friedman et al., 1981; MacGregor et al 1980 Moses et al., 1999; Visseren et al., 1996; Vissler, 1988), and, once infected, such ECs upregulate adhesion molecules expression, enabling increased binding of circulating leucocytes (Richardson et al., 1997; Span et al., 1991; Yang et al., 1994). Infected ECs also have less anticoagulant properties and instead are prone to a procoagulant phenotype (Etingin et al., 1990; Hajjar et al., 1987; Vercellotti, 1998).

Herpesviruses can also infect and affect vascular SMCs. RatCMV infection leads to a doubling of SMC proliferation in the rat model of aortic allograft transplantation (Lemstrom et al., 1994). One potential mechanism whereby it can achieve this is through the inhibition of the tumor supressor gene p53 (Epstein et al., 1996). The p53 gene product inhibits cells cycle progression and cell proliferation. An early gene product of ratCMV has been shown to inhibit p53 activity, thereby disinhibiting cell proliferation (Epstein et al., 1999).

Herpesviruses can also affect lipid metabolism. HSV-1 stimulates macrophages to take up cholesterol esters like foam cells (Hajjar et al., 1985). Furthermore HCMV can increase the uptake by SMCs of oxidised LDL (Zhou et al., 1996).

1.5.2.3 Inflammatory cytokines

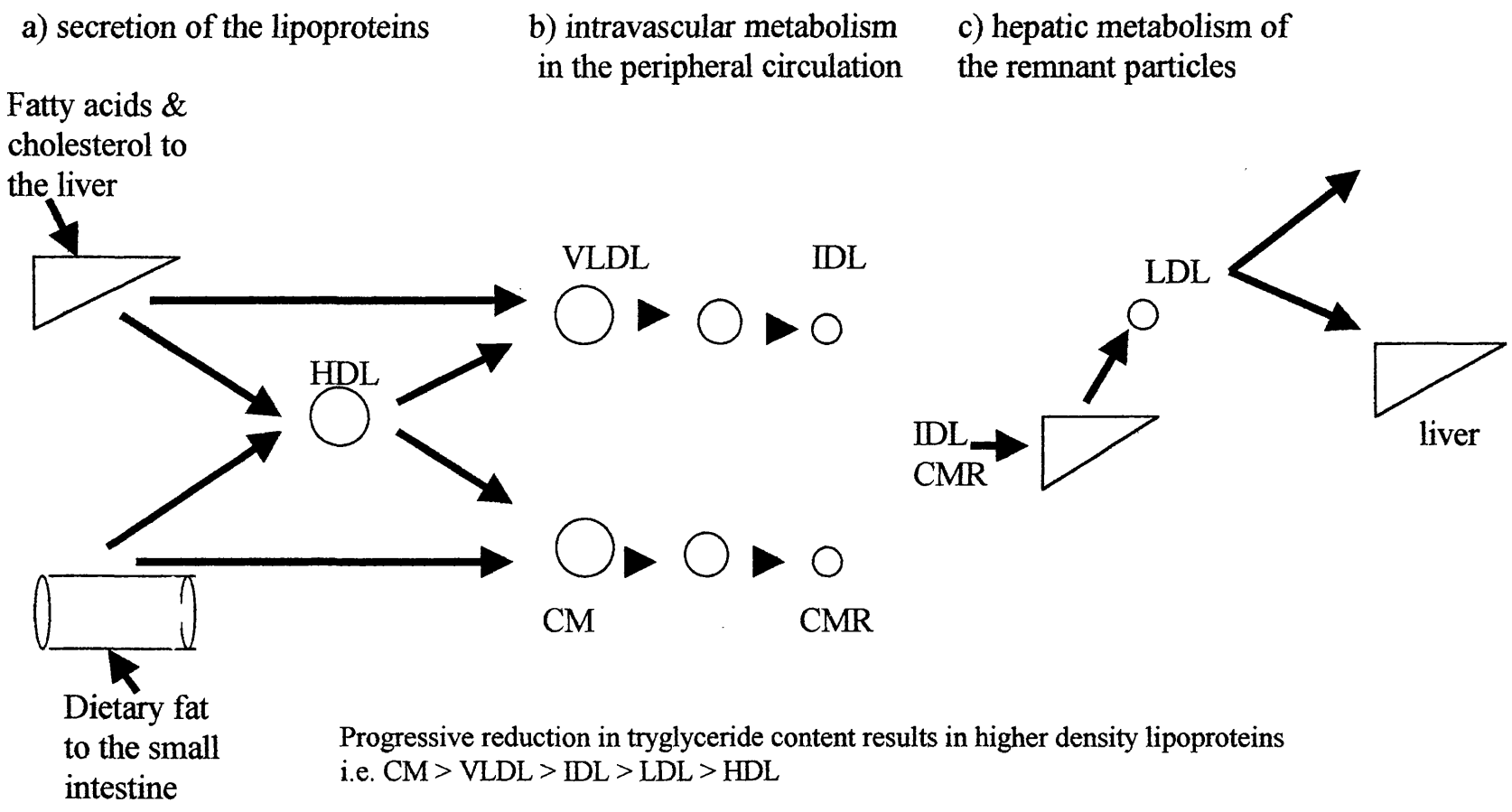
Inflammatory cytokines involved in atherosclerosis are found in cell cultures infected with herpesviruses. HCMV infection upregulates IL-6 mRNA from human ECs (Almeida et al., 1994). Kaposi's cells (infected with HHV-8) proliferate and

secrete IL-6 in response to Oncostatin M (OSM), an IL-6 related pro-inflammatory cytokine (Miles et al., 1992). Raised serum levels of IL-6 are associated with atheroma (Ridker et al., 2000) (see section 1.2). Oncostatin M is found within macrophages in aortic aneurysms, signifying its involvment in chronic inflammation. In both *in vivo* and *in vitro* studies its effect on ECs as regards the expression of inflammatory cytokines and adhesion molecules mimic those found in atherosclerotic models (Modur et al., 1997). Oncostatin M also has profound effects on vascular SMCs acting in synergy with interleukin-1β (Bernard et al., 1999). Thus herpesviruses can promote many of the cytokine pathways involved in atherogenesis.

1.6 LIPOPROTEIN METABOLISM IN MAN

Plasma lipids are poorly soluble in water and therefore their transport depends on being transported as complex proteins known as lipoproteins. Lipoproteins are rendered soluble by their surrounding apolipoproteins. One of these, apolipoprotein E (ApoE), is constitutively part of very low-density lipoproteins (VLDLs) synthesised by the liver (figure 1.4). In the peripheral tissues VLDLs are catabolised and release their triglyceride to become intermediate density lipoproteins (IDL). IDLs are avidly taken up by hepatocytes that recognise their ApoE. ApoE is therefore primarily responsible for internalising and catabolising VLDLs and IDLs. IDL and chylomicrons are made into LDL by the liver. In man most of the cholesterol is carried as LDL as opposed to HDL. LDL plays a role in atherogenesis (section1.1) and man is therefore susceptible to the disease. The gene for ApoE lies on chromosome 19q12-13.2 and is polymorphic (Mahley and Angelin, 1984).

Figure 1.4. Lipoprotein metabolism in man



Progressive reduction in tryglyceride content results in higher density lipoproteins
i.e. CM > VLDL > IDL > LDL > HDL

ApoE therefore comes in three isoforms E3, E4 and E2 in decreasing order of prevalence (Utermann, 1988). Based on positive charge these isoforms have different binding strengths to the hepatocyte. Therefore individuals with E2/2 have the weakest binding affinity for apoE and the resulting high circulating levels of VLDLs and IDLs gives rise to type III hyperlipoproteinaemia. Type III hyperlipoproteinaemia is typified by premature atherosclerosis.

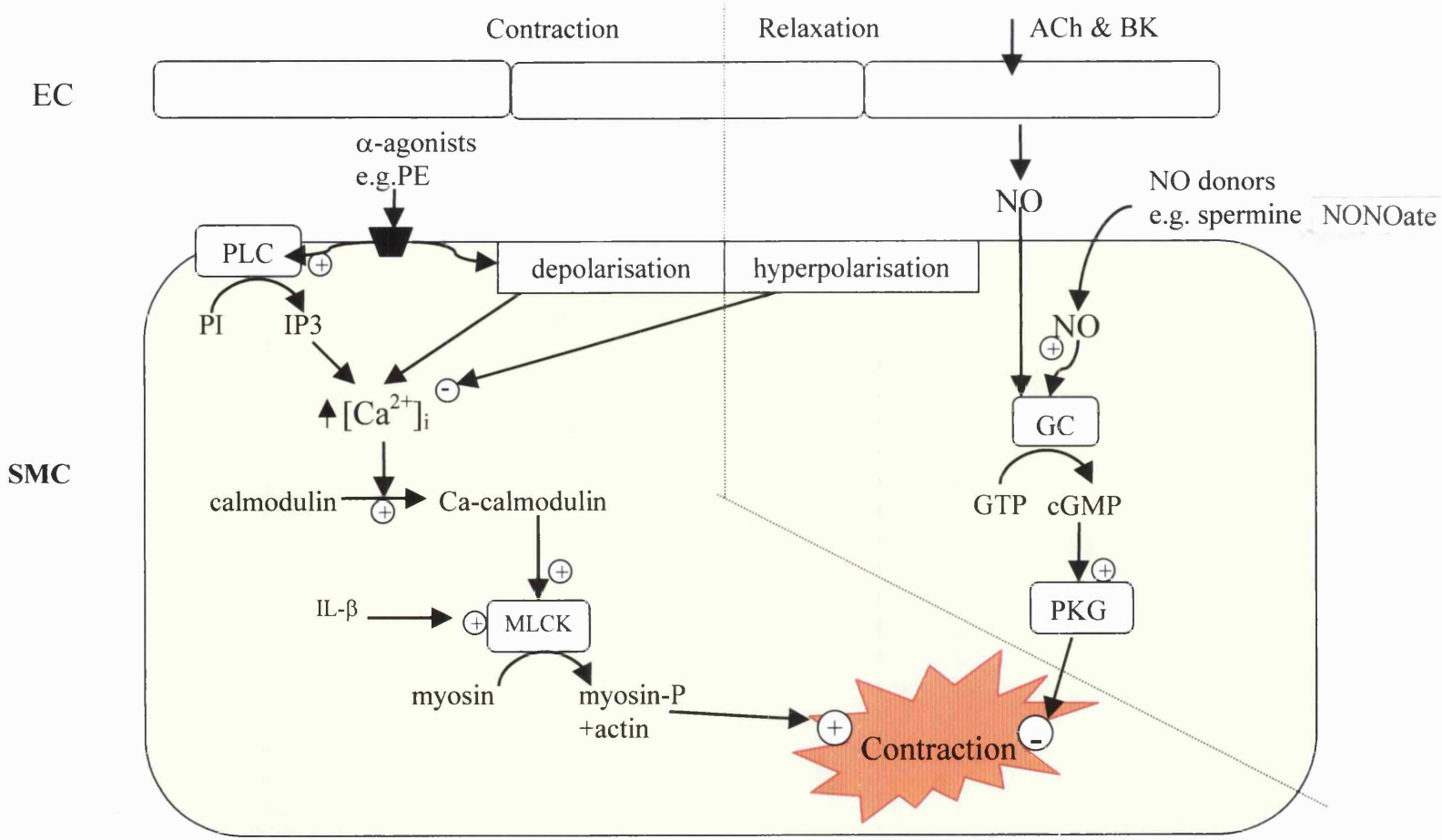
1.7 ENDOTHELIAL DYSFUNCTION IN MAN

Endothelial dysfunction is the hallmark of cardiovascular risk factors (Ross, 1993). Endothelial dysfunction is not a single phenomenon but a composite of abnormal procoagulant activity, endothelial cell apoptosis (Dimmeler et al., 1998), secretion of proinflammatory growth factors and cytokines (Ross, 1993), increased adhesion molecule expression (Lemaire et al., 1998) and subsequent monocyte adhesion, enhanced vasoconstrictor responses, as well as the impaired vasodilator responses. These events occur early in atherogenesis and precede the development of atheroma. Therefore if herpesviruses were to be involved in atherosclerosis it would be expected that herpesvirus infection would influence some of these measures of endothelial function. Two of these components of endothelial function, namely vascular reactivity and adhesion molecule expression, have already been shown to be affected by herpesvirus infection and therefore these will be discussed in greater detail.

1.7.1 Impaired endothelium-dependent relaxation responses

The finding that acetylcholine only produced vasodilation in the presence of an intact endothelium led to the identification of an endothelium-derived relaxing factor, nitric oxide (NO) (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987). NO is synthesised in the endothelium by NO synthase (Ignarro et al., 1987; Palmer et al., 1987) from L-arginine, and its release mediates smooth muscle cell relaxation (figure 1.5). Competitive antagonists of L-arginine, such as N^G monomethyl-L-arginine (L-NMMA), cause a reduction of endothelial NO. Systemic infusion of L-NMMA in man has shown that basal NO release is important in maintaining blood pressure by dilating resistance vessels (Vallance et al., 1989). When given to isolated vascular beds L-NMMA reduces acetylcholine induced vasodilation. Individuals at risk from atherosclerosis from hypertension have impaired constrictor respones to L-NMMA (Calver et al., 1994), as well as impaired endothelium-dependent relaxation responses to certain exogenous drugs, such as acetylcholine and bradykinin (Kelm et al., 1996; O'Kane et al., 1994) signifying a reduced availability of NO. Individuals at risk from atherosclerosis from other classical risk factors have similarly been shown to have impaired endothelium dependent relaxation, the degree of impairment being proportional to the degree of risk, although conflicting results have been reported (Calver et al., 1994; Calver et al., 1992; Chowienczyk et al., 1992). Thus if herpesviruses were to play a role in atherosclerosis they may be expected to have some effect on endothelium-dependent vascular responses, and this might be a useful surrogate marker for a pro-atherogenic effect.

Figure 1.5. Control of vascular smooth muscle by the endothelium.
Drugs which cause contraction do so by increasing intracellular calcium $[Ca^{2+}]_i$ by various means. This is counteracted by agents including NO which mediate relaxation. See list of abbreviations.



1.7.2 Enhanced vasoconstrictor responses

As the vasodilation of NO is functionally antagonised by the action of vasoconstrictor agents (figure 1.5), it is not surprising that augmented constrictor responses have been seen in certain situations in man. Acetylcholine has been shown to induce constriction in atherosclerotic coronary arteries (Ludmer et al., 1986). In angiographically normal coronary arteries both vasodilator and constrictor responses were seen in response to intracoronary ACh but the number of risk factors for coronary artery disease was the best predictor of a constrictor response (Vita et al., 1990). A constrictor response to ACh in coronary arteries has been associated with age (Yasue et al., 1990) and the risk of developing atherosclerosis in cardiac transplants (Fish et al., 1988). Augmented constrictor responses can therefore be seen as a manifestation of endothelial dysfunction and are another mechanism by which herpesvirus infection could enhance atherosclerosis.

NO not only mediates vascular tone but also has a range of anti-atherosclerotic effects such as the inhibition of platelet aggregation and adhesion (Radomski et al., 1987), adhesion molecule expression and leucocyte adhesion (Takahashi et al., 1996), as well as smooth muscle cell proliferation and migration (Garg and Hassid, 1989). Thus NO has a plethora of effects on endothelial function and hence atherosclerosis.

1.7.3 Endothelial adhesion molecule expression

Leucocyte–endothelial cell interactions are an early event in atherogenesis (Ross, 1993) (see section 1.2). The initial low-affinity interaction between leucocyte and EC is manifested as rolling. Rolling cells then become more firmly adherent and

finally transendothelial migration (diapedesis) can occur. Each stage of leucocyte recruitment rolling, adherence and migration is mediated by a different family of adhesion molecules, including the selectins, the β-integrins and the immunoglobulin superfamily (see Panes for review (Panes et al., 1999)). The characteristics of the adhesion molecules discussed in this thesis are summarised in Table 1.2.

1.7.3.1 Adhesion molecules in man

Adhesion molecules have been found in the human atherosclerotic plaque (O'Brien et al., 1996), and in transplanted hearts with accelerated atherosclerosis (Steinhoff et al., 1995). Levels of soluble, circulating forms of adhesion molecules, shed from the surfaces of activated EC, have been found to correlate with the presence of atheroma (Oishi et al., 2000).

1.7.3.2 Infection and adhesion molecules

Adhesion molecules are involved in the pathobiology of other infectious organisms whose lifecycles involve the endothelium in man. Thus ICAM-1 functions as a receptor for both the rhinoviruses and polioviruses and facilitates viral uncoating and entry into the host cell (Bella and Rossman, 1999; Belnap et al., 2000). West Nile Virus, a flavivirus and cause of the outbreak of encephalitis in New York in 1999, causes increased expression of E-selectin, ICAM-1 and VCAM in human endothelial cells as identified by FACS (Shen et al., 1997). Chlamydial heat shock protein 60 induces E-selectin, ICAM-1 and VCAM in human endothelial cells (HUVECS) (Kol et al., 1999). Adhesion molecules are therefore involved in many infections of endothelial cells in man.

Table 1.2. Characteristics of adhesion molecules involved in atherosclerosis

Adhesion molecule	Location	Expression		Ligand	Function	Regulation
		Constitutive	*Inducible*			
Selectins						
L-selectin	All leucocytes	Yes	No	P & E-selectin	Rolling	
P-selectin	EC & platelets	Yes	Yes	L-selectin	Rolling	Released from Weibel-Palade bodies, partly transcription.
E-selectin	EC	No	Yes	L-selectin	Rolling	Transcription
Integrins						
CD11a/CD18	All leucocytes	Yes	Yes	ICAM-1	Adherence	
CD11b/CD18	Monocytes	Yes	Yes	ICAM-1	&	
α4β4 (VLA)	Lympho-& monocytes	Yes	No	VCAM	Emigration	
α4β7	Lymphocytes	Yes	No	VCAM		
Immunoglobulins						
ICAM-1	EC & monocytes	Yes	Yes	CD11a/b,CD18	Adherence	Transcription
VCAM	EC	No	Yes	α4β1, α4β7	& Emigration	Transcription

1.7.3.3 Herpesviruses and adhesion molecules

HSV-1 infection has been shown to upregulate P-selectin in HUVECs and so increase cellular adhesion, an effect which is blocked by anti-P-selectin antibodies (Etingin et al., 1991). Human CMV has been shown to upregulate ICAM-1 in human endothelial cells *in vitro* (Burns et al., 1999). HCMV infected endothelial cells can activate T-cells to both proliferate and produce inflammatory cytokines, and this effect is dependent on endothelial ICAM-1 (Waldman et al., 1998). Thus herpesvirus infection could potentially be enhancing atherosclerosis through their influence on adhesion molecule expression on endothelial cells.

1.8 ATHEROSCLEROSIS IN HIV POSITIVE INDIVIDUALS

As HIV positive individuals are more prone to infections (including herpesviruses), it might be predicted that such individuals were more susceptible to atherosclerosis if there was a causal link between herpesviruses and atherogenesis. Isolated case reports of accelerated atheroma in HIV positive individuals have been accruing since the advent of highly active anti-retroviral therapy (HAART) allowed these patients to live longer. Children seem particularly vulnerable to this form of accelerated atheroma (Bharati et al., 1989). Although one study has shown an increase in atheroma in HIV positive individuals compared to HIV negative controls (Constans et al., 1995), it is still not known for certain whether there is a true increase in atherosclerosis in those who are HIV positive compared to the general population. It has however been proposed that the dyslipidaemia secondary to protease inhibitors, which form part of HAART, may account for some of this apparent increase

(Behrens et al., 1998; Gallet et al., 1998; Henry et al., 1998; Vittecoq et al., 1998). A syndrome of dyslipidaemia, insulin resistance, central obesity and peripheral lipodystrophy occurs in about 30-50% of those commenced on protease inhibitor therapy within 3-10 months of starting therapy (Carr et al., 1998; Walli et al., 1998). It appears to be relatively drug-specific (Periard et al., 1999; Segerer et al., 1999) and may possibly reflect the drug's ability to react not only with HIV protease, its intended target, but also with key cellular proteins that regulate lipid metabolism (Carr et al., 1998). Moreover, there appears to be a genetic propensity for those with the ApoE4 allele to succumb to this disorder (Behrens et al., 1999), although this alone cannot account for the large variation in severity and clinical presentation of protease-inhibitor associated side-effects.

Doubt exists however whether the dyslipidaemia alone can account for any increase in atherosclerosis in this population. What is more likely is that any increase in atheroma is probably multifactorial in origin and that the increased infectious burden in these patients (consequent upon persistent immunosuppression) could also be playing a part.

1.8.1 Evidence for herpeviruses in atherogenesis in HIV positive individuals

The strongest evidence for infection playing a role in atherogenesis in those with HIV infection comes from post-mortem studies. Tabib et al found that the histology of atheroma in young patients infected with HIV is intermediate between that of conventional atherosclerosis and the accelerated form of the disease found in cardiac transplant recipients (Tabib et al., 2000). As it is known that the risk of accelerated atheroma in transplants is greatest in those seropositive for HCMV (see section 1.5),

they postulated that a common herpesvirus infection in this population could be promoting atheroma formation.

1.9 PREVIOUS ANIMAL MODELS OF ATHEROSCLEROSIS AND HERPESVIRUS INFECTION

Marek's disease virus (MDV) is the cause of a debilitating and often fatal illness of commercial fowl. Although MDV is now classified as an alphaherpesvirus on the basis of its DNA sequence, it shares many features of gammaherpesviruses and was previously classified as such (see section 1.4.3.). The virus replicates in a lytic fashion in B cells before becoming latent in T cells by incorporation into the host's genome (Delecluse et al., 1993). Occasional cells undergo transformation into T cell lymphomas. Within one affected chicken all lymphomas have MDV incorporated into the same site indicating the clonal origin of the lymphomas. However between chickens there is no consistent site of incorporation (Ross, 1999). Affected chickens suffered from neurodegeneration, lymphoproliferative disease and atheroma. Fabricant showed as early as 1978 that pathogen-free chickens infected with Marek's disease virus (MDV) developed atheroma over a period of 32 weeks (Fabricant et al., 1978). The lesions that developed were similar to those of humans, developing at branching sites of the large elastic vessels and being fibroproliferative with cholesterol accumulation within the vessel walls (Minick et al., 1978). Cholesterol feeding increased the level of cholesterol accumulation (Hajjar et al., 1986) (Fabricant, 1985; Fabricant et al., 1983). Later work however challenged the finding that atheroma occurred without cholesterol supplementation (Njenga and Dangler, 1996). One reason for the differing results with the MDV-chicken model may be

that infection has to be with live infected cells, injected intrathecally, a technically difficult procedure.

Two models of atherosclerosis and herpesvirus infection have been developed in the rat. In the first rat aortic allograft model, smooth muscle cells proliferation and intimal thickening was increased in rats infected with ratCMV intraperitoneally at the time of grafting compared to uninfected controls (Lemstrom et al., 1993). Moreover ratCMV infection was associated with enhanced expression of certain growth factors known to be involved in atherosclerosis (Lemstrom et al., 1994). In the second rat carotid artery injury model, both injury and immunosuppression were necessary to enhance the atheroma that formed, and CMV DNA was not found in uninjured arteries or immunocompetant rats (Persoons et al., 1994). In both of these models the virus was inoculated via the non-physiological intraperitoneal route, and as mechanical vascular injury was involved, the process was not truly *de novo* atherogenesis but transplant or restenosis atherosclerosis. Therefore although informative and in some ways more convenient than the avian Marek's Disease Virus model, the ratCMV model still has several disadvantages.

As the genetics and immunology of the mouse are better known than the rat, a murine model would be an advantagous. However strains of mice that are generally susceptible to atheroma are relatively resistant to herpesvirus infection. Murine CMV has been demonstrated to cause early lesions and raised serum LDL levels in Balb/c mice (a strain relatively resistant to atherosclerosis but susceptible to herpesvirus infection) (Berencsi et al., 1998). However because of the nature of mCMV (see section 1.4.2.) the animals still required immunosuppression by

gamma-irradiation to allow mCMV to replicate and cause a significant number of inflammatory lesions. In addition, the animals require a high cholesterol diet before lesions were seen. If very young suckling C57Bl and Balb/c mice are used immunosuppression is not required as mCMV can replicate in these mice (Dangler et al., 1995). Cholesterol supplementation will increase the number of lesions but is not a prerequisite for lesion development (Dangler et al., 1995). Nevertheless, in both these murine models only very early lesions were seen (inflammatory cells plus some lipid accumulation) with no progression to mature plaques. What has been lacking therefore is a mouse model that has no need of immunosuppression, mechanical injury or an atherogenic diet, and that produces the full range of atheroma from early fatty streaks to mature plaques.

1.10 MURINE MODELS OF ATHEROSCLEROSIS

1.10.1 Dietary murine models of atherosclerosis

Mice are generally resistant to developing atherosclerosis because they carry the majority of their cholesterol as HDL rather than LDL. The work of Paigen et al established that certain strains were more susceptible to diet induced atheroma as follows: C57BR/cdJ > C57L/J > SM/J > C57BL/6J > SWR/J > C58/J > 129/J > DBA/2J > AKR/J > Balb/c > NZB/BINJ, HRS/J > A/J, C3H/HeJ, SJL/J, CBA/J (Breslow, 1996; Paigen et al., 1990). The C57Bl mouse has emerged as the first established model of murine atherogenesis. Early atheromatous lesions appeared at points of shear stress in the aortic wall after 14 weeks on an atherogenic diet (i.e. 1.25% cholesterol as opposed to 0.04% in normal chow) (Paigen et al., 1987). This model was subsequently used to investigate the underlying mechanisms of

atherogenesis. Thus although dietary antioxidants did not reduce atherogenesis (Munday et al., 1998), injections of the pro-inflammatory cytokine IL-6 did increase atherosclerosis (Huber et al., 1999). Correlations were also found between the level of HDL and total cholesterol with the amount of atheroma present (Kunjathoor et al., 1996; Paigen et al., 1987). One disadvantage of this model was the small size and early stage of the induced lesions, but this was overcome with the advent of transgenic mouse strains.

1.10.2 Transgenic models of disordered lipid metabolism

Gene targeting allowed the generation of mice lacking certain genes, so called-'knock-out' strains. Such mice developed more atheroma which was composed of both early and late plaques (Breslow, 1996; Smith and Breslow, 1997). Two of these models - the LDL-receptor deficient (LDLR-/-) mouse (Ishibashi et al., 1994) and the ApolipoproteinE-deficient (ApoE-/-) mouse (Plump et al., 1992; Zhang et al., 1992) - have been widely used and give comparable results (see (Knowles and Maeda, 2000) and (Smith and Breslow, 1997) for review).

The ApoE-/- mouse was independently developed in the laboratories of Maeda and Breslow by homologous recombination in embryonic stem cells (Piedrahita et al., 1992; Plump et al., 1992). ApoE in chylomicrons, VLDL and LDL is the ligand recognised by the LDL receptor responsible for uptake of these lipoproteins. Thus absence of either apoE or the LDL receptor leads to high LDL levels in the mouse and atheroma Whereas the LDLR-/- mouse has twice the usual serum cholesterol and only develops significant lesions on a high cholesterol diet, the ApoE-/- mouse has 3 to 5 times the

serum cholesterol and can spontaneously develop lesions on a normal chow diet. The ApoE-/- mouse has lesions of similar cell type to man, which do progress to form mature plaques (Reddick et al., 1994). Lesions are also at similar anatomical sites to man, being around the aortic root and at sites of shear stress such as the coronary cusps, branches of the great vessels, origins of intercostal arteries and iliac bifurcation (Nakashima et al., 1994). Moreover lesions can be quantified by reliable methods (Tangirala et al., 1995). As a model of atherosclerosis the ApoE-/- mouse has considerable advantages over the C57Bl mouse on a high cholesterol diet, in that it not only avoids dietary variables between studies, but it also develops plaques of the whole range of maturity and at more sites along the aorta.

1.10.3 The role of inflammation in atherosclerosis as explored in the ApoE-/- mouse

The ApoE-/- mouse has been used in a number of studies investigating the role of inflammation in atherosclerosis but few studies have examined the effect of infection on atherogenesis. For example the importance of monocytes/macrophages in atherosclerosis has been established by breeding mice deficient in the genes for both macrophage colony stimulting factor (MCSF) and ApoE, and showing a reduction in atheroma (Smith et al., 1995). Another relevant cytokine, monocyte chemoattractant protein (MCP-1), works through the CC chemokine receptor-2 (CCR2) on monocytes/macrophages and T-cells. When CCR2-/- mice are cross-bred with ApoE -/- mice there is also a reduction in atherogenesis (Boring et al., 1998). Conversely, when irradiated mice have their bone marrow replaced with cells over-expressing MCP-1 there is an increase in atheroma (Aiello et al., 1999). Thus ApoE-/- mice can

be seen to be a useful tool in exploring the role of various inflammatory cytokines in atherogenesis. The studies with infection are less clear. In 1997 Moazed et al showed that *Chlamydia pneumoniae* could infect ApoE-/- mice despite it not being a natural infection and be found within the arterial wall (Moazed et al., 1997). However owing to a outbreak of murine hepatitis virus during this study no conclusions as to its effect on atheroma could be drawn. The same authors later showed that repeated injections of the organism caused a modest increase in atheroma when the ApoE-/- were fed a high cholesterol diet (Moazed et al., 1999). Other groups have disputed the suggestion that infectious agents are necessary for murine atherogenesis. ApoE-/- mice that were also deficient of a gene that allows them to respond to bacterial lipopolysaccharide (LPS) (ApoE/ lps^{d}-/-) showed no difference in the amount of atheroma compared to the single ApoE knockout. In addition, ApoE-/- mice kept in sterile conditions had similar amounts of atheroma as mice exposed to normal ambient pathogens (Wright et al., 2000). However, these mice were kept on a Western diet which may have accelerated atheroma formation over and above any effect of infection. Additionally, although infectious agents appear not to be necessary for atherogenesis these results do not exclude a role for infectious agents in modifying atherogenesis in the presence of other risk factors.

1.10.4 Diabetic murine models of atherogenesis

Type 1 diabetes mellitus (insulin-dependent) is a well known risk factor for coronary atherosclerosis, increasing the risk of atherosclerosis in men by three-fold and in women by seven-fold (Krolewski et al., 1987; Swerdlow and Jones, 1996). Conventional risk factors do not account for all of this increase in atherosclerotic risk (Morrish et al., 1991). The increase in Type 2 diabetes (insulin-independent)

may account for a large proportion of the increase in atherosclerosis in developing countries (Pearson, 1999).

Genetic models of diabetes exist (see (Shafrir et al., 1999) for review), but various manipulations can make wild-type mice diabetic. Injections of streptozocin (STZ) can, by causing an autoimmune destruction of the beta islet cells of the pancreas, make mice chronically hyperglycaemic and a model for Type 1 diabetes. When fed an atherogenic diet such diabetic mice develop atheroma (Kunjathoor et al., 1996). STZ given to ApoE-/- or LDLR-/- mice leads to an acceleration of their atherogensis (Keren et al., 2000; Tse et al., 1999). Dietary murine models more closely resembling Type 2 diabetes in man have also been developed. C57Bl mice fed a diet high in sucrose as well as cholesterol became hyperinsulinaemic with impaired glucose tolerance (Surwit et al., 1988). These mice had atheroma, but less so compared to mice on the atherogenic diet (Schreyer et al., 1998). Further studies revealed that it was the fat content of the diet, rather than the total caloric content, that was most important in inducing diabetes (Surwit et al., 1995). In summary, there appears to be an interaction between diabetes and hypercholesterolaemia in the mouse model that may reflect the situation in man.

1.11 EVIDENCE FOR ENDOTHELIAL DYSFUNCTION IN ANIMAL MODELS

1.11.1 Impaired vasodilation responses

Impaired responses are seen in diet induced animal models of atherosclerosis (Busse and Fleming, 1996; Ohara et al., 1993); in the genetically predisposed Watannabe heritable hyperlipidaemic rabbit (Taguchi et al., 1995) as well as primates (Frieman

et al., 1986). Rats infected with rat cytomegalovirus (ratCMV), a β–herpesvirus, show reduced endothelium-dependent responses, albeit in their resistance and not conductance arteries (conductance arteries being more prone to atherosclerosis than resistance arteries) (Eerdmans et al., 1996). Although these animals did not go on to develop atheroma, ratCMV has been shown to induce atheroma in the rat allograft model (Lemstrom et al., 1994). *Chlamydia pneumoniae* has more recently been shown to cause impaired vascular responses in the ApoE-/- mouse and can enhance atherosclerosis in this model (Liuba et al., 2000; Moazed et al., 1999). Thus the precedent exists for infections that predispose towards atheroma to be linked to impaired endothelium-dependent responses.

1.11.2 Enhanced vasoconstrictor responses

Enhanced vasoconstrictor responses have been seen in the hypercholesterolaemic rabbit (Henry, 1990; Seto et al., 1993; Verbeuren et al., 1986) and primate (Heisted et al., 1984). In the pig model a high cholesterol diet and injection of pro-inflammatory cytokines results in atherosclerotic lesions in porcine coronary arteries. Examination revealed that areas with early intimal thickening exhibited enhanced vasoconstriction to serotonin and histamine both *in vivo* and *in vitro* whereas normal areas of coronary artery did not (Katsumata et al., 1997; Shimokawa et al., 1996). Such findings thus reflect the situation in man, in that in certain cases enhanced vasoconstrictor responses signify endothelial dysfunction and a propensity to developing atheroma (see section 1.7.2).

1.11.3 Adhesion molecules in animal models of atherosclerosis

In vivo studies in the rat have shown that *Helicobacter pylori* induces leucocyte adhesion via ICAM-1 (Yoshida et al., 1993). Similarly ratCMV has been shown to increase adhesion molecule expression on endothelial cells, leucocyte adherence and accumulation of subendothelial lipid in the rat (Span et al,1991a;Span et al,1991b). Parallel *in vitro* studies have shown that ratCMV infection leads to different patterns of up-regulation of E-selectin, VCAM and ICAM-1 on endothelial cells (Sedmak et al., 1994; Yilmaz et al., 1996). Intercellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM) have been shown to be upregulated at lesion prone sites (Iiyama et al., 1999; Nakashima et al., 1998) and by hypercholesterolaemia in ApoE-/- mice (Iiyama et al., 1999). Moreover, genetic deficiency of these molecules in mice protects against atherosclerosis (Nageh et al., 1997). Mononuclear cell rolling has been shown to be P-selectin and VCAM dependent in ApoE-/- mice and inhibition of ICAM-1 attenuates macrophage homing to lesions (Patel et al., 1998; Ramos et al., 1999).

1.12 SUMMARY

In summary, previous work has highlighted the importance of chronic inflammation in the pathophysiology of atherosclerosis although the exact role of infection in causing such inflammation remains obscure. The herpesviruses, because of their biological properties, are potential candidates for affecting atherogenesis; this is particularly so in the cases of the β- and γ-herpesviruses. Human studies have proved inconclusive, and therefore investigation of a link between herpesvirus

infection and atherosclerosis has been pursued through the use of animal models. The development of such models would not only allow a direct link between herpesvirus infection and enhanced atherogenesis to be proven, but would also pave the way for underlying mechanims to be explored. This in turn could lead to advances in the understanding of how herpesvirus infection interacts with other established risk factors for atherosclerosis, in particular hypercholesterolaemia and diabetes.

1.13 OVERALL HYPOTHESIS: THAT HERPESVIRUS INFECTION PLAYS A ROLE IN ENHANCING ATHEROSCLEROSIS

The initial studies described in this thesis were designed to explore a definitive causal link between herpesvirus infection and atherogenesis in a murine model. Subsequent studies then went on to investigate whether certain herpesvirus infections in humans were associated with atherosclerosis or markers of endothelial dysfunction in those patients more prone to herpesvirus infections (HIV infected), and in a subgroup of patients (diabetics) more prone to atherosclerosis.

1.13.1 Apolipoprotein E deficient mouse/ MHV-68 model: this study set out to test the hypotheses that:

i) Herpesvirus infection enhances atherogenesis in the ApoE-deficient mouse.

ii) Infection with a pro-atherogenic herpesvirus would cause impaired responses to endothelium-dependent vasodilators and enhanced responses to vasoconstrictors.

iii) Infection with a pro-atherogenic herpesvirus would cause an upregulation of adhesion molecule expression in a murine endothelial cell line.

1.13.2 C57Bl/ MHV-68 model with dietary modification: this model was established in order to test the hypotheses that:

i) Infection with a pro-atherogenic herpesvirus would induce atherosclerosis in the presence of an atherogenic diet.

ii) Infection with a pro-atherogenic herpesvirus would induce atherosclerosis in the presence of a diabetogenic diet.

1.13.3 Retrospective post-mortem survey in HIV positive individuals: This study set out to explore the issue as to whether a human herpesvirus infection was associated with accelerated atherosclerosis, by using a surrogate marker of infection in susceptible individuals. The hypothesis here was that atheroma would be more common in those HIV infected individuals previously infected with HHV-8 as manifested by the presence of Kaposi's sarcoma.

1.13.4 HCMV and HSV-1 serology in Type 1 diabetics: this epidemiological study was designed to test the hypotheses that:

i) Previous infection with either HCMV or HSV-1 was associated with enhanced atherosclerosis as determined by coronary artery calcification.

ii) Previous infection with HCMV/HSV-1 was associated with impaired endothelial vascular responses.

iii) These associations were more marked in the presence of an additional risk factor for atherosclerosis, namely diabetes.

Chapter 2: METHODS

2.1 CELL STOCKS & CULTURE

2.1.1 Cell Culture

NIH 3T3 cells were a gift from Peter Collins at Glaxo Wellcome, UK. BHK-21 cells were donated by Dr Stacey Efstathiou of Cambridge University, UK. Murine sEnd.1 cells were given by Dr. Guy Whitely from St. George's Hospital, UK. Table 2.1 shows the media used for the propagation of these cell lines.

Table 2.1

Cells	Media
NIH 3T3	DMEM
sEnd-1	DMEM
BHK-21	GMEM

Dulbeccos Modified Eagle's medium (DMEM) without sodium pyruvate and Glasgow Modified Eagle's medium (GMEM) were both obtained from Gibco Life Technologies. All media were supplemented with 1mM glutamine and 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Growth medium for sEnd-1 cells was supplemented with 10% foetal bovine serum (FBS) and maintenance media with 5% FBS. NIH 3T3 cells were grown in DMEM supplemented with 10% newborn bovine serum (NBS) and maintained on medium supplemented with 2% heat inactivated NBS.

2.1.2 Cryopreservation of cells

Cells were pelleted at 1000g in a bench centrifuge for 5 minutes and resuspended at a concentration of $5x10^6$ -10^7 cells/ml in a mixture of 20% NBS:GMEM or 20% FBS:DMEM as appropriate plus 10% DMSO (Sigma). Cells were then aliquoted into 1ml cryotubes and kept for 2 hours at –20°C and then –70°C overnight and finally stored in liquid nitrogen.

2.2 VIRUS

2.2.1 Virus stocks

MHV-68 was generously donated by Dr Stacey Efstathiou of Cambridge University, UK. All virus growth and titration was carried out in Type II cabinets.

2.2.2 Murine Herpesvirus-68: Virus growth

Virus was propagated in under-confluent BHK-21 cell monolayers in tissue culture (T125) flasks. Growth media was removed and 3ml of maintenance media containing virus at a sufficient concentration to provide a multiplicity of infection (moi) of 0.001was added to each flask. The virus was left to adsorb for 1hour at 37°C and 5% pCO_2, tipping the flasks at 15 minute intervals. Then 18 ml of maintenance media was then added to each flask and the flasks kept at 37°C and 5% pCO_2 until over 80% cytopathic effect (cpe) had occurred. Cells were then shaken or scraped off, pooled from all T125s and spun in an autoclaved centrifuge tube on a 14JA rotor at 1,600g for 20 minutes at 4°C to separate down the cellular virus as a pellet. The supernatant was spun again at 16,000g for 90 minutes at 4°C to spin

down the extracellular virus. Meanwhile the pellet containing the cellular virus was resuspended in 3ml of sterile PBS and placed in a sonicating glass tube. This was sonicated at 4°C for 10 minutes until the suspension resembled milk. This was then centrifuged at 1300g for 5 minutes at 4°C and the supernatant aliquoted into prelabelled cryotubes at 200-500µl/tube. Tubes were kept on ice and immediately frozen at –80°C. Extracellular virus was resuspended in 100µl per original T125 flask of sterile PBS using a P1000 Gilson. This was then aliquoted into prelabelled cryotubes on ice and frozen immediately at –80°C.

Aliquots were defrosted as required and the same aliquots from the same stock used for each experiment. Defrosted aliquots were re-titrated.

2.2.3 Murine Herpesvirus-68: Virus titration

Virus dilutions were made up in maintenance media and kept on ice. 2ml of dilutions 10^{-7}, 10^{-8} and 10^{-9} added to sterile universals. To each universal $8x10^{6}$ NIH-3T3 cells were added in a volume less than 1ml of maintenance media. The universals were left to shake at 37°C for 30 minutes to allow for virus absorption. 8ml of 0.8% medium viscosity carboxymethyl cellulose (Sigma) made up in maintenance media was then added to each tube and inverted twice. The contents were then divided equally between two 60mm Petri dishes and left to incubate at 37°C 5% CO2 for over 72 hours. The cells were then fixed by adding 3 ml of formal saline (10% v/v formaldehyde and 0.85% w/v NaCl) and leaving them for over 30 minutes. Cell monolayers were then washed in water and stained with 0.7%

crystal violet (1:1 methanol: water) for 10 minutes. Cell monolayers were then washed again and left to dry before plaques and syncitia were counted.

2.2.4 Virus isolations

Frozen lung specimens were homogenised on ice in 2ml of 2% DMEM (GIbcoBRL) in 50ml Falcon tubes. (Between specimens the homogeniser was washed in sterile PBS, 70% ethanol and then PBS). Cellular debris was then removed by centrifugation at 1300g for 5 minutes and the tube placed immediately on ice. The supernatant was removed and serial dilutions made in maintenance medium (2% DMEM). 100µl of each dilution was left to adsorb on just confluent 3T3 monolayers at 37°C for 1 hour as for virus titrations. Monolayers were overlaid with 1:6 4% agarose in 2% DMEM and kept in a humidified atmosphere at 37°C and fixed when plaques appeared between 3-5 days.

2.3 ANIMAL EXPERIMENTATION

2.3.1 Animal husbandry

ApoE-/- mice were purchased from Jackson Laboratory (Bar Harbour, Maine) and originated from Dr.N.Maeda, University of Carolina (http//jaxmice.jax.org) ApoE-/- mice were bred at the Royal Veterinary College, London under Specific Pathogen Free conditions or in a positive-pressure isolator. Age matched C57Bl mice were purchased from Charles River, Kent. Mice were kept in similar size groups and allowed food and water ad libitum.

Mice were screened regularly for opportunistic infections and kept in sex and age-matched groups. All experiments were carried out in accordance with the Animals & Scientific Procedures Act (1986).

2.3.2 Diets

All diets were obtained from Lillico Biotechnology, irradiated and in 0.5kg bags and stored at –20°C prior to use. Atherogenic diet (diet A) consisted of standard RM3 diet plus 20% fat, 1.25% cholesterol, 0.5% sodium cholate. The Diabetogenic diet (diet D) consisted of 35% saturated fat, 20% protein and 31% sucrose.

2.3.3 Antiviral drug: 2'-deoxy-5-ethyl-b-4'-thioridine (EtdU).

2'-deoxy-5-ethyl-b-4'-thioridine (EtdU) was a generous gift from P.Sadler. 100mg of EdtU was dissolved in 50ml of filtered water and kept at 65°C for 1 hour. This was stored at RT and made up to 300ml with filtered water prior to use. The final concentration was 0.33mg/ml.

2.3.4 Animal infection technique

Mice were infected at 3-4 weeks of age. Infecting the animals was carried out under light halothane anaesthesia to maximise the accuracy of the dose and minimise discomfort. Intranasal infection was carried out with $5x10^5$ pfu in 20 µl of PBS administered using a P20 Gilson. For an intraperitoneal infection $5x10^5$ pfu in 200µl was administered in the lower outside left-hand quadrant of a mouse held and tipped head down in the left hand to avoid injuring the gut using a 1ml syringe and 23 gauge (blue) needle. Mice were observed until they were moving around normally before

being replaced in their cage. Mice were later culled under terminal anaesthesia by cardiac puncture or by cervical dislocation, all mice from the same experiment being culled in the same way.

2.4 IMAGE ANALYSIS TECHNIQUES & PROTOCOLS

2.4.1 Tissue preparation for image analysis

Surrounding adventitial tissue was removed from the aorta in cold PBS using a dissection microscope. Aortas were then placed in 70% ethanol for 5minutes, saturated Oil Red-O (Sigma) in 70% ethanol for 90 minutes, 70% ethanol for 5 minutes and then distilled water for 5 minutes. Aortas were then mounted en face on frosted slide (Becton) in glycerol-gelatin mountant (Sigma). A coverslip was firmly pressed over the tissue and the slide left to dry overnight. Coverslips were sealed with clear nail varnish.

2.4.2 Photography

All images were taken under identical conditions using a Leica MZ6 microscope and Contax167MT camera in a dark room. The magnification was set at x0.63. The first frame on a film was always a 1cm graticule marked with a number later used to identify the film. The second frame was always the same aorta which subsequently will be referred to as the 'standard aorta'. All images of aortas were taken with the aortas in the same alignment under dark phase photography with a 16 second manual exposure. All slides from a single experiment were photographed using the same batch of film and developed (automated) on the same day by an external company (CPL, London).

2.4.3. Scanning

Processed films were then scanned into a jpeg format at 650dpi using a Nikon Coolscan III by the same operator.

2.4.4. Processing

JPEG images were imported into Adobe Photoshop LE 4.0. A single operator then drew around the area of atheroma without knowing the identity of the specimen. The whole aorta was then similarly drawn around. The areas of atheroma, normal aorta and background were then filled in so the image appeared in three uniform colours. These images were exported in GIF89a format. GIF images were then imported into ImageStat 1.0 programme and the area of red atheroma determined by this image analysis programme. The area of atheroma was expressed as a percentage of the total aortic area.

2.5 ANALYTICAL TECHNIQUES

2.5.1 Histology

Aortas were fixed overnight in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1mol/L phosphate buffer (PB, pH7.4), washed in PB and postfixed in 1% osmium tetroxide in PB for 90 minutes. Tissues were washed in PB and dehydrated through a graded series of ethanols with a final change in propylene oxide. This procedure was followed by infiltration and embedding in epoxy resin. Sections (1μm) were cut and stained with toluidine blue.

2.5.2 Immunohistochemistry

Frozen aortic sections (10μm thick) were air dried and fixed in 100% ethanol. SEnd.1 cells were grown over sterile, scored coverslips (BDH) in 6 well culture dishes. The medium from cell monolayers was carefully removed and the monolayer was washed twice in PBS, fixed in 100% ethanol for 10 minutes and then rinsed and the coverslips removed and stored in PBS.

All specimens were then washed in Tris-buffered saline and 0.3% Tween (TBST, pH 7.4). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes (200μl of hydrogen peroxide and 12ml of methanol). Specimens were then washed 3 times with TBST and blocked with 10% serum in TBST for 10 minutes. This was removed and the a 1/250 dilution of the primary antibody (anti-MHV-68 polyclonal rabbit serum donated by Dr Stacey Efstathiou of Cambridge University, UK.) in TBST was added. Sections were incubated at room temperature for 1 hour in a humidified atmosphere. Specimens were then washed 3 times in TBST. Horseradish peroxidase anti-rabbit antibody (1/100 dilution DAKO) was used as the secondary antibody and incubated on the sections or monolayers for 30 minutes before being washed 5 times with TBST. Horseradish peroxidase activity was detected with the use of DAB solution (DAKO). The reaction was stopped with tap water, the specimens were counterstained with Mayer's haemotoxylin, dehydrated in serial ethanols with a final step in xylene and mounted in DPX (BDH). Negative controls were set up without the primary antibody.

2.5.3 Serum preparation

Whole blood specimens were left overnight at 4°C and then centrifuged at 4°C for 4 minutes at 1000g. The supernatant was pipetted off and respun for 3 seconds at 16,000g at 4°C. This supernatant was then removed and kept at –20°C.

2.5.4 Cholesterol and glucose measurements in both mice and human subjects.

Total cholesterol, HDL-c and triglyceride measurements on serum samples were performed using an enzymatic colorimetric method ; using cholesterol esterase, cholesterol oxidase and 4-aminoantipyrine (Allain et al., 1974; Fossati and Prencipe, 1982; Sugiuchi et al., 1995). LDL-c was calculated using the Friedewald formula (Friedwald et al., 1972). Serum glucose measurements were performed using the Integra method whereby hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate. This in turn is oxidised by NAD+ and catalysed by glucose-6-phosphate dehydrogenase to form NADH which is measured colormetrically.
Tests were done by automation in the Biochemistry Department of The Middlesex Hospital, London directed by Dr. Buckley-Sharpe.

2.5.5 Anti-MHV-68 Serology

Anti -MHV-68 serology was performed by Dr. Alber as described by Alber *et al* except the 96 well plates were coated with purified MHV-68 (Alber et al., 1995).

2.5.6 T-cell proliferation studies

T-cell proliferation studies were performed by Dr Dagmar Alber by culturing lymphocytes isolated from the spleens or para-aortic lymph nodes of infected or

control ApoE-/- mice in the presence of UV-inactivated MHV-68 or influenza antigen (control antigen). Assays were set up as previously described (Alber et al., 1995). The stimulation index (SI) was calculated as SI=mean cpm (test)/mean cpm (control), where cpm=counts per minute.

2.6 MOLECULAR BIOLOGY TECHNIQUES

2.6.1 RNA extraction

Specimens in 1ml of TRIZOL (GibcoBRL) in eppendorfs were defrosted at room temperature (RT) and vortexed with 200µl of chloroform and left to stand at RT for 2 minutes. Tubes were then centrifuged at 16,000g for 15 minutes. (In the case of lung specimens this step was repeated with 0.5ml of Chloroform:Phenol (Sigma) and then processed as follows). The clear upper layer was then pipetted into a sterile eppendorf vortexed with 0.5ml of isopropanol and left to stand at RT for 10 minutes. Tubes were then centrifuged for 15minutes at 16,000g at 4°C. The supernatant was removed and the pellet washed in 1ml of 75% ethanol. RNA was either stored at –80°C or proceeded to be run on a RNA gel as follows.

RNA pellets in ethanol were centrifuged at 16,000g for 5minutes at 4°C, the supernatant removed and the pellet dried on ice. The pellet was then resuspended in 9µl of DEPC-treated water and mixed with an equal volume of RNA loading buffer (Sigma). The mixture was then heated kept at 65C for 15 minutes and then placed immediately on ice for 5 minutes, before being loaded onto the RNA denaturing gel.

2.6.2 RNA denaturing gel

A 1% denaturing agarose gel was made up as follows. To 1.5g of agarose 128ml of DEPC water and 15ml of 10x MOPS-EDTA-Sodium acetate buffer (Sigma) were added. The mixture was heated until the agarose had dissolved and left to cool for 5 minutes. 8ml of 37% formaldehyde (Sigma) was then added in a fume hood and the mixture poured into the gel rig and allowed to set. 1xMOPS buffer was used as the running buffer and the gel was run at 5-10V/cm for 90 minutes.

2.6.3 Blotting

The RNA gel was removed and placed on a wick of Whatman paper suspended over a bath of 20xSSC (GibcoBRL). Hybond N+ membrane (Amersham Pharmacia Biotech) was placed on top of the gel and the edges covered with cling film. Three sheets of Whatman paper saturated in 20xSSC were then placed above the membrane followed by 3-cm depth of folded tissue paper and hand towel. The blot was then weighted down and left overnight. The membrane was then removed and the RNA covalently linked to it using a UV Stratalinker 2400.

2.6.4 Primer design

At the time of experiment 1 the RT-PCR reactions were performed by Dr. Dagmar Alber. All subsequent RT-PCR reactions for Northern blot analysis were performed by myself.

Nucleotide sequences for the relevant adhesion molecule mRNA were obtained from the PubMed data base. Primers were designed with the help of Primer 3 programme and checked using the Williamstone programme. A blast search was performed using Tehcet for Windows to ensure the primers would not hybridise to other DNA sequences. The primers used were; for ICAM-1 (forward CGAAGCTTC-TTTTGCTCTGC and reverse ATGGGAGCTAAAGGCATGG), for VCAM (CTG-ACCTGCTCAAGTGATGG and ATGTTTCGGGCACATTTCC), for P-selectin (TGAAGCCATCAAGTGTCCAG and AAGTGGTGTTCGGACCAAAG) and for E-selectin (ATCTGGCATCTGGGATAACG and GGAAAGGTCCAGAAGCACTG). Primers for β-actin and the viral DNA-binding-protein were designed by Dr Dagmar Alber (β-actin GACATGGAGAAGATCTGGCA and GCTCGAAGTCTAGAG-CAACA) and (DNAbp AGAGCTACTACACCAACGTG and TCACGTACAGGAC-AGAGTTG). The control ribosomal S18 probe for hybridisation experiments was obtained from Dr James Leiper.

2.6.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR experiments in Chapter 4 were performed by Dr. Dagmar Alber, whilst RT-PCR reactions for probes for Northern blots for Chapter 5 were carried out by myself.

For the purposes of generating probes for Northern blots, sEnd.1 cells in 6 well plates were exposed to 500μl of 10μg/ml of LPS (*Salmonella typhosa* - a gift from Neale Foxwell) diluted in growth media, and were suspended in TRIZOL after 2 hours. RNA was extracted and the pellet resuspended in 10μl of DEPC. 2μl

(approximately 4μg total RNA) of this solution was used for the RT-PCR reaction, using GibcoBRL reagents. 1μl of Oligo(dT) was added to 2μl of RNA and made up to 12μl with DEPC. The mixture was heated to 70°C for 10 minutes and chilled on ice. To this was added 4μl of 5xFirst Strand Buffer (250mM Tris-HCl pH 8.3, 375 mM KCl, 15mM MgCl2), 2μl of 0.1M DTT and 1μl of 10mM dNTP mix (10mM each of dATP,dGTP, dTTP and dCTP). These reagents were mixed gently and kept at 42°C for 2 minutes. 1μl of SUPERSCRIPT II (Reverse Transcriptase) was then added, and the mixture then incubated at 42°C for 50 minutes. The reaction was inactivated by heating to 70°C for 15 minutes and then placing on ice.

2μl of the first strand reaction mixture was then used for the subsequent PCR. To this DNA template the following were added: 10μl of PCR Buffer (200mM Tris-HCl pH 8.4, 500mM KCl); 3μl of 50mM $MgCl_2$; 2μl dNTP mix; 1μl of each primer; 1μl Taq DNA polymerase (5U/μl); and 80μl of distilled water. The mixture was denatured at 94°C for 3 minutes, and then 38 cycles of 94°C for 30seconds, 55°C for 30 seconds and 70°C for 30 seconds were carried out. Finally the mixture was held at 72°C for 10 minutes and then kept at 4°C.

The PCR product was mixed with Gel Loading Solution (Sigma) in a 6:1 ratio, and loaded onto a 2% agarose gel with ethidium bromide. Bands were visualised under UV and cut out with a sterile scalpel. Gel extraction was carried out using a QIAQuick Gel Extraction Kit (Qaigen).

Dr. Alber used Perkin-Elmer reagents with an anchored oligo(dT). The PCR conditions were 94°C for 4 minutes and then 30 cycles at 94°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute. The final cycle was 72°C for 7 minutes.

2.6.6 Labelling of probe

DNA probes were labelled using a Random Primed DNA labelling kit (Roche Molecular Pharmaceuticals). 2µl of DNA probe eluted by the QIAQuick Extraction kit was added to 10µl of distilled water and denatured by heating to 110°C for 10 minutes before being chilled on ice. To this 1µl of dATP, dTTP and dGTP (0.5mM in Tris-buffer) were added plus 2µl of hexanucleotide mix (in 10x buffer) and 2µl of Redivue ^{32}P dCTP (Amersham Pharmacia Biotech), and 1µl of Klenow enzyme (2U/µl). The mixture was mixed by pipetting and transferred to a water bath at 37°C for 1 hour.

The probe was cleaned by passing it down a Nick Column (Amersham Pharmacia Biotech). The storage buffer was allowed to run through the column followed by an equal volume of TE buffer (pH 8). The column was then set up above the first of a series of three sterile eppendorfs. The probe was removed from the ice and placed in the centre of the column and washed through with 400µl of TE buffer. This was repeated twice more with the same volume of buffer with the Column over the remaining two eppendorfs. Each tube was checked for activity using the Geiger Counter, and the second most active tube selected. This probe was then denatured by heating to 110°C for 10 minutes and chilled immediately on ice.

2.6.7 Hybridisation

The Hybond N+ membrane bearing the RNA was prehybridised in a roller tube with 5-10ml of ExpressHyb (Clontech) at 68°C for 30 minutes. Then the ExpressHyb was poured out, and 3-5ml of fresh ExpressHyb was mixed with the probe in a Falcon

tube and added to the roller tube. The blot was then hybridised for 1 hour at 68°C. The hybridising solution was then drained out and the blot washed twice with Wash 1 and Wash 2 for 15-20 minutes each at 25°C and 50°C respectively. Wash 1 consisted of 2x SSC, 0.05% SDS and wash 2 of 0.1x SSC, 0.1% SDS. The blot was then wrapped in cling film. For the probes against mRNA of adhesion molecules the blots were placed on an Imaging Plate BAS-MP (Fuji Photo Film Co. Ltd) for 36 hours, and for the control S18 probe only for 2-5 minutes, before quantification. Films of adhesion molecule blots were taken by placing blots on BIOMAX-MS film (Kodak Scientific Imaging Film) and keeping at –80°C for 36-48 hours. Meanwhile blots for the control probe were placed on X-OMATAR film (Kodak Scientific Imaging Film) at –80°C for 2-4 hours.

2.6.8 Sequencing of probes

Probes were sequenced using the pre-mix in the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction kit. 8µl of premix was added to 1µl of DNA probe and 3.2 µl of a single primer at 1pmol/µl and made up to 20µl with distilled water. This was repeated using the reverse primer. Reaction mixtures were vortexed and kept on ice. The following cycling reaction was carried out for 25 cycles (Techne or Biometra thermocyclers overlaying with silicone oil as necessary): denatured at 94°C for 30 seconds, annealed at 55°C for 18 seconds and allowed to extend at 60°C for 4minutes. Reaction mixtures were then pipetted into eppendorfs containing 2µl of sodium acetate and 50µl of 95% ethanol, vortexed and left on ice for 10 minutes. Pellets were precipitated by centrifuging for 30 minutes at 16,000 g at 4°C. The supernatant was then removed taking care not to disturb the pellet. The

pellet was washed in 250µl of 70% ethanol and centrifuged at the same speed and temperature for 5 minutes. The supernatant was removed and the pellet vacuum-dried for 30 minutes before being stored overnight at -20°C. Pellets were suspended to 4µl of RNA sample loading buffer (Sigma), heated to 95°C for 10 minutes and cooled immediately on ice. Sequences were loaded onto a gel prepared by Dr J Sainta–Maria or Miss CTL Tran, and read by an ABI Prism 377 DNA Sequencer. Sequences were checked visually against the expected nucleotide sequence for the appropriate mRNA.

2.6.9 SEnd.1 cell protocol for Northern blots

Cells were grown as described in Methods section 2.1, in 10%FBS:DMEM (GibcoBRL). No reduction in serum was used in the maintenance media for the virus, so that serum changes could not be affecting adhesion molecule expression. Cells were plated out at $5x10^5$ cells/well in 6 well culture dishes. The next day, with cells just under-confluent, the media was removed and the required concentration of virus was added in 200µl of media. Culture dishes were kept covered in a humidified atmosphere at 37°C, 5%CO2. After 2 hours, 500µl of media was added to prevent the cells drying out. At predetermined time points media was removed, cells were rinsed with PBS and immediately resuspended in 1ml of TRIZOL (GibcoBRL) and stored at –80°C. Cells used as a negative control received 500µl media only. Cells used as a positive control received 500µl of 10µg/ml of LPS (*Salmonella typhosa* - a gift from Neale Foxwell) diluted in growth media, and were suspended in TRIZOL after 2 hours.

2.7 ORGAN BATH TECHNIQUES & PROTOCOLS

2.7.1 Vascular ring preparation for organ bath experiments

Mice were anaesthetised with halothane and blood removed by cardiac puncture. The descending thoracic aorta was then carefully removed and placed in oxygenated Krebs' at room temperature, (Krebs composition in mmol/l=NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 glucose 12). Loose connective tissue was removed and two to three 3-4mm rings were cut and mounted on stainless steel hooks. Rings were then suspended in vertical organ baths containing 25mls of Krebs' at 37°C and gassed with a mixture of 95% oxygen and 5% carbon dioxide. The hooks were connected to transducers to measure isometric tension. Rings were allowed to equilibrate at a tension of 0.3g for 1 hour and washed at 20 minute intervals. Rings were repeatedly washed until they had returned to this optimal resting tension after each stage in the experimental protocol. Rings which failed to contract to a minimum of 0.4g in response to 10^{-5}M PE were excluded. Any ring which failed to relax by at least 50% to 10^{-6}M ACh was taken as not having an intact endothelium and was also excluded.

2.7.2 Drugs

All concentrations are expressed as the final concentration in the organ bath. Drugs were obtained from the following sources; acetylcholine (Ach) (Sigma), spermine-NONOate (RBI), sodium nitroprusside (SNP) (Sigma), glyceryl trinitrate (GTN) (David Bull Laboratories, Warwick, UK), U44619 (U19) (Biomol) and phenylephrine (PE) (Sigma).

2.8 HUMAN STUDIES

2.8.1 Subjects

All subjects were recruited as for another study carried out by Dr Helen Colhoun. I took no part in gathering this data (Colhoun et al., 2000)..

Briefly participants were recruited from a cohort of 400 subjects (199 type 1 diabetic and 201 non-diabetic subjects). Type 1 diabetes was defined as diabetes with age of onset ≤ 25 years, with continuous use of insulin within one year of diagnosis. The sampling frame for the diabetic patients was the diabetes registers of 5 London Hospitals (all patients aged 30-55 years were invited to take part). Those with renal failure or on renal replacement therapy were excluded, but otherwise the diabetic patients were representative of the diabetic population. The non-diabetic participants were a random sample from the patient registers of two London general practices, stratified to have the same age and sex distribution as the diabetic subjects. Subjects were recruited without regard to their history of coronary heart disease or diabetic complications. All were aged between 30 and 53 years, and 50% were female. Of this cohort of 400 subjects, 157 (39%) took part in the *in vivo* endothelial function testing, 44 had moved address and were uncontactable, 15 were ineligible (3 were pregnant and 12 had co-existing medical conditions mostly co-existing infections) and 184 refused. In total, there were 88 (56.1%) type 1 diabetic (54 men and 34 women) and 69 (43.9%) non-diabetic subjects (34 men and 35 women). Of the 400 original participants, the response rates for this study were slightly higher amongst diabetic men (52%) compared to non-diabetic men (36%), diabetic women (36%) and non-diabetic women (33%). The cohort recruited to the present study did

not differ in risk factor profile (blood pressure, body mass index, lipids), diabetes duration, control or complications from the overall sample. None of the participants agreeing to take part were on nitrate therapy. Diabetic subjects who had hypoglycaemia within 24 hours prior to the study, pregnant women and those with cancer, psychiatric illness or acute infection were excluded. The majority of women (diabetic and non-diabetic) were studied in the follicular phase of their menstrual cycle. All participants gave their informed consent. The study had the approval of the local ethics committee and was conducted over a period of 14 months.

2.8.2 Serology for Herpes Simplex Virus and Human Cytomegalovirus

All human serology testing was carried out by Miss Dawn Andrew in the Department of Virology, Royal Free Hospital and Medical School, London. I took no part in generating the raw data.

Briefly HCMV testing was done using a BioElisa CMV IgG (Biokit) assay. All reagents were prepared as per instructions. 100µl of each diluted specimen and diluted controls were placed in wells coated with HCMV antigen. Blank wells were left empty. The plate was then sealed and incubated at 37°C for 1 hour. The contents of the wells were then aspirated and rinsed 4 times with 300µl of the diluted washing solution. 100µl of diluted conjugate was then added to each well except the blank, and the plate sealed and incubated for 30 minutes at 37°C. Towards the end of this period 280µl of chromogen (TMB) was mixed with 14ml of substrate buffer per plate. The wells were then aspirated and washed four times as before. 100µl of

substrate-TMB solution was added to each well including the blank well. The plate was incubated at room temperature in the dark for 30 minutes. The reaction was then stopped by adding 100μl of stopping solution into each well. The absorbance of each well was then read at 450nm after blanking the reader with the blank well. The mean absorbance of the low positive calibrator was taken as the cut off value. All specimens that tested in duplicate with an absorbance equal to or greater than the cut off value were considered positive for IgG HCMV. Equivocal tests were re-tested in duplicate and if still neither positive nor negative removed from the subsequent statistical analysis. All specimens were tested for IgG anti-HSV-1 antibodies by an identical method but using an ETI-HSV-1K-G kit (DiaSorin).

2.8.3 Human *in vivo* endothelial function studies

All human *in vivo* studies were carried out by Dr. Norman Chan as part of a previous study. I took no part in the generation of the raw data.

Briefly, forearm blood flow in response to endothelium-dependent vasodilator acetylcholine (ACh) and bradykinin (BK), and the endothelium-independent vasodilator (exogenous NO donor) glyceryl trinitrate (GTN) were measured by venous occlusion plethysmography. Additionally, basal NO-dependent vascular tone was assessed using L-N^G-monomethyl-L-arginine (L-NMMA, an NO-synthase inhibitor) with response to norepinephrine (α-adrenergic receptor agonist) assessed as a comparative control. Blood pressure in the right arm was measured and venous non-fasting blood was taken. A 27-gauge stainless steel needle (Cooper's Needle Works, Birmingham, UK) sealed to an epidural catheter (Portex, Hythe), was

inserted into the brachial artery of the non-dominant arm under local anaesthesia (1% lignocaine). Forearm blood flow was recorded simultaneously in both arms by venous occlusion plethysmography, calibrated to measure absolute blood flow with electrically temperature-compensated strain-gauges attached to the upper part of the forearms. During measurements, upper arm collecting cuffs were inflated to 40 mmHg for 10 out of every 15 seconds and circulation to the hands was excluded by inflating the wrist cuffs to 200 mmHg. Basal blood flow was recorded after 25 minutes of rest following insertion of arterial cannula. Forearm blood flow were measured in response to intrabrachial infusion of ACh (Sigma; doses of 25, 50, 100 nmol/L, each dose for 3 minutes); BK (Clinalfa, Laufelfingen, Switzerland; doses of 10, 30 and 100 pmol/min, each dose for 3 minutes); GTN (David Bull Laboratories, Warwick, UK; of 4, 8, 16 nmol/min, each dose for 5 minutes); norepinephrine (Levophed; Sanofi Winthrop Ltd, Guildford, UK; 60, 120, 240 pmol/min, each dose for 5 minutes); and L-NMMA (Clinalfa, Laufelfingen, Switzerland; 1, 2, 4 μmol/min, each dose for 5 minutes). Each drug infusion was separated by a 10-minute saline washout period. The order of vasodilator infusions (ACh, BK, GTN) was randomised. Flow was recorded for approximately 10 seconds in every 15 seconds, and the mean of the last four measurements of each recording period was used for data analysis. Blood flow was expressed as mls of blood per 100 mls of forearm volume per minute (ml/100ml/min).

3.8.4 Electron Beam Computed Tomography (EBCT)

All subjects had coronary artery calcification (CAC) score assessed by EBCT approximately 12 months prior to the *in vivo* vascular function study under the direction of Dr. Helen Colhoun and Dr. Norman Chan of the Department of

Epidemiology and Public Health. The detailed method and protocol of EBCT assessment of this cohort has been described in detail elsewhere (Colhoun et al., 2000).

Briefly an Ultrafast CT scanner (IMATRON C-150XL) was used to quantify the amount of CAC. Two sets of 20 transverse tomograms of 3mm thickness were obtained from the lower margin of the bifurcation of the right main branch of the pulmonary artery to the apex of the heart, with the subject breath-holding. A radiologist placed a region of interest around each potentially calcified lesion (peak density >130 Hounsfield units) within the right, circumflex, left anterior descending and left main stem coronary arteries. The area and peak density of each lesion was measured. A density score of 1-4 was defined based on the peak density of the lesion, and the calcification score was then calculated as the product of the area of the lesion and its density score. All scans were scored by the same radiologist who was blinded as to the sex and diabetic status of the subject.

2.9 STATISTICAL ANALYSIS

2.9.1 Organ bath studies

Relaxation responses to either ACh or SNP were expressed as the percent relaxation from the total amount of pre-contraction produced by PE EC90 or U19 EC70. Student's unpaired t-Tests were performed for the absolute force needed to achieve the EC90 of contraction, as well as the dose of vasoconstrictor used to reach this. Student t-Tests were also used to compare the EC50s and maximum relaxation responses between infected and control animals. The latter were also compared by 2 way ANOVA.

2.9.2 Assessment of atheroma

The mean and standard error of the mean of percentage atheroma was determined at each time point. Statistical analysis was carried out using Student's t-Test to 95% confidence limit and by two-way ANOVA test

2.9.3. Human serology data

Statistical analysis of the human serology data was carried out by Dr Helen Colhoun and Dr Norman Chan of the Department of Human Epidemiology and Public Health, University College London.

All the data were analysed using STATA 6.0. Comparison of background characteristics between the sexes and between diabetic and control groups was made using multiple linear regression to adjust for age and sex where appropriate. The response to drug was calculated, as described previously (Calver et al., 1992), as:

$$\frac{\text{flow in drug arm/ flow in control arm during drug infusion}}{\text{flow in drug arm/ flow in control arm at baseline}}$$

This was summarised across the three doses as the area under the dose response curve (Matthews et al., 1990). The inverse of this ratio was used for calculating the area under the curve for constrictors. Differences in the area under the curve for each drug between seropositive and seronegative subjects was tested for statistical significance using multiple linear regression to adjust for age, sex, diabetes and then other risk factors. Social class was defined using the Registrar General's

Classification of Occupation as being manual (social classes III-manual, IV and V) and non-manual (I, II and III-non-manual).

2.10 SUMMARY OF THE RELATIVE CONTRIBUTIONS

Many parts of this thesis were in collaboration with others whom it is important to acknowledge (see Acknowledgements). Here is a breakdown of my and their contributions to the methodology.

Establishing the ApoE-/- MHV-68 model was done in conjunction with Dr. Dagmar Alber and my supervisors Prof. Kenneth Powell and Prof. Patrick Vallance, at University College London. All cell culture, virus growth, titration and isolation has at some time been performed by myself, but at the time of the studies in Chapter 4 was performed by Dr. Alber. Dr. Alber also performed the subsequent immunohistochemistry on aortic slices, anti-MHV-68 serology and T-cell proliferation studies. The immunohistochemistry on sEnd.1 cells was performed by myself under her supervision. I was solely responsible, with Mr Mike Gahan, for developing the image analysis protocols, and did all such analysis for the studies in Chapter 4. Histology was done by Mr Dave Goodwin, UCL. All organ bath studies, molecular biology techniques (except for the RT-PCR in Chapter 4 which was done by Dr. Alber) and their associated virus growth and titrations were performed by myself. Human ELISA testing was done by Miss Dawn Andrew at the Royal Free Hospital and Medical School, although I have used ELISA kits for HHV6/7 which is not included in this thesis. Human plethysmography, coronary calcification quantification and its subsequent statistical analysis was done by Dr. Norman Chan and Dr. Helen Colhoun, in the Department of Epidemiology & Public Health, University College London.

Chapter 3: Developing the MHV-68/ApoE-/- murine model

3.1 INTRODUCTION

Developing the ApoE-/- MHV-68 murine model for atherogenesis entailed setting up the ApoE-/- mouse colony as well as establishing and optimising a method of image analysis. Previous methods used to quantify the amount of atheroma in the C57Bl mouse had used a variation of the method by Paigen et al (Paigen et al., 1987), whereby the proximal aorta was embedded and sections cut across the aortic root, and the area of lipid stained with Oil Red-O determined by image analysis. This method has been subsequently used to assess the amount of atheroma in ApoE-/- mice (Moghadasian et al., 1997; Reddick et al., 1994; Roselaar et al., 1996; Zhang et al., 1992), although it does ignore the sizeable proportion of total aortic atheroma in the descending aorta. For this reason, various groups have developed a method similar to that previously used in rabbits, whereby the fresh aorta is dissected and cut longitudinally to expose its luminal, atheromatous surface. The tissue could be then be either stained with Oil Red-O (Tangirala et al., 1995), or simply photographed after fixation in formal-sucrose (Palinski et al., 1994), with the area of lesions then being determined by image analysis. Although drawing on the work of others, the image analysis technique described in this chapter were specifically designed for this thesis and represents a novel, reliable method for determining the area of atheroma in the aorta of a mouse.

3.2 ESTABLISHING THE APOPLIPOPROTEIN-E-DEFICIENT MOUSE COLONY

Four pairs of mice were imported from Jackson, Maine, USA in January of 1998 into Heathrow airport. They were then transported under MAFF licence to the Biological Services Unit at the Royal Veterinary College, London. Mice were bred in a positive pressure isolator, and when sufficient mice were available a core nucleus of breeding pairs were kept in a separate isolator. Mice for experimentation were transferred in sealed boxes to a negative pressure isolator and kept there for the duration of the experiment. All bedding, water and mouse chow was autoclaved prior to being brought into the isolators. Mice were regularly culled and screened for opportunistic mouse pathogens by the Royal Veterinary College. Additionally Dr. Dagmar Alber screened mice for HSV-1 and MCMV. Mice were culled if they developed malpositioned teeth causing them to fail to gain weight, or congenital hydrocephalus (5% incidence).

3.3 OPTIMISATION OF IMAGE ANALYSIS

3.3.1 Microscopy

In order to photograph the aorta along its entire length a x0.8 objective was added to the Leica MZ6 stereo-dissecting microscope, and the lowest (x0.63) magnification used. The majority of aortas still could only be photographed using two frames, and a prominent feature (branching vessel or atheromatous plaque) was used to divide each half aorta at image analysis. When the area of atheroma was determined on a single aorta photographed in either one or two frames, no difference was found.

3.3.2 Photography

Dark phase microscopy was used to allow the normal aortic wall to be distinguishable from the background. This meant that the exposure time had to be prolonged to 16 seconds. To reduce incident light, all images were taken in a dark room and a time cable was used to reduce camera shake. All images for a single experiment were taken with the same batch of Kodak Ektachrome 100SW film. Tungsten (160T) Kodak film resulted in poorer red coloured images. The first frame of each film was a 1cm graticule. The microscope was focused on this graticule and not changed for the rest of that film. All photographs of aortas from an individual study or a single time point were processed on the same day by CPL, London.

3.3.3 Scanning and image analysis

Films scanned by Medical Illustration (UCL) by different operators proved to give inconsistent images. Therefore all images were scanned into JPEG format by a single operator using a Nikon Coolscan III at 650 pixels/inch. The image analysis programme ImageStat 1.0, specially written for these studies by Mike Gahan, could only use images in GIF format. JPEG images were therefore imported into Adobe Photoshop 4.0 LE and GIF89a exported. These GIF images were then imported into ImagStat 1.0 and the area of atheroma determined. Details of this programme have been made available at http://www.ucl.ac.uk/~ccamrg/imagests.html. To test the image analysis programme a series of photographs were taken of one, two, three and four identical red circles that fitted into the field of view. These images were processed, scanned, converted into GIF format by Adobe and analysed using

ImageStat 1.0. The resulting areas are shown in figure 2.1; the coefficients of variation for each incremental increase in area were small (<1.5%).

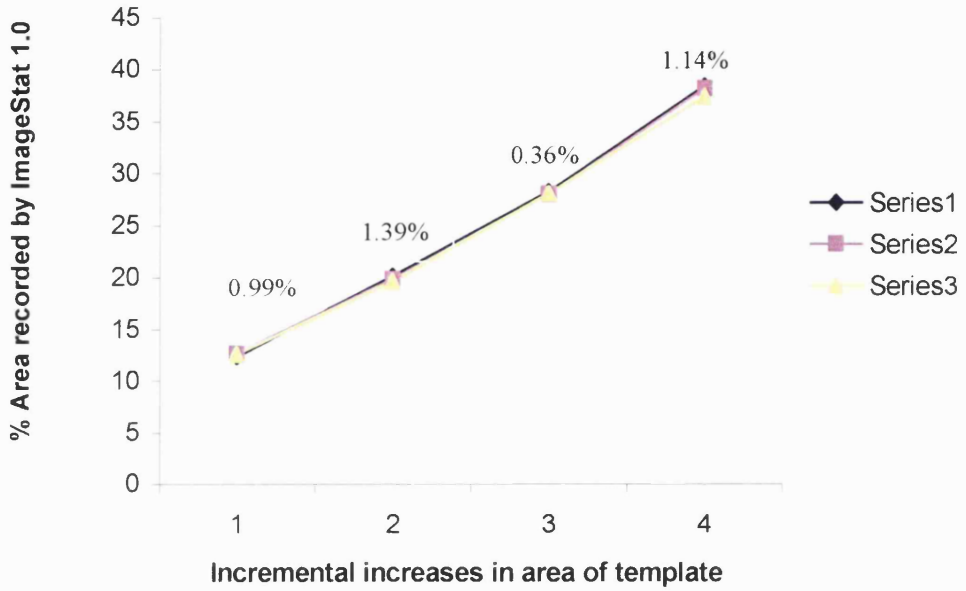


Figure 3.1. Percentage areas recorded by the image analysis programme ImageStat 1.0. A single template of one, two, three and four red circles was photographed three times. The figure beside each point is the coefficient of variation (%) for those three values.

The ImageStat 1.0 programme allows the operator to set a range of colours for the background and then another (white) for the normal aortic wall, and finally a range of reds for the atheromatous plaques. It was noted that the infected aortas sometimes had a pink rather than the normal white aortic wall. The image analysis programme would therefore count any pink pixels as atheroma despite the fact that they were not part of the red plaques, so potentially over-calculating the amount of atheroma in the infected group. For this reason an additional step was incorporated into the image analysis protocol, whereby the areas of red atheromatous plaques were drawn around (in Adobe Photoshop) by a single operator unaware of the identity of the images.

The whole aorta was similarly outlined, and the areas of atheroma, normal aorta and background filled in with a uniform colour. The images had therefore been reduced to an image of three uniform colours. These images were then GIF89a exported and subsequently analysed by ImageStat 1.0 as before.

As this additional step had brought in a human variable, the following was standardisation procedure was performed. On each film the second frame was of the same 'standard aorta'. This standard aorta was drawn around and processed as above along with the other images on that film. The coefficient of variation for the percentage of atheroma on the standard aorta between films calculated as 8.3%.

3.4 DISCUSSION

The image analysis system developed specifically for this thesis has distinct advantages and disadvantages. Its advantages are that it allows the whole aorta to be assessed, and is relatively cheap and repeatable. Its disadvanatges are that is time consuming and ignores the depth and histology of the lesions. In addition, as adventitial fat also stains red with Oil Red O, it must be scrupulously removed prior to staining, which introduces some inter-operator variability at dissection. To avoid this, in the following experiments those dissecting did not know the identity of the specimens and each person dissected an equal number of control and infected aortas. This image analysis system was used for all subsequent animal studies and proved a novel, dependable method of atheroma quantification.

The ApoE-/- mouse took time to become established and was expensive to maintain. However it did provide cohorts of age-matched animals that developed atheroma at a common and predictable rates. Interestingly, the amount of atheroma in the mice in this colony developed at slightly later age than initial reports (Nakashima et al., 1994; Tangirala et al., 1995), possibly because the mice in our studies were kept free of ambient mouse pathogens, and may therefore have been exposed to fewer pro-inflammatory stimuli.

Chapter 4: MHV-68 infection accelerates atherosclerosis in the ApoE-/- mouse

4.1 INTRODUCTION

The classic risk factors for atherosclerosis account for only 50% of its incidence (Crouse, 1984). Infectious agents have been proposed as additional risk factors (Cook and Lip, 1996; Danesh et al., 1997; Ellis, 1997) with members of the herpesviruses family being strong candidates. Herpesviruses have been proposed as potential initiators of arterial injury (Benditt et al., 1983; Fabricant et al., 1978; Yamashiroya et al., 1988), endothelial dysfunction and local inflammation, all of which might trigger or exacerbate atherosclerosis (Ross, 1993). However, current evidence for a role of herpesviruses in vascular disease is conflicting, with data both for and against their involvement ((Epstein et al., 1996; Ridker et al., 1998; Speir et al., 1998) and see section 1.5).

As epidemiological studies in man have proved inconclusive, the role of animal models has attained greater prominence. The ApoE-/- mouse is a well-established model of atherosclerosis in man, and develops both high serum cholesterol levels and atheroma on a normal diet ((Nakashima et al., 1994; Plump et al., 1992; Zhang et al., 1992) and see section 1.10.2). Meanwhile MHV-68 is a naturally occurring mouse pathogen (Blaskovic et al., 1980; Stewart et al., 1998; Sunil-Chandra et al., 1994) that causes an arteritis in immune-deficient animals (Weck et al., 1997).

4.2 HYPOTHESIS

The hypothesis put forward is therefore that infection with MHV-68 in the ApoE-/- mouse would accelerate atherosclerosis.

4.3 METHODS

4.3.1 Virus propagation

Virus growth, titrations and isolations were carried out as described in Methods section 2.2.

4.3.2 Animals

Experimental mice were housed and infected as described in Methods section 2.3.

4.3.3 Quantification of atherosclerotic lesions

Aortas were prepared and analysed as described in Methods section 2.4.

4.3.4 Histology, Serology , Cell mediated immunity, Detection of viral message by RT-PCR and Localisation of virus by immunohistochemistry

These techniques were performed as described in Methods section 2.5.

4.3.5 Serum cholesterol levels

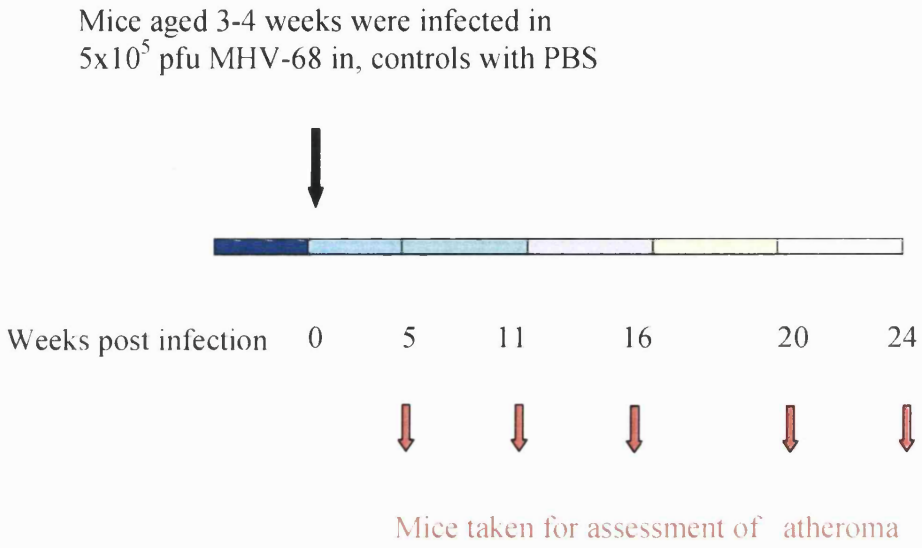
Serum was prepared from whole blood and total cholesterol measured spectroscopically as described in Methods section 2.5

4.4 Experimental protocols

4.4.1 Experiment 4.1: Twenty-eight 3-4 week old ApoE-/- mice were infected with $5x10^5$ pfu of MHV-68 intranasally (in) meanwhile controls (n=27) received an

equal volume of sterile PBS. Mice were culled at 5, 11, 16, 20 and 24 weeks post infection. Aortas were removed for quantification of atheroma by image analysis

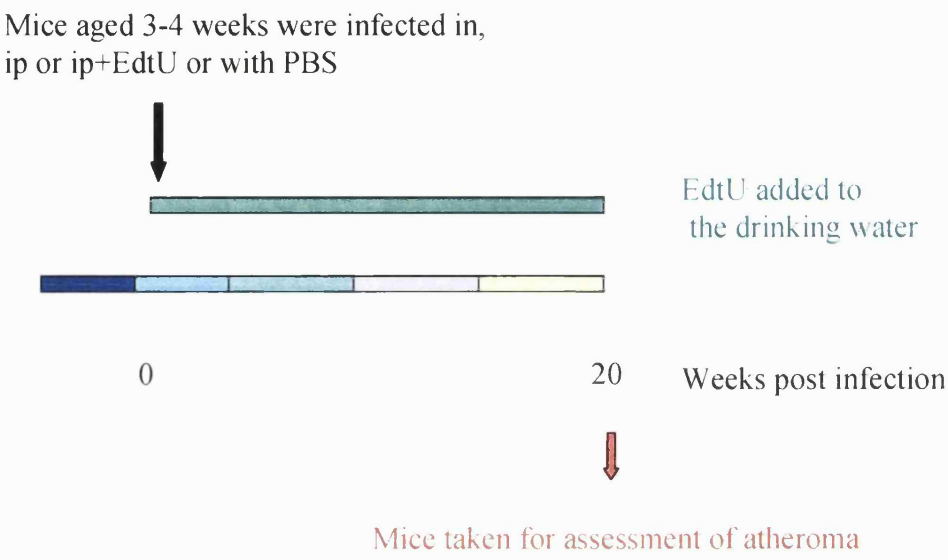
Figure 4.1. Schematic representation of this protocol used in Experiment 4.1



4.3.2 Experiment 4.2:

Fifteen 3-4 week old ApoE-/- mice were infected intraperitoneally (ip) with $5x10^5$ pfu of MHV-68. Eight of these mice received the antiviral drug 2'-deoxy-5-ethyl-b-4' thioridine (EdtU) in their drinking water at a concentration of 0.33mg/ml, from the day of infection. These mice were culled at 20 weeks and the amount of atheroma in their aortas compared to the control and infected groups in experiment 4.1.

Figure 4.2. Schematic representation of this protocol used in Experiment 4.2



4.4 RESULTS

4.5.1 Virus isolation from lungs of ApoE-/- mice infected with MHV-68

Intranasal infection of ApoE-/- mice (n=3) with MHV-68 caused a subclinical infection. Virus titres isolated from the lungs 5 days post infection were similar to those measured in C57Bl mice, the parental strain, indicating that ApoE-/- mice were equally susceptible to MHV-68 infection (ApoE-/- = $5.52x10^5$+/- $3.5x10^5$sem, C57Bl =$4.4x10^4$ pfu/g).

4.5.2 Lesion development in mice infected with MHV-68

Macroscopically typical atherosclerotic lesions were seen in the aorta at 24 weeks after infection. These plaques were yellow-white in appearance and projected into

the lumen of the aorta (figure 4.3(a)). There was considerably more atheroma in the aorta from the infected animal compared to the control (figure 4.3 (b) and (c)). Aortas from C57Bl mice infected with the same dose of MHV-68 for 24 weeks did not stain with Oil Red-O, and no macroscopic lesions of atheroma were seen (figure 4.3 (d)).

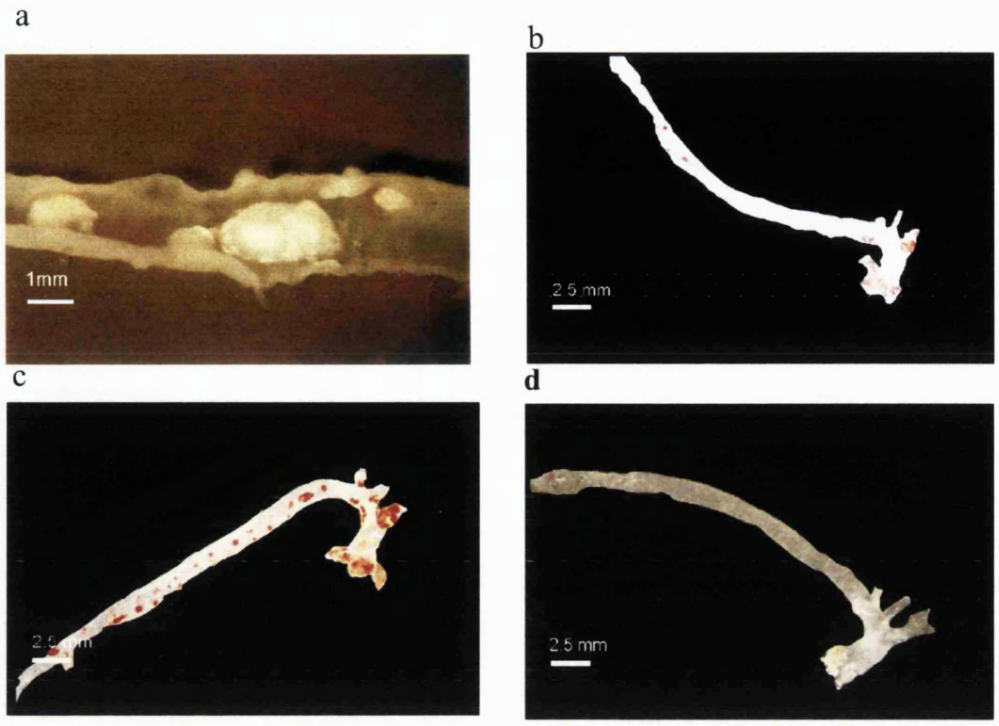


Figure 4.3. Atherosclerotic lesions in aortas of of ApoE-/- mice. (a) Unstained aorta of an infected mouse culled at 2 weeks post infection, showing the raised atheromatous plaque. (b) Stained aorta of a control mouse at 24 week post infection with 6.4% total atheroma. (c) Stained aorta of an infected mouse at 24 weeks post infection with 22.2% atheroma. (d) Stained aorta of a C57Bl mouse 24 weeks post infection with no atheroma for comparison.

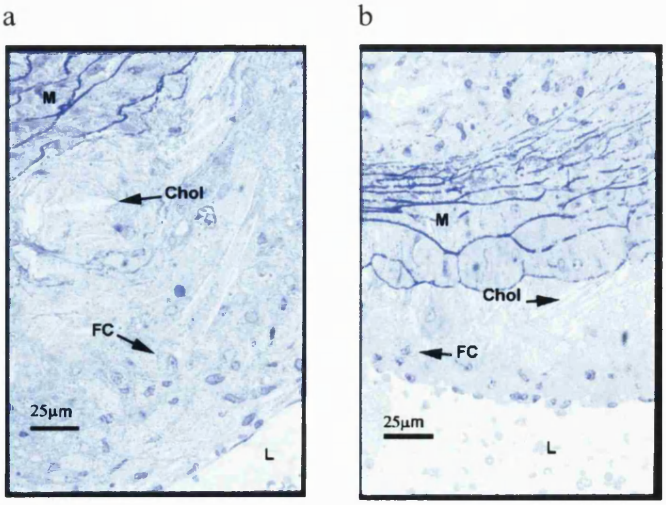


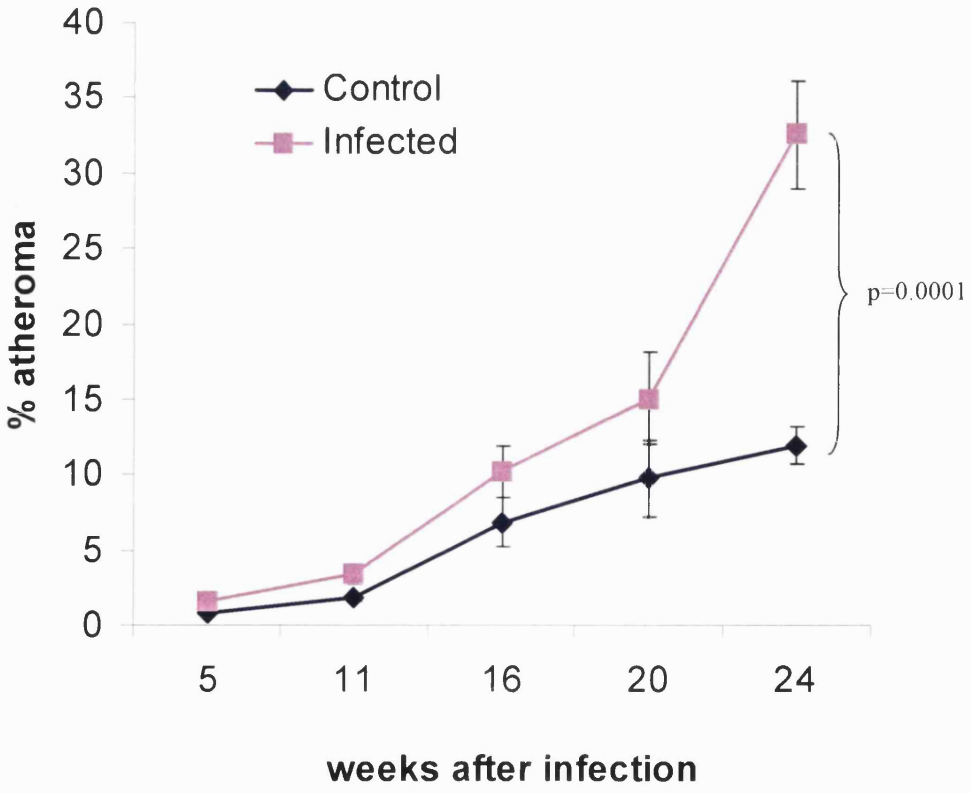
Figure 4.4. Histology if atherosclerotic lesions in ApoE-/- mice at 24 weeks post (a) inoculation with PBS (control) and (b) MHV-68. L=lumen, M=media, FC=foam cell Chol= cholesterol crystal.

Histology of lesions taken from mice 24 weeks after infection showed a typical appearance (figure 4.4). There was thickening of the vessel wall with disruption of the elastic fibres and deposition of cholesterol crystals. The increase in smooth muscle cells (SMCs), the large number of foam cells, and the presence of inflammatory cells were also typical of atherosclerotic lesions in these animals (Nakashima et al., 1994).

4.5.3 MHV-68 accelerates atherogenesis over time in the ApoE-/- mouse

The increase in atheroma was time dependent in both the infected and control groups in Experiment 4.1. Compared to controls mice infected with MHV-68 showed an enhancement in atherosclerosis which was highly significant by 2 way ANOVA ($p=0.0001$, figure 4.5).

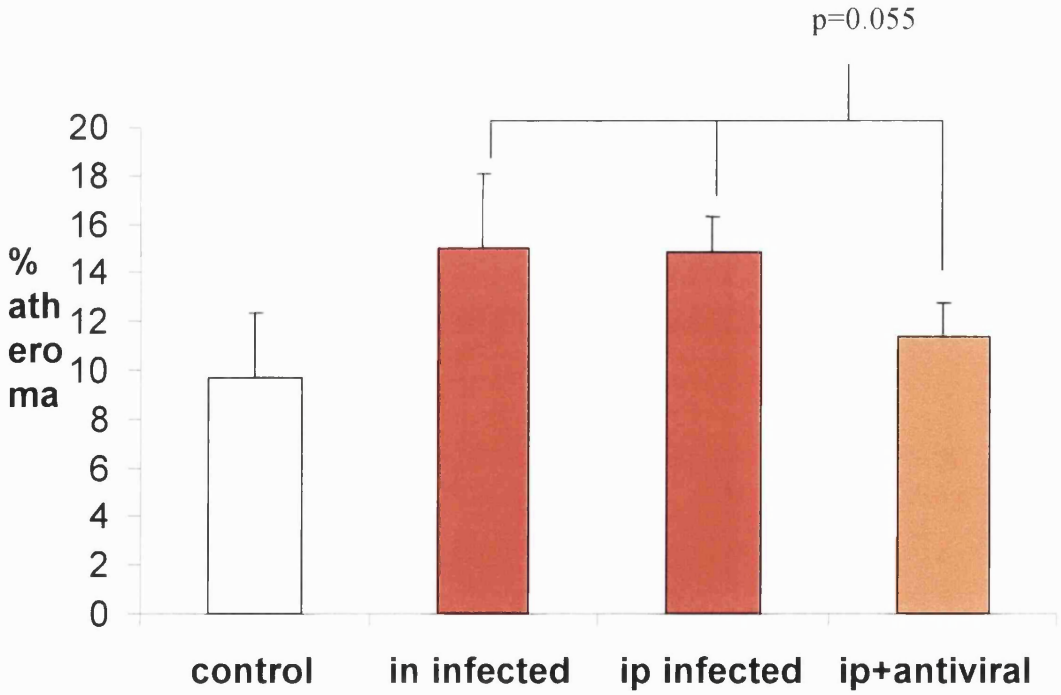
Figure 4.5. Acceleration of atherosclerosis in ApoE-/- mice. The percentage of atheroma increases in control (n=27) over 24 weeks. This increase is accelerated if mice are infected with MHV-68 (n=28, $p=0.0001$ by 2 way ANOVA).



4.5.4 Effect of antiviral treatment on atherogenesis in ApoE-/- mice infected with MHV-68.

The enhancement in atheroma was similar in mice infected either intranasally or intraperitoneally when examined at 20 weeks after infection. Compared to ip infected mice, mice which received an antiviral drug, 2'-deoxy-5-ethyl-β-4'-thiouridine (EtdU), from the day of inoculation had a mean 67% reduction in their area of atheroma ($p>0.05$, figure 4.6).

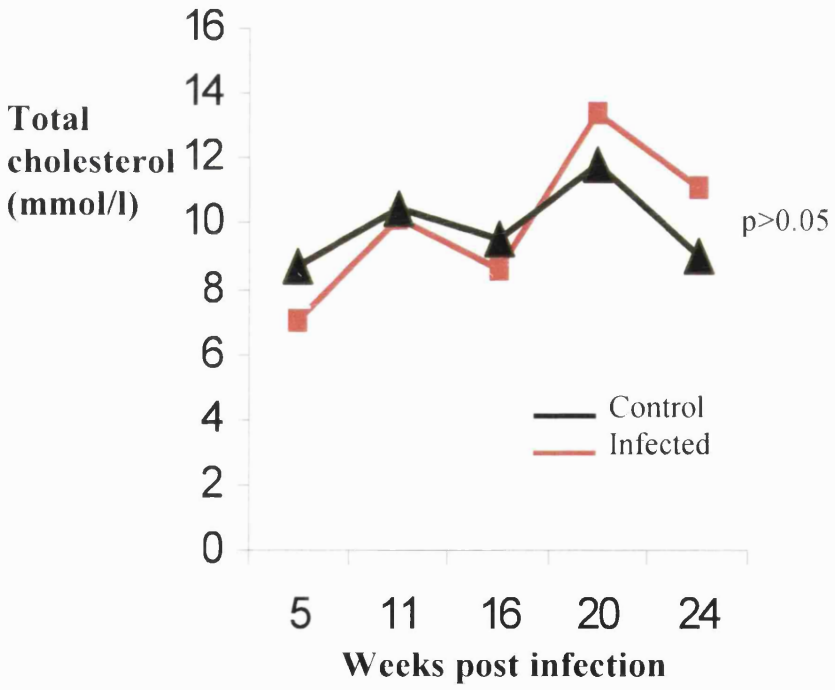
Figure 4.6. Reduction in MHV-68 accelerated atherosclerosis with the addition of the antiviral drug EdtU to the drinking water on the day of inoculation. Control (n=6) , infected in (n=7), infected ip (n=7), infected ip with the addition of EdtU (n=8). Comparing both infected groups together against the group with antiviral drug ($p=0.055$ by Students t-Test).



4.5.5 Total cholesterol levels in ApoE-/- mice infected with MHV-68

Total cholesterol levels were examined to see if MHV-68 infection increased cholesterol levels and thereby promoted atherogenesis. Total cholesterol levels were not significantly different at any time point during the course of the experiment and were appropriate for the ApoE-/- strain (figure 4.7).

Figure 4.7. Total cholesterol measurements in ApoE-/- mice. Showing control (n=27) and infected (n=28) mice.



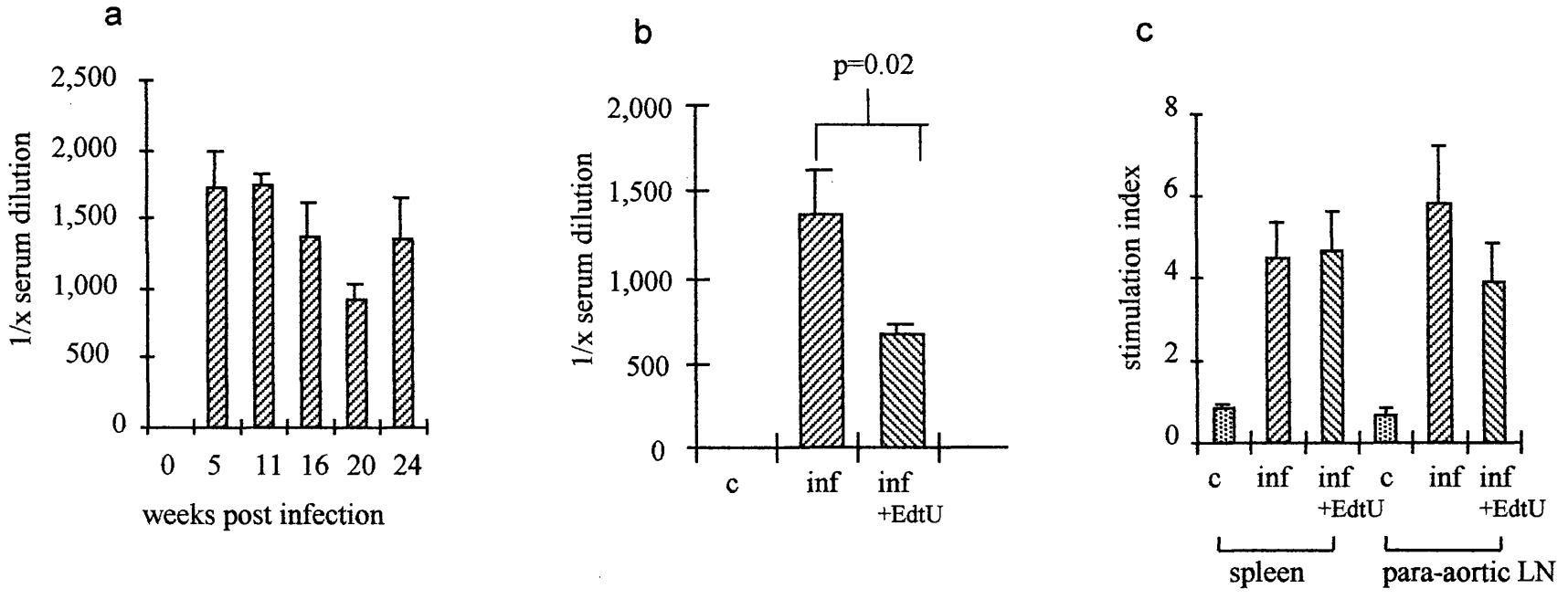
4.5.6 Antibody and T cell proliferation response in infected ApoE-/- mice

To ensure that all mice in the infected group had been exposed to MHV-68, and that no cross infection of the control group had taken place, two aspects of the immune response to MHV-68 were examined, namely the anti-MHV-68 antibody and T-cell responses. These studies were performed by Dr D. Alber.

There was no significant difference in antibody response as measured by ELISA in serum samples of mice culled at any time point after infection. The antibody response was significantly lower in the infected plus anti-viral mice as compared to the infected mice (figure 4.8 (a) and (b)).

The cell-mediated immune response was measured by *in vitro* T-cell proliferation assays in lymphocytes isolated from the spleens of infected mice. There was no significant difference between the proliferative response of splenocytes isolated from Experiment 4.1 (infected in, data not shown), and Experiment 4.2 (infected ip) and infected but treated with the antiviral drug EdtU (figure 4.8 (b)). Uninfected mice did not mount a MHV-68 specific immune response. Lymphocytes did not proliferate in response to control influenza antigen (data not shown). Figure 4.8.c.

Figure 4.8. MHV-68 specific immune response in ApoE-/- mice. (a) IgG anti-MHV-68 response for Experiment 4.1, where mice were culled at various time points post infection (n=4-7). (b) IgG response for Experiment 4.2 for control, infected ip and infected ip plus EdtU (n=6-9). (c) T-cell proliferation response of lymphocytes isolated from control and infected mice at 20 weeks post infection (n=3-5).



4.5.7 Detection of viral message in aortas of infected ApoE-/- mice and in sEnd.1 cells

In order to establish whether replicating MHV-68 was present in the aortas of infected mice, aortas were harvested 5 to 10 days post infection and total RNA isolated. Viral mRNA was detected by RT-PCR. Two bands corresponding to the mRNA for major capsid protein and DNA binding protein were detected in infected but not control aortas. No viral message was detected in whole blood-derived RNA samples at this time point. To establish whether MHV-68 could infect endothelial cells, a murine endothelial cell line (sEnd.1) was infected with MHV-68 at moi of 0.1 for 3 days. Viral mRNA was detected by RT-PCR in infected but not control cells (figure 4.9).

4.5.8 Detection of viral protein by immunohistochemistry

To determine whether MHV-68 could directly infect aortic tissue, an *in vitro* system was used. Dissected aortas from C57Bl mice were cultured for 24 hours and infected with 10^3 pfu MHV-68, or mock-infected with media. Aortas were harvested 3 days after infection. Virus protein was localised by immunohistochemistry predominantly on the luminal side of the aorta. No viral protein was detected in the mock infected aorta (figure 4.10 (a) and (b)).

To determine whether viral protein was detectable in endothelial cells, sEnd.1 cell monolayers were infected with moi 1 *in vitro*. Viral antigen was detected by immunohistochemistry in infected but not control cells (figure 4.10 (c) and (d)).

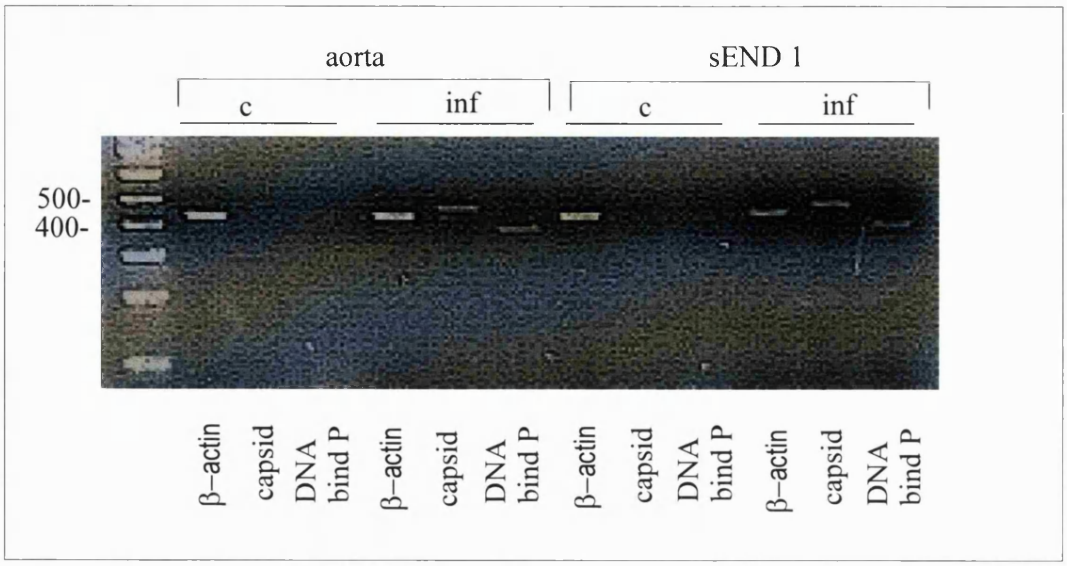


Figure 4.9. Detection of replicating MHV-68 by RT-PCR. Data from aortas of infected mice and infected sEnd.1 cells. Total RNA from a control mouse (c) and a mouse infected (inf) 5 days previously; total RNA from control (c) and infected (inf) sEnd.1 cells. Replicating MHV-68 was detected by the presence of DNA binding protein (DNA bind p) and viral major capsid protein (capsid). β-actin was used as positive control. The size of the relevant bands in base pairs is indicated by the 1-kb DNA ladder on the left.

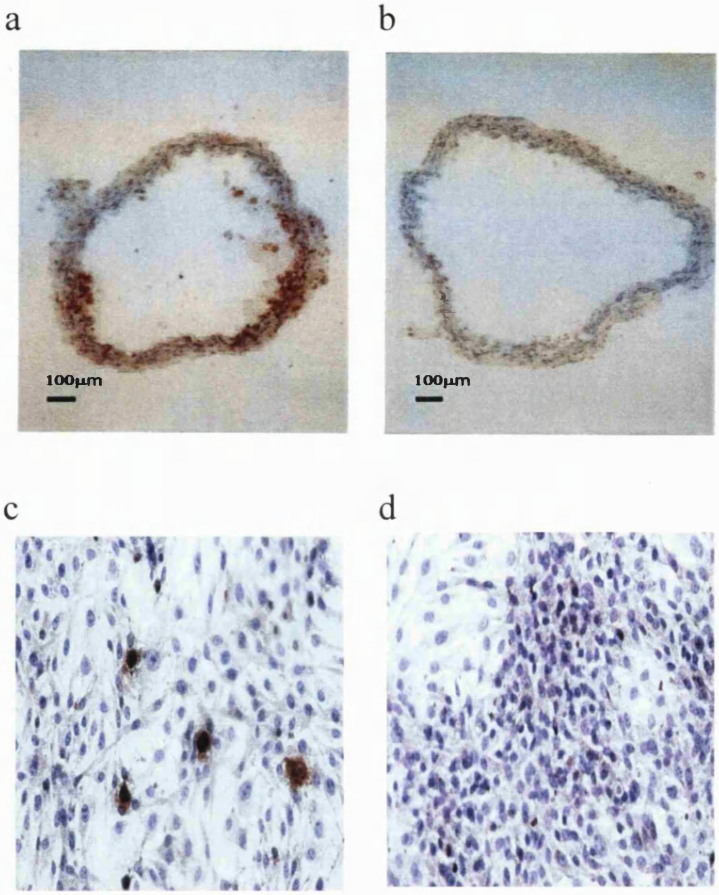


Figure 4.10. Localisation of MHV-68 antigen by immunohistochemistry.
Aortas from C57Bl mice were infected *in vitro* by MHV-68 (a) and mock-infected with PBS (b). Send.1 cells were similarly infected (c) or mock-infected with medium alone (d). Immunohistochemistry was carried out using rabbit anti-MHV-68 & HRP anti-rabbit antibodies, so infected cells appear brown.

4.6 DISCUSSION

The results presented here demonstrate that infection of ApoE-/- mice with MHV-68 accelerates atheroma formation. Previous work in the ApoE-/- mouse has shown that repeated inoculations with *Chlamydia pneumoniae* causes a slight increase in atheroma formation, and that the organism is found within the plaques (Moazed et al., 1999; Moazed et al., 1997). In the present study we show that a single inoculation with MHV-68 is sufficient to significantly enhance atherosclerosis, without fundamentally altering lesion histology. Furthermore, virus was detected within the aorta before atheroma development. Antiviral drug treatment failed to significantly reduce lesion development.

These findings are not consistent with MHV-68 being a mere 'innocent bystander' (Nicholson and Hajjar, 1998) in atherosclerosis, but suggest a direct role for MHV-68 in atherogenesis.

4.6.1 Evaluation of the ApoE-/- mouse-MHV-68 model of atherosclerosis

The quantification of atheroma by Oil Red-O is a well established method of assessing atherosclerosis in a murine model (Groot et al., 1996; Palinski et al., 1994; Tangirala et al., 1995). The acceleration in atheroma formation observed was present early on in the time course but became more marked at 20-24 weeks after inoculation, well after the peak viraemia of 5-7 days post inoculation (Dutia et al., 1997; Sarawar et al., 1997). Evidence of MHV-68 infection, by RT-PCR detection within the aortic wall but not in the circulating blood, was seen at day 5 but not at later stages after inoculation. Thus MHV-68 infection seems to have a continuing effect on the amount of atherogenesis long after evidence of an acute infection has subsided. Histological analysis of mature lesions in both control and infected

animals showed no change in cellular composition between the two groups. So MHV-68 infection appears to accelerate rather than fundamentally change the atheroma.

In these studies the route of infection did not significantly affect the acceleration of atherogenesis over a 20 week time period. Possible differing patterns of latency with in and ip infection might pootentially have affected atherogenesis, but this was not found to be the case (Simas and Efstathiou, 1998; Weck et al., 1999).

Antiviral drug treatment failed to significantly reduce lesion development. EdtU is a nucleoside analogue, which inhibits viral replication without preventing the establishment of virus latency (Stewart et al., 1998). This may explain why the drug failed to completely inhibit virus-accelerated atherosclerosis. Alternatively, the drug could have some unidentified anti-inflammatory property, or be inhibiting endogenous murine viruses, and so reduce atherosclerosis by this mechanism. Future experiments could explore this by including a group of uninfected mice that received the anti-viral drug alone. The effect of EdtU in reducing the acceleration in atherogenesis just failed to reach statistical significance. This may be because larger groups were required or because the experiment was terminated at 20 weeks and not 24 weeks post infection. Furthermore, the antiviral drug may have given more conclusive results if it had been given prior to rather than on the day of infection. In the ratCMV aortic allograft model prophylactic treatment with an antiviral drug reduced transplant-arteriosclerosis, whereas the same drug had little effect if used to treat the infection 7 days after inoculation (Lemstrom et al., 1994).

Despite these criticisms the MHV-68-ApoE-/- mouse model extends and supports the early work of Fabricant et al (Fabricant, 1985; Fabricant and Fabricant, 1999; Fabricant et al., 1978; Fabricant et al., 1983; Fabricant et al., 1981), whilst for the first time demonstrating that a herpesvirus can induce atherosclerosis in a mammalian model.

4.6.2 Comparison with other studies of infection and the ApoE-/- mouse

Previous studies have shown some effect of infection on atherogenesis in ApoE-/- mice. *Chlamydia pneumoniae* caused a slight increase in atheroma in these mice, and the organism was found within plaques (Moazed et al., 1999; Moazed et al., 1997). However, this model had several important differences; firstly mice developed a severe rather than subclinical pneumonia, secondly multiple inoculations were used, and thirdly the increase in atheroma appeared to decrease between 16 and 20 weeks post inoculation suggesting that it may have been a reversible phenomenon secondary to the acute systemic inflammation induced by the pneumonia. Thus the finding that a single dose of MHV-68 repeatedly and dependably caused an increase in total aortic atheroma in the ApoE-/- mouse without causing overt clinical disease is a significant advance in the field of infection and atherosclerosis.

4.6.3 Antibody response and serology

As discussed in section 1.5 the seroepidemiological evidence linking herpesvirus infection and atherosclerosis in man is inconclusive (Adam et al., 1987; Grattan et al., 1989; Ridker et al., 1998; Zhou et al., 1996). However all these studies relied on

a single serology test that therefore gave no indication of how recently the infection had been acquired or of how many times the virus had reactivated. This point is amply illustrated by the ApoE-/- MHV-68 model where the antibody response against MHV-68 is the same at 5 weeks post infection (when very little atheroma was visible) as it is a 24 weeks (when a large amount of atheroma was present). Thus single serology measurements give no accurate picture of enhanced atherogenesis secondary to herpesvirus infection, and seroepidemiological studies may therefore give false negative results.

4.6.4 Possible mechanisms for virus-induced accelerated atherosclerosis

MHV-68 may be enhancing atherosclerosis by various mechanisms, which can be broadly categorized into indirect and direct effects on the arterial wall. One indirect mechanism is by increasing total serum cholesterol. For example, a high cholesterol diet is known to accelerate atherosclerosis in the ApoE-/- mouse. Total serum cholesterol measurements were not significantly different between control and infected groups in Experiment 4.1. In the Marek's disease model, despite similar total serum cholesterol levels, changes in lipid metabolism were detected (Fabricant, 1985). The unaffected total cholesterol levels reported in this study do not therefore exclude a more subtle viral effect on lipid metabolism. However fundamental changes in total cholesterol does not appear to be the main mechanism whereby MHV-68 infection is enhancing atherosclerosis.

A direct mechanism whereby MHV-68 could be influencing atherosclerosis is by targeting the endothelial cell in the arterial wall. In Ross's 'response to injury'

model for atherosclerosis, noxious stimuli interact at the endothelial cell to promote atherosclerosis. C57Bl/6J mice infected with MHV-68 for 24 weeks but without receiving a high cholesterol diet did not develop macroscopic plaques. Therefore MHV-68 infection alone would seem not to be able to induce atherosclerosis, but only to accelerate it in the presence of a genetic predisposition or altered lipid metabolism.

It has been shown by RT-PCR that MHV-68 replication occurs within the aortic wall *in vivo* at day 5 after infection, and replication can occur in an endothelial cell line (sEnd-1 cells) *in vitro*. Therefore MHV-68 could be promoting atherosclerosis by infecting the endothelial cell monolayer and so causing an element of endothelial dysfunction, which in turn is known to promote atherogenesis. The question as to whether MHV-68 is causing endothelial dysfunction, and thereby enhancing atherogenesis, is more fully addressed in Chapter 5.

4.6.5 Conclusion

In summary the results of this study show that infection with a γ–herpesvirus MHV-68 accelerates atherosclerosis in the ApoE-/- mouse. It is not known whether this effect relates to general systemic inflammation promoting atherogenesis, or to a more specific effect of herpesvirus family viruses in general or γ–herpesviruses in particular acting locally on the vessel wall. Preliminary results suggest that the virus can infect endothelial cells both *in vitro* and *in vivo*, and may therefore possibly induce endothelial dysfunction and so promote atherogenesis in conjunction with other known risk factors for the disease.

Chapter 5: Exploring the mechanism underlying MHV-68 enhanced atherogenesis in the ApoE-/- mouse

5.1 INTRODUCTION

Infection with MHV-68 enhances atherosclerosis in the ApoE-/- mouse model. Moreover MHV-68 has the ability to infect the aorta *in vivo* and endothelial cells *in vitro* (see Chapter 4 and (Alber et al., 2000)). Therefore infection of the endothelium has the potential to cause endothelial dysfunction and may be the underlying mechanism whereby MHV-68 infection accelerates atherogenesis (Ross, 1993). There are several established measures of endothelial dysfunction (see section 1.11), and this Chapter focuses on two such measures, namely altered reactivity to certain vasoactive drugs and upregulation of adhesion molecule expression. The former comprises two components; impaired vasodilatation and enhanced vasoconstriction.

5.1.1 Impaired endothelium-dependent relaxation

The classical risk factors for atherosclerosis have been shown to cause impaired endothelium-dependent relaxation in both humans and animal models ((Busse and Fleming, 1996; Calver et al., 1992; Chowienczyk et al., 1992; Ohara et al., 1993; Taguchi et al., 1995) and see section 1.7 and 1.11). Two infections which promote atherosclerosis in animal models have also been shown to cause reduced vasodilation responses in arterial rings *in vitro*; that is ratCMV in the rat and *Chlamydia pneumoniae* in the ApoE-/- mouse (Eerdmans et al., 1996; Liuba et al., 2000). Thus

MHV-68 infection could be causing impaired endothelium-dependent relaxation in the ApoE-/- mouse model.

5.1.2 Enhanced vasoconstriction

Enhanced vasoconstrictor responses have been shown to be associated with atherosclerosis in man (Ludmer et al., 1986; Vita et al., 1990) and in two animal models of atherosclerosis - the hypercholesterolaemic swine (Fukumoto et al., 1997; Shimokawa et al., 1996) and rabbit (Verbeuren et al., 1986). Although there have been no reported cases of infections causing increased constrictor responses, it is nevertheless another potential indicator of endothelial dysfunction, albeit less widely reported than impaired vasodilatation.

5.1.3 Adhesion molecules

Expression of adhesion molecules on the endothelial cell surface mediates monocyte adherence to the site of endothelial injury, and is an early event in atherogenesis (Ross, 1993). In man adhesion molecule expression is associated with atheromatous plaques (Steinhoff et al., 1995), and levels of soluble adhesion molecules in plasma correlate with the presence of atheroma (Hwang et al., 1997; Oishi et al., 2000). In the ApoE-/- mouse adhesion molecules are upregulated by hypercholesterolaemia and shear stress (Iiyama et al., 1999; Nakashima et al., 1998), and are clearly involved in monocyte homing to lesions (Patel et al., 1998; Ramos et al., 1999). Genetic deficiency of these molecules in mice protects against atherosclerosis (Nageh et al., 1997). Both HSV-1 and HCMV have been shown to upregulate adhesion molecule expression in HUVECs (Burns et al., 1999; Etingin et al., 1991), and furthermore to induce the production of inflammatory cytokines from

activated T-cells (Waldman et al., 1998). Thus MHV-68 infection could potentially be enhancing atherosclerosis in the ApoE-/- mouse by influencing adhesion molecule expression on the endothelium. Adhesion molecule expression is controlled by transcription (Springer, 1990; Springer, 1994)and therefore Northern blots were used to look at mRNA levels in a murine endothelial cell line (sEnd.1).

5.2 HYPOTHESIS

i) That infection with a pro-atherogenic herpesvirus would cause impaired responses to endothelium-dependent vasodilators, and/or enhanced vasoconstrictor responses in aortic rings *in vitro*.

ii) That infection with a proatherogenic herpesvirus would cause an upregulation of adhesion molecule expression in a murine endothelial cell line.

5.3 METHODS

5.3.1 Animal husbandry and infection

Animal husbandry and infection was carried out as described in Methods section 2.3.

5.3.2 Vascular ring preparation for organ bath studies

Aortic rings were prepared as described in Methods section 2.7.

5.3.3 Drugs

Drugs used in organ baths were described in Methods section 2.7.2. Spermine was found to be more practical in terms of cleaning the organ baths compared to SNP and was therefore used as the endothelium independent dilator in all experiments after Experiment 5.2.

5.3.4 Virus culture and titration

Virus culture and titration were carried out as described in the Methods section 2.2 Virus was aliquoted into vials at the required concentration and stored at –80C. Aliquots were defrosted as required and the same stock used for each experiment. Defrosted aliquots were re-titrated. In addition experiments 5.3, 5.4 and 5.5 all used virus from the same working stock.

5.3.5 Northern blots using a murine endothelial cell line

Northern blots from a murine endothelial cell line (sEnd.1) were prepared as described in Methods section 2.6.

5.3.6 Statistics

Statistics used are described in Methods section 2.9.

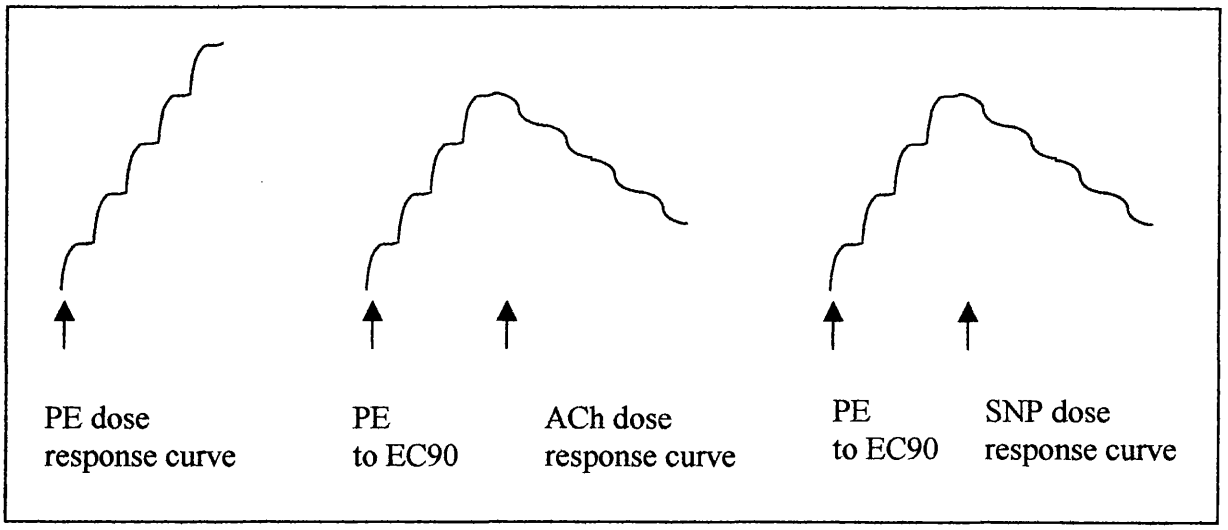
5.4 EXPERIMENTAL PROTOCOLS

5.4.1 Experiment 5.1. Do young C57Bl mice show impaired endothelium-dependent relaxation at peak viraemia?

Nineteen female 3-4 week old C57Bl mice were used (9 infected and 10 controls). Mice were culled on day 5 post infection. Aortic rings were exposed to cumulative concentrations of PE (10^{-9}–10^{-6}M), until a plateau was reached.. After re-equilibrating, aortic rings were then pre-contracted with cumulative doses of PE to achieve 90% of this maximal contraction (EC90). Once a stable contraction had been achieved a cumulative concentration response curve to ACh (10^{-9}–10^{-6}M) was performed. After re-equilibrating once more, the vessel was again pre-contracted to an EC90, and a concentration response curve to sodium nitroprusside (an

endothelium independent vasodilator) was similarly performed (10^{-10}–10^{-7}M). A schematic outline of the protocol used is illustrated below.

Protocol for Experiment 5.1



5.4.2 Experiment 5.2. Do older mice show impaired endothelium-dependent responses if infected at a young age or compared to C57Bl mice?

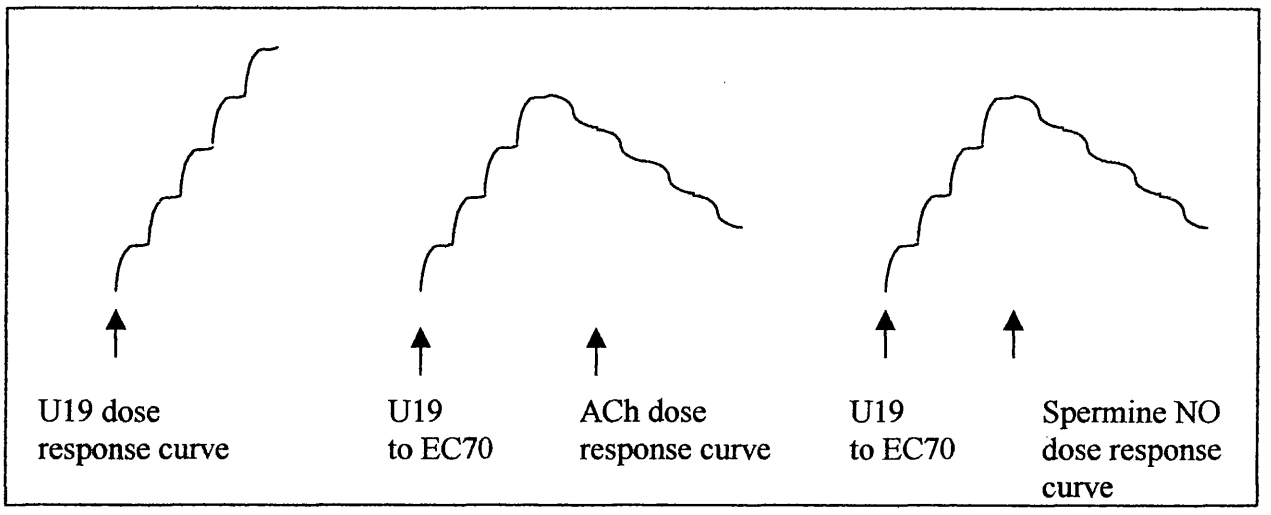
Three groups of 8 month old mice of both sexes were used: C57Bl mice (n=9); ApoE-/- mice (n=7); and ApoE-/- mice infected at 4 weeks old (n=11), (Appendix A4). The same protocol as Experiment 5.1 was used although four of the C57Bl mice, six of the ApoE-/- controls and four of the ApoE-/- infected mice received spermine-NO as the endothelium independent vasodilator; in the remaining animals sodium nitroprusside was used.

5.4.3 Experiment 5.3. Do young ApoE-/- mice show impaired endothelium-dependent relaxation or enhanced vasoconstriction at peak viraemia?

Eleven female 7-8 week old ApoE-/- mice were used: 6 infected and 5 controls. Mice were culled on day 5 post infection. The protocol used was the same as that

used above, but in addition a further ring from each animal was exposed to cumulative doses of U46619 (U19), a thromboxane mimetic. After re-equilibration this vessel was pre-contracted to an EC70 with U19 and dose response curves obtained for both ACh and spermine-NO as illustrated below.

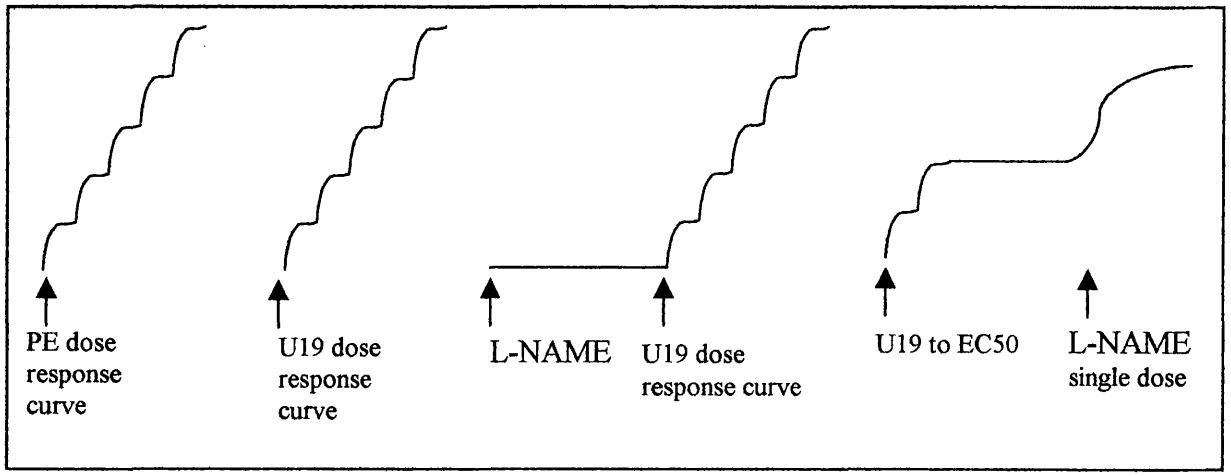
Protocol for additional ring in Experiment 5.3.



5.4.4 Experiment 5.4. Do young C57Bl mice show enhanced vasoconstriction at peak viraemia?

Fourteen female 6-8 week old C57Bl mice were used, n=7 infected and n=7 controls. Mice were culled 5 days post infection. Four rings from each thoracic aorta were obtained. One ring was exposed to cumulative doses of PE, one to U19 and one to U19 after 30 minutes incubation with L-NAME ($3x10^{-6}M$). The fourth ring was precontracted to an EC50 with U19 and then the contraction to a single dose of L-NAME ($10^{-4}M$) recorded (as illustrated over the page).

Protocol for Experiment 5.4, using 4 separate rings from each animal.



5.4.5 Experiment 5.5. Does infection with MHV-68 increase adhesion molecule RNA in sEnd.1 cells?

SEnd.1 cells were infected with moi 10 of MHV-68 and harvested at various time points - namely at 30 minutes and then at 1, 2, 3, 4, 5 and 6 hours after infection. An moi 10 was chosen as suitable, as it would probably result in the majority of the cells being infected. Identical wells of cells treated for 2 hours with 10μg/ml LPS were used as the positive control. At each time point post-infection cells were compared to identical wells of cells treated with a change in media only. Cells were washed and resuspended in TRIZOL. RNA was extracted and run on a 1% denaturing gel. The gel was blotted overnight on to Hybond N+ membrane and covalently linked under a UV Stratalinker. The blots were hybridised with probes for two adhesion molecules ICAM-1 and P-selectin. Each blot was used three times. One blot was hybridised with a probe against mRNA for the viral DNA binding protein. The control probe for all blots was the S18 ribosomal subunit. Results were expressed as a percentage of the adhesion molecule expression at 30 minutes post infection.

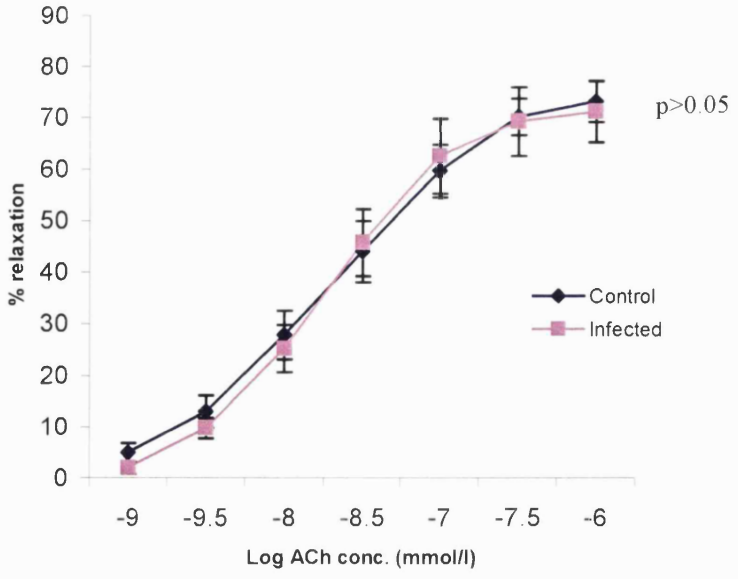
5.5 RESULTS

5.5.1 Experiment 5.1. Effect of infection on endothelium-dependent relaxation on day 5 post-infection in C57Bl mice

There was no difference in the concentration response curves to acetylcholine (ACh, endothelium-dependent vasodilator) in C57Bl mice at 5 days post infection with MHV-68 (figure 5.1).

No significant differences were found in the contractile responses to phenylephrine (PE) (Appendix A2 (i)), or to the vasodilator response to sodium nitroprusside (SNP) (Appendix A1 (i))

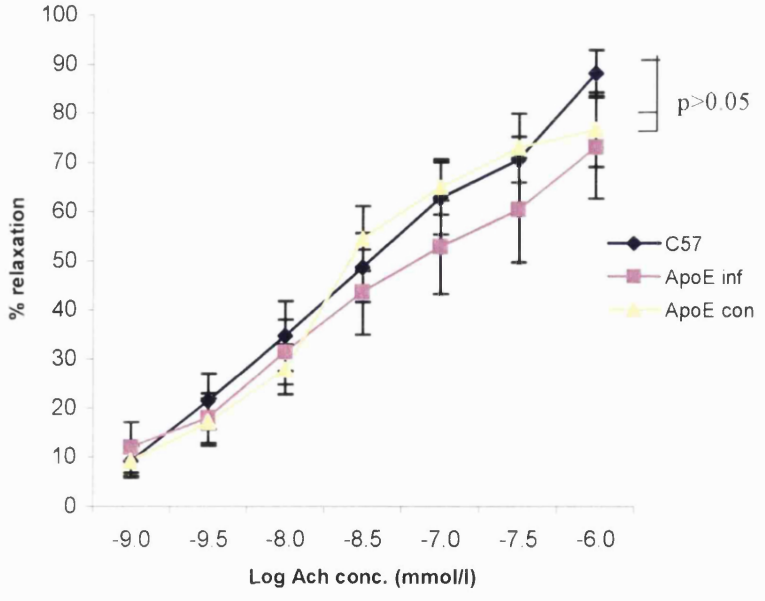
Figure 5.1. Concentration response curves for ACh. For C57Bl mice 5 days after being infected with 10^5 pfu MHV-68 (n=9) compared to controls (n=10). Non-significant ($p>0.05$) by 2 way ANOVA, and by t-Test on EC50s and t-Test on maximum relaxation achieved.



5.5.2 Experiment 5.2. Effect of mouse strain and infection on endothelium dependent relaxation in older mice

There was no significant difference between the concentration response curves to ACh (endothelium-dependent vasodilator) between older C57Bl and ApoE-/- mice, nor between older ApoE-/- infected with MHV-68 at a young age and uninfected control ApoE-/- mice (figure 5.2). There was no difference either in the contractile responses to phenylephrine (Appendix A2 (ii)), or in the concentration dose response curves to an endothelium-independent vasodilator (Appendix A1 (ii) and (iii)).

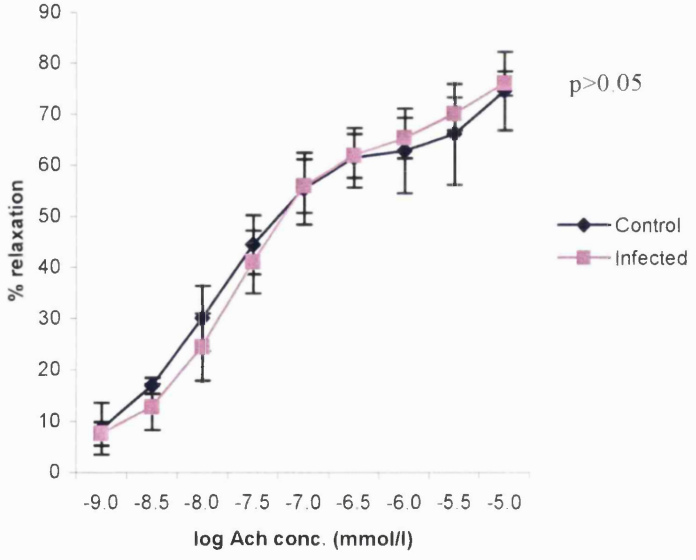
Figure 5.2. Concentration response curves to ACh in older mice. 20 rings from C57Bl mice (n=9), 13 rings from ApoE-/- uninfected (control) mice (n=7) and 21 rings from ApoE-/- mice infected with MHV-68 at 4 weeks old (n=11). Non-significant (p>0.05) by 2-way ANOVA, and by t-Tests on EC50s and maximum relaxation achieved.



5.5.3 Experiment 5.3. Effect of infection on vascular responses in young ApoE-deficient mice day 5 post infection

There was no significant difference in concentration response curves to ACh in young ApoE-/- mice on day 5 post infection (figure 5.3), nor was there any significant difference to spermine-NO (Appendix A1 (iv)).

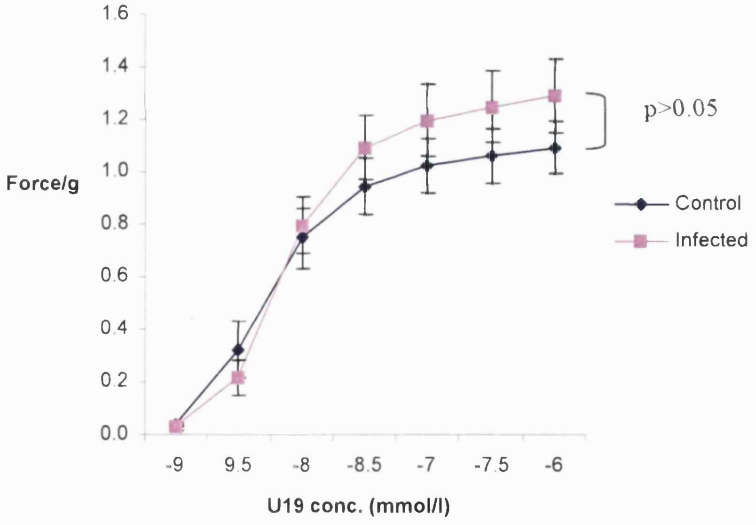
Figure 5.3. Concentration response curves to ACh. For young ApoE-/- mice (n=5) at 5 days post infection with MHV-68 compared to control ApoE-/- mice (n=5). Non-significant ($p>0.05$) by 2-way ANOVA, and by t-Test on EC50s and maximum relaxation achieved.

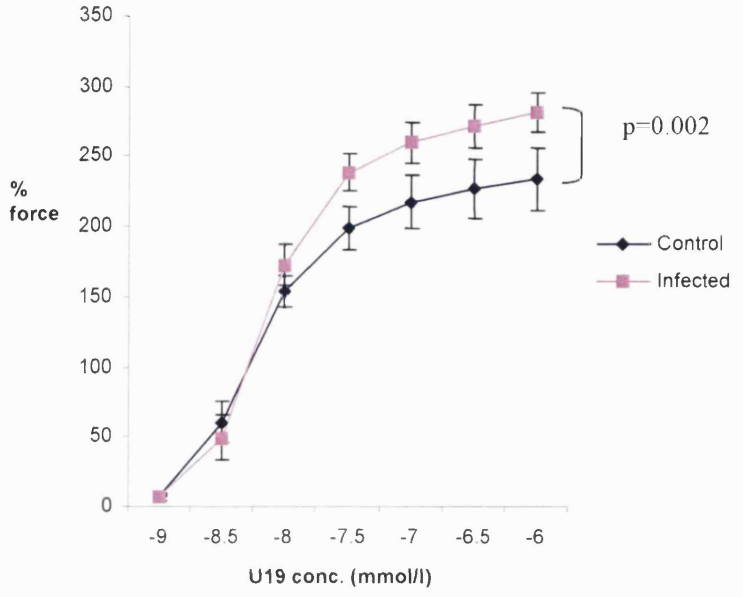


No significant difference in contractile responses to 50mM of potassium were seen between the two groups (control=0.45g+/-0.05, infected =0.45g+-0.06). An increase in contractile responses to phenylephrine (PE) was seen although this did not reach significance (Appendix A2 (iii)). There was a trend, albeit still insignificant, by 2-

way ANOVA, for contractile responses to U19 in terms of force generated to be enhanced in the infected group. This reached statistical significance when the contractile responses were normalised and expressed as a percentage of the potassium responses (figure 5.4).

Figure 5.4. Concentration response curves to U19 in young ApoE-/- mice. ApoE -/- infected with MHV-68 (n=5), compared to control ApoE-/- mice (n=5). Data are expressed as (i) absolute force generated, and (ii) percentage of the force generated in response to 50mM potassium chloride. In (i), non-significant ($p>0.05$) by 2-way ANOVA, and by t-Test for EC50s and maximum force generated. In (ii), significant ($p=0.002$) by 2-way ANOVA, and non-significant ($p>0.05$) by t-Test on EC50s and maximum force generated.



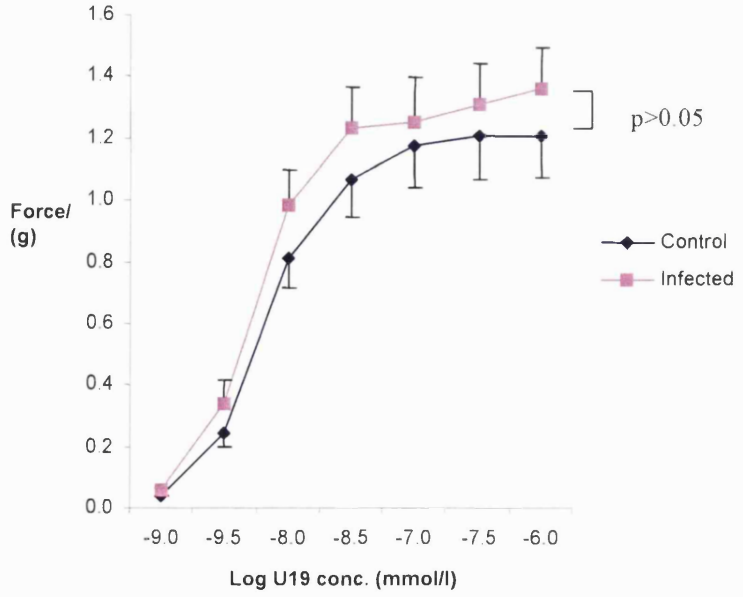


5.5.4 Experiment 5.4-Effect of infection on vascular constriction in young C57Bl mice

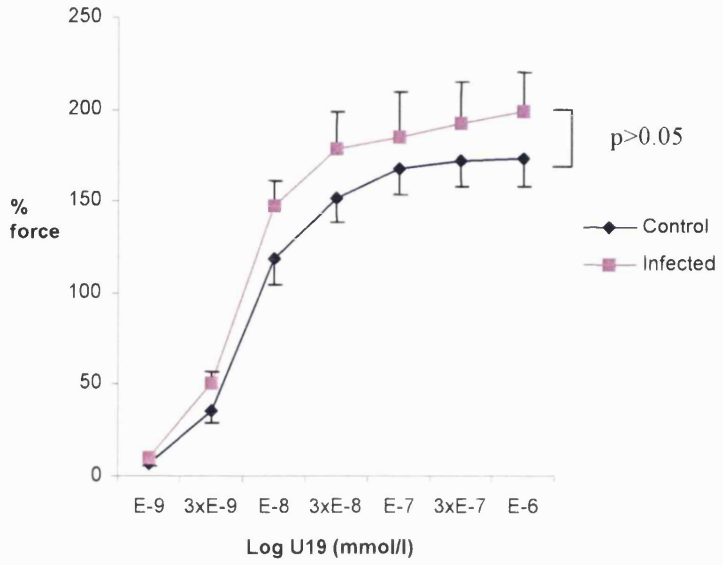
No significant differences were seen between infected and control mice in terms of response to a second dose of 50mM potassium chloride (controls=0.65+/- 0.08g (n=7), infected=0.60+/- 0.08g (n=7)). There was no significant difference in contractile responses to cumulative doses of PE (Appendix A2 (iv)) or to U19; however, in the case of U19 normalised to the potassium response there was a tendency for there to be an increase in contractile response in the infected group (figure 5.5). There was no difference in U19 curves performed in the presence of L-NAME or in the tension produced to a single dose of 10^{-4}M L-NAME after partial contraction with U19 (Appendix A3 (i)-(ii)).

Figure 5.5. Concentration response curves to U19 for young C57Bl mice. C57Bl mice infected with MHV-68 (n=7) are compared to control C57Bl mice (n=6). Data are expressed as (i) absolute force generated and (ii) percentage of the force generated in response to 50mM potassium chloride. Non-significant ($p>0.05$) by 2-way ANOVA, and by t-Test on EC50s and maximum force generated.

(i)



(ii)



5.5.5 Results of RT-PCR

Clear, distinct bands at 300bp were obtained by RT-PCR for ICAM-1, VCAM, E-selectin and P-selectin. All probes had the correct sequence when tested. (Appendix A5).

5.5.6 Experiment 5.5. MHV-68 infection does not cause an upregulation in mRNA for ICAM-1 or P-selectin

Successful Northern blots were made for all four adhesion molecules under investigation (figure 5.6). Message for viral DNA binding protein became apparent at 3 hours post infection (figure 5.6(b), suggesting that viral replication was taking place within the chosen time frame. Successful experiments were repeated three times for ICAM-1 and P-selectin only. No significant upregulation was seen for either adhesion molecule (figure 5.7).

Figure 5.6. Experiment 5.5-specimen Northern blots. These show (a) mRNA for ICAM-1, E-selectin and ribosomal subunit S18 (control) as recorded on photographic film and (b) mRNA for viral DNA binding protein and S18 as recorded by a phosphoimager. (For clarity, only infected lanes are shown).

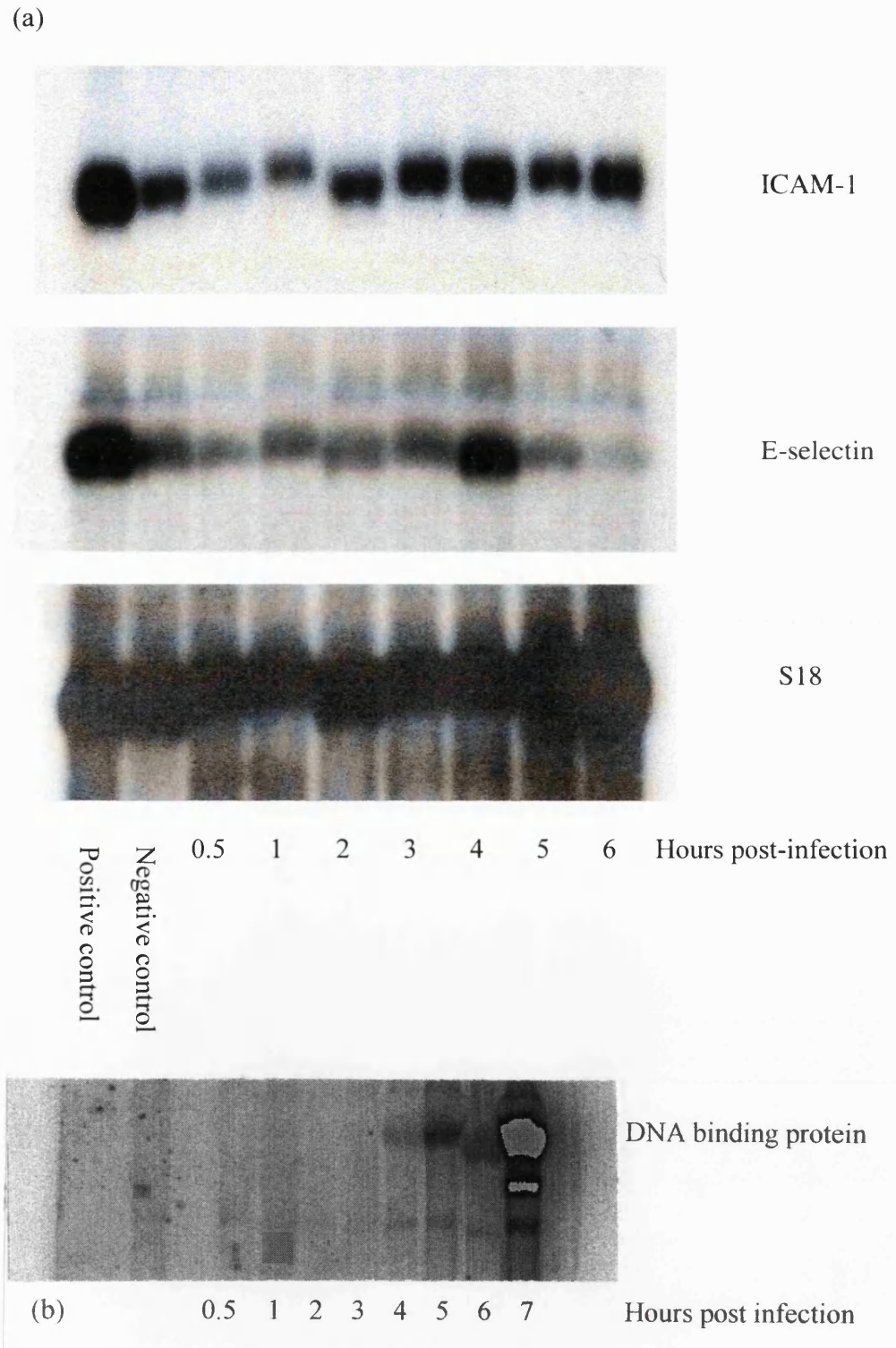
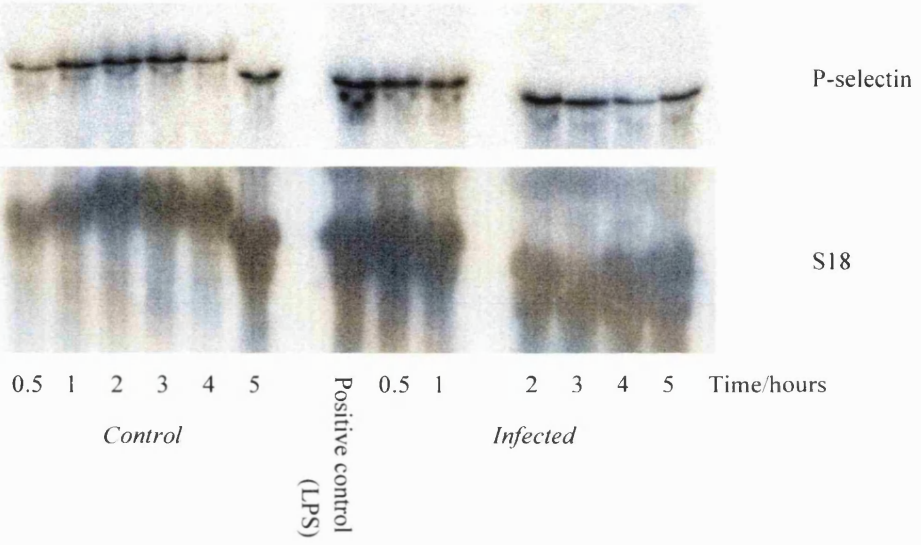
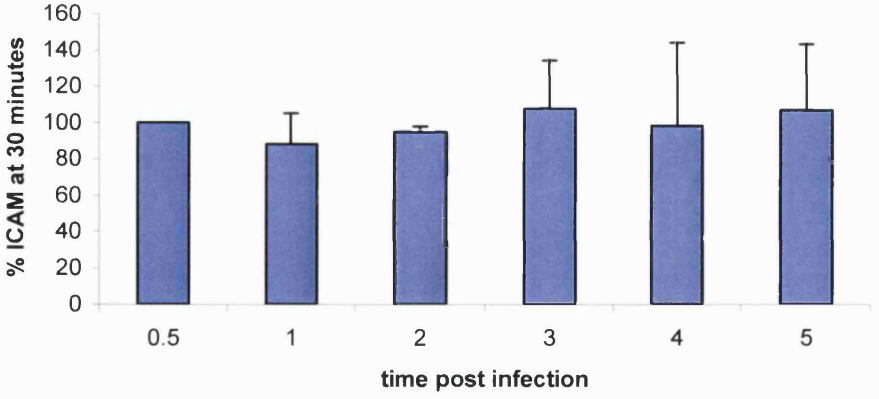
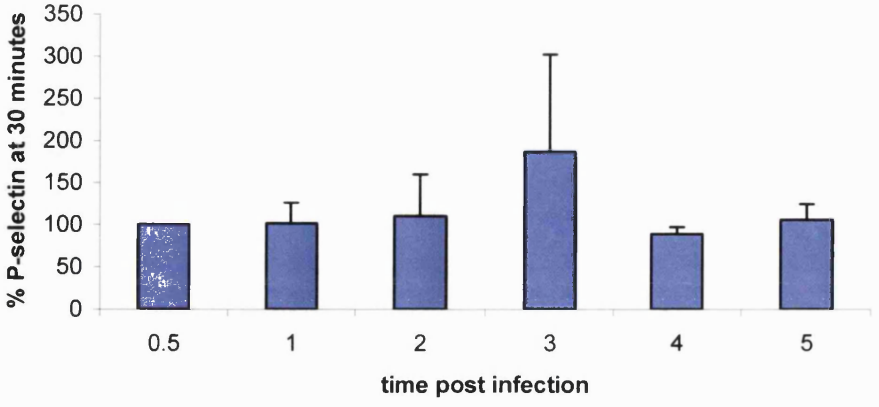


Figure 5.7: Experiment 5.5. (a) A specimen Northern blot showing mRNA expression for P-selectin in sEnd.1 cells with MHV-68 infection over time compared to identical wells of cells that received a change in media only with their respective S18 controls as seen on a phosphoimager. No significant difference was found when the experiment was repeated (n=3) for (b) ICAM-1 or (c) P-selectin.







5.6 DISCUSSION

The studies presented in this Chapter indicate that impaired endothelium-dependent relaxation does not appear to be the mechanism underlying MHV-68 enhanced atherogenesis in this murine model. MHV-68 infection does not cause impaired endothelium-dependent relaxation in either the young C57Bl or ApoE-/- mouse at a time when the virus is known to be replicating within the aortic wall. In older mice there was no difference in endothelium-dependent relaxation between control ApoE-/- mice and ApoE-/- who had been infected several months previously. Moreover impaired endothelium-dependent relaxation does not correlate with atherogenesis per se in the mouse as there was no difference in endothelium-dependent responses between older C57Bl and ApoE-/- mice, despite there being a marked difference in the amount of aortic atheroma present.

These studies do however show an association between MHV-68 infection and enhanced contractile responses to U19, a thromboxane mimetic. Young ApoE-/- mice had an increased contractile response to U19 on day 5 post infection which was not seen in the infected C57Bl mice. This enhanced contractile response is most likely to be due to some characteristic of the ApoE-/- mouse not shared by its background strain, possibly high serum cholesterol levels. Further studies are needed to explore the mechanisms underlying this enhanced contractile response.

There was no upregulation in the adhesion molecules ICAM-1 or P-selectin in the murine endothelial cell line sEnd.1 with MHV-68 infection.

5.6.1 Lack of impaired endothelium-dependent relaxation in ApoE-/- compared to C57Bl mice

The finding that no endothelium-dependent relaxation can be demonstrated in the ApoE-/- compared to the C57Bl mouse is unexpected given the marked atherogensis that occurs in the ApoE-/- compared to the C57Bl mouse and the substantial body of evidence linking impaired endothelial dependent relaxation and atherogenesis (see section 1.11.1). However this finding is supported by other authors who similarly report no difference in endothelium-dependent responses between ApoE-/- and C57Bl mice on a normal diet (Bonthu et al., 1997; Deckert et al., 1999; Kauser et al., 2000). Thus Deckert et al found no appreciable differences between C57Bl and ApoE-/- female mice aged 29 weeks in their responses to NE, ACh or SNP (Deckert et al., 1999). Bonthu et al found no significant differences between the vascular responses to U19, ACh (endothelium-dependent, receptor-dependent vasodilator), SNP and A23187 (calcium ionophore, receptor-independent, endothelium-dependent vasodilator), in mice of both sexes, aged 16 to 19 weeks (Bonthu et al., 1997). This finding was confirmed in male mice aged 35 weeks (Kauser et al., 2000). There is only one report of impaired endothelium-dependent responses in ApoE-/- mice compared to C57Bl, and that was using ADP and A23187 (Williams et al., 2000). This directly contradicts the findings of others (Bonthu et al., 1997; Deckert et al., 1999) and the reason for this is unclear but may lie in the different sexes used in the various studies.

There have been only two reports of impaired endothelium-dependent responses in the ApoE-/- compared to the C57Bl mouse. In the first instance both the ApoE-/-

and C57Bl mouse had been on a high cholesterol diet (Barton et al., 1998; Deckert et al., 1999). In the second case impaired responses where seen in the double ApoE-/- LDL-/- receptor knock out mouse compared to the C57Bl mouse on a normal diet (Bonthu et al., 1997). Therefore it appears that impaired vasodilation responses are either absent or difficult to demonstrate in murine models of atherosclerosis unless quite marked differences in mouse metabolism occur.

5.6.2 No evidence of impaired endothelium dependent relaxation in mice infected with MHV-68

As endothelium-dependent vasodilation has **not** been clearly linked to murine atherogenesis in previous studies, it is perhaps not surprising that a pro-atherogenic infection of MHV-68 does not cause impaired endothelium-dependent relaxation. However two animal models of infection-enhanced atherosclerosis have been reported as causing such reduced responses, they are the rat infected with ratCMV and the ApoE-/- mouse infected with *Chlamydia pneumoniae* (Eerdmans et al., 1996; Liuba et al., 2000).

Although ratCMV causes endothelium-dependent relaxation in the immunosuppressed rat, this model is different in several important respects to the the mouse. Firstly rats become extremely unwell in the ratCMV model; secondly the reduced vascular responses are found in resistance not conductance vessels; thirdly the responses appear not to be NO-mediated (Eerdmans et al., 1996). In contrast, in mouse aorta endothelium-dependent responses have been found to be mediated

mainly by NO (Barton et al., 1998; Bonthu et al., 1997; Kauser et al., 2000). Thus the rat model is very unlike the ApoE-/-/MHV-68 model.

The comparison with *C. pneumoniae* in the ApoE-/- mouse, in the study of Liuba et al, is more intriguing. ApoE -/- mice were not unwell at the time of this study, the dose has been shown to promote atherogenesis in this model (Liuba et al., 2000) and infected mice showed impaired endothelium-dependent responses to methacholine which was inhibited by L-NAME and therefore NO-mediated. The reason for the discrepancy between the *C.pneumoniae* and MHV-68-ApoE-/- models is not clear. However, it should be noted that whereas MHV-68 is a natural infection of mice and requires a single inoculation *C.pneumoniae* is not, and therefore three inoculations were necessary before a reduction in endothelium-dependent responses were seen.

5.6.3 Infection with MHV-68 causes enhanced contractile responses in the ApoE-/- mouse

Infection with MHV-68 caused an enhanced response to the constrictor U19, a thromboxane mimetic in ApoE-/- but not C57Bl mice. Thus there appears to be some interaction between a characteristic of the ApoE-/- not shared by the C57Bl mouse and MHV-68 infection. This could be the spontaneous hypercholesterolaemia in the ApoE-/- mouse, but this remains to be proven. Further experiments would be needed to test this directly.

The underlying mechanism whereby MHV-68 infection could be increasing contractile responses is unclear. It could be endothelium-dependent or -independent, NO-mediated or mediated by other vasoactive substances and further studies would be required to test these possibilities. As well as the stimulated NO release with

ACh, there is a basal NO release that can be quantified by the magnitude of the contractile response to a competitive inhibitor of NOS (e.g. L-NAME) in partially pre-constricted vessels (Rubanyi et al., 1997). The basal NO-mediated response has been found to be significantly lower in ApoE-/- mice compared to C57Bl mice on a normal diet (Kauser et al., 2000). Thus, to determine whether the enhanced constrictor response is due to a decreased basal release of NO it would be necessary to repeat Experiment 5.4 in ApoE-/- mice.

In addition to NO, other vasoactive substances are important in determining arterial calibre (see figure 1.5). In the *Chlamydia*-ApoE-/- model there is some evidence for vasoactive prostanoids being important in infected but not control animals. Thus L-NAME inhibited methacholine induced vasodilatation in both control and infected animals whereas incubation with diclofenac as well as L-NAME caused a partial restoration in methacholine-induced relaxation in infected but not control aortic rings (Liuba et al., 2000). Diclofenac is an inhibitor of both constitutive COX-1 and inducible COX-2 enzymes. The diclofenac-induced improvement in relaxation in the *Chlamydia*-ApoE-/- model could be explained by the inhibition of production of vasoactive prostanoids such as thromboxane A2 by infection. The finding reported in Experiment 5.3 - that U19 causes enhanced responses in infected but not control animals - would seem to suggest that a greater sensitivity to thromboxane analogues might underlie the increased contractile responses. However the contractile responses to PE also appeared to be increased and may have only not reached significance becauses of their greater variability compared to U19 responses. Therefore a greater senstivity to all/several vasoconstrictors may exist in the MHV-

68 infected mouse aorta. Further studies with other vasoconstrictors would be needed to test this theory directly.

Alternatively, the increased contractile response to U19 with MHV-68 infection could be mediated by endothelium-independent changes in the underlying smooth muscle. Such a mechanism is seen in the porcine/inflammatory cytokine model. Swine develop coronary lesions after a high cholesterol diet and chronic treatment with pro-inflammatory cytokines. Porcine coronaries treated with interleukin-1β show an increase in intimal thickness and increased contraction to serotonin and histamine (Fukumoto et al., 1997; Shimokawa et al., 1996). *In vivo* studies in this model using inhibitors of NO synthase revealed that a reduction in basal NO could only account partly for the increased constriction. The remaining underlying mechanism would therefore have to be some aspect of smooth muscle constriction. One such aspect is the phosphorylation of myosin light chain (MLC) kinase, an increase in which would lead to enhanced constriction. Studies both *in vivo* and *in vitro* in the porcine model have demonstrated a correlation between serotonin-induced hyperconstriction and MLC kinase phosphorylation (Katsumata et al., 1997). A similar association between enhanced serotoninergic responses and MLC kinase phosphorylation has been observed in Watannabe heritable hyperlipidaemic rabbits (Seto et al., 1993). Thus further studies are needed in our ApoE-/- murine model to determine whether increased MLC kinase phosphorylation could account for the findings with MHV-68 in the ApoE-/- mouse (see figure 1.5).

The animal models in which enhanced constriction responses are seen as a prelude to atheroma development all have one another risk factor in addition to

hypercholesterolaemia. These are chronic inflammatory cytokines in the porcine model, mechanical injury in the rabbit, and MHV-68 infection in the ApoE-/- mouse. Therefore it is not inconceivable that the C57Bl mouse would also show enhanced constriction if given both MHV-68 infection and a high cholesterol diet.

5.6.4 No evidence for adhesion molecule mRNA upregulation in sEnd.1 cells

Previous studies have examined adhesion molecule expression in endothelial cells and their methods and results are summarised in Table 5.4. Using either Northern blots to detect mRNA or flow cytometric analysis to detect actual surface protein expression it can be seen that adhesion molecule expression is an early event in endothelial cells infected *in vitro* with a range of organisms. Although no significant upregulation in message was seen in these studies this does not exclude an upregulation in protein expression, and the experiment needs to be repeated using flow cytometric analysis.

There are several reasons why an upregulation in adhesion molecule mRNA was not seen in Experiment 5.5. Firstly, the time course may have been too short. Messenger RNA for viral DNA binding protein is only present at 3 hours post infection and is still dramatically increasing at 6 hours. Therefore the experiments need to be repeated with a longer time course to see if adhesion molecule expression is upregulated at a later stage. Against this argument is the finding of Burns et al (Burns et al., 1999) that HCMV infection causes an upregulation of mRNA for ICAM-1 which is maximal at 2 hours post infection with an moi only 0.1 (1% of that

used in Experiment 5.5). This upregulation was mediated by immediate early (IE) viral gene products transactivating the ICAM-1 promoter. Transcription of the IE genes occurs immediately on viral entry into the cell and therefore precedes DNA binding protein transcription. If therefore MHV-68 used the same mechanism, an upregulation of adhesion molecule mRNA expression would have been expected within the time course of Experiment 5.5.

A second reason for there being no significant upregulation in adhesion molecule mRNA is the possibility that the constitutive adhesion molecule expression in the endothelioma cell line used was already high pre-infection. In the reported studies using Northern blots to assess adhesion molecule mRNA the constitutive level of control mRNA was very low. Thus in the study of Burns et al the negative control (mock infected HUVECs) was 100 times less than the ICAM-1 mRNA induced by the positive control (TNFα-treated HUVECs after 4 hours) (Burns et al., 1999); similarly, in Krull et al the control HUVECs had adhesion molecule mRNA levels between 10-100 times less than the positive control (IL-1β treated HUVECs after 4 hours) (Krull et al., 1999). As the positive control in Experiment 5.5 was only 4-5 times the negative control, this therefore could also explain why no significant upregulation was seen.

In summary impaired endothelium-dependent vasodilation appears not to be the mechanism underlying MHV-68-induced enhancement of atherogenesis in the mouse. However such impaired responses do not seem to occur in the mouse model on a normal diet. MHV-68 infection did increase contractile responses to U19 and

possibly PE. Whilst the underlying mechanism for this is unclear, on the basis of findings in other models of infection and atheroma it could be due either to a reduction in basal NO release from the endothelium, or to changes in other endothelium derived vasoactive substances, or to smooth muscle cell changes secondary to infection/inflammation. The finding that adhesion molecules are not upregulated in a murine cell line does not exclude this still being a potential mechanism in the mouse.

Table 5.4. Comparison of *in vitro* studies on adhesion molecule expression in endothelial cells.

Adhesion molecule	Cell	Inducing agent	Dose/moi	Detection technique	Time to maximum upregulation (hours)
ICAM-1	HUVEC	HCMV	0.1	Northern blot (Burns et al., 1999)	2
E-selectin *ICAM-1* *VCAM*	HUVEC	HSP60	5μg/ml	FACS (Kol et al., 1999)	6 24
ICAM-1 *VCAM* *E-selectin*	HUVEC	*Chlamydia pneumoniae*	$6.5x10^4$ ifu/ml	Northern blot (Krull et al., 1999)	2
P & E-selectins	eEnd.2 sEnd.1 tEnd.1	TNFα	100U/ml	Northern blot Immunoblot (Weller et al., 1992)	2 3
ICAM-1 *VCAM* *E-selectin*	HUVEC	WNV	5	FACS (Shen et al., 1997)	2-4
ICAM *VCAM* *E-selectin*	HDMEC	HSV-1	2.5	FACS (Kim et al., 2000)	4
P-selectin	HUVEC	HSV-1	1	FACS (Etingin et al., 1991)	18-24

HUVEC = human umbilical vein endothelial cell, HDMEC = human dermal microvascular endothelial cells. FACS = flow cytometric analysis, HSP60 = Chlamydial & human heat shock protein 60, WNV = West Nile Virus, a flavivirus, TNFα = tumour necrosis factor alpha, eEnd.2 = embryonic endothelioma, sEnd.1 = skin endothelioma, tEnd.1 = thymus endothelioma murine cell lines.

Chapter 6: Exploring the interaction of diet and herpesvirus infection in the murine/MHV-68 model

6.1 INTRODUCTION

MHV-68 infection increases atherogenesis in the ApoE-/- mouse (with spontaneous hyperlipidaemia) but not in the background C57Bl strain (with low serum cholesterol levels)(see Chapter 4). This would suggest that both MHV-68 infection and the presence of hyperlipidaemia are necessary for atheroma to develop in the mouse (Alber et al., 2000). Therefore the question arises as to whether MHV-68 infection could accelerate atherosclerosis in the diet-induced atherosclerosis C57Bl model.

Previous studies have examined the effect of an atherogenic or a diabetogenic diet on atheroma formation in the C57Bl mouse (see sections 1.10.1 and 1.10.4).

An atherogenic diet contains 1.25% total cholesterol, compared to a normal chow diet content of 0.04% cholesterol. Atheroma covering about 1% of the total aortic area was found to develop in C57Bl mice after 14 weeks on such an atherogenic diet, with female mice tending to have larger lesions than male mice, although this did not reach significance (Paigen et al., 1987). Paigen et al developed a more sensitive method for quantifying the small amount of atheroma present, which was mostly at the aortic root. Cross-sections were taken at the aortic root and a mean lesion size was determined from the average of several sections each 80 micrometers apart (Paigen et al., 1987). Subsequent authors have used a similar method and a wide variation in lesion sizes have been reported for various durations of diet up to 1 year

(Boisvert and Curtiss, 1999; Huber et al., 1999; Munday et al., 1998; Schreyer et al., 1998; Tangirala et al., 1995). Whether these differences are accounted for by variations in the methods of image analysis or by the fact that animals were kept in non-sterile conditions with different ambient pathogens remains unclear. Nevertheless the C57Bl mouse on atherogenic diet is an established model for diet-induced atherosclerosis.

A diabetogenic diet contains a high proportion (37%) of sucrose as well as 35% saturated fat (Schreyer et al., 1998). C57Bl mice on such a diet develop hyperinsulinaemia with impaired glucose tolerance, a picture of Type 2 diabetes (Surwit et al., 1995; Surwit et al., 1988). Atheroma of the aortic root is also a feature although this is less marked compared to mice on the atherogenic diet (Schreyer et al., 1998). Either diet therefore produces an animal model of atherogenesis in which the interaction of MHV-68 infection and conventional risk factors can be explored.

6.2 HYPOTHESIS

This Chapter set out to test the hypothesis that infection with MHV-68 would accelerate atherogenesis in the C57Bl mouse on an atherogenic or diabetogenic diet. In addition it sought to establish whether this enhancement was reduced by the antiviral drug 2'-deoxy-5-ethyl-b-4'-thioridine (EdtU).

6.3 METHODS

6.3.1 Animal experimentation

Animal husbandry and infection, diet and antiviral drug administration were carried out as described in Methods section 2.3.

6.3.2 Virus growth and titration was performed as described in Methods section 2.2.

6.3.4 Image analysis was carried out as described in Methods section 2.4. All images were processed by an operator blinded to the treatment groups. As only a small amount of atheroma was present compared to Chapter 4, during image processing (section 2.4.4) the individual plaques were not drawn around but the image imported unaltered into ImageStat 1.0 and all atheroma quantified automatically using the same image master.

6.3.5 Cholesterol and glucose measurements.

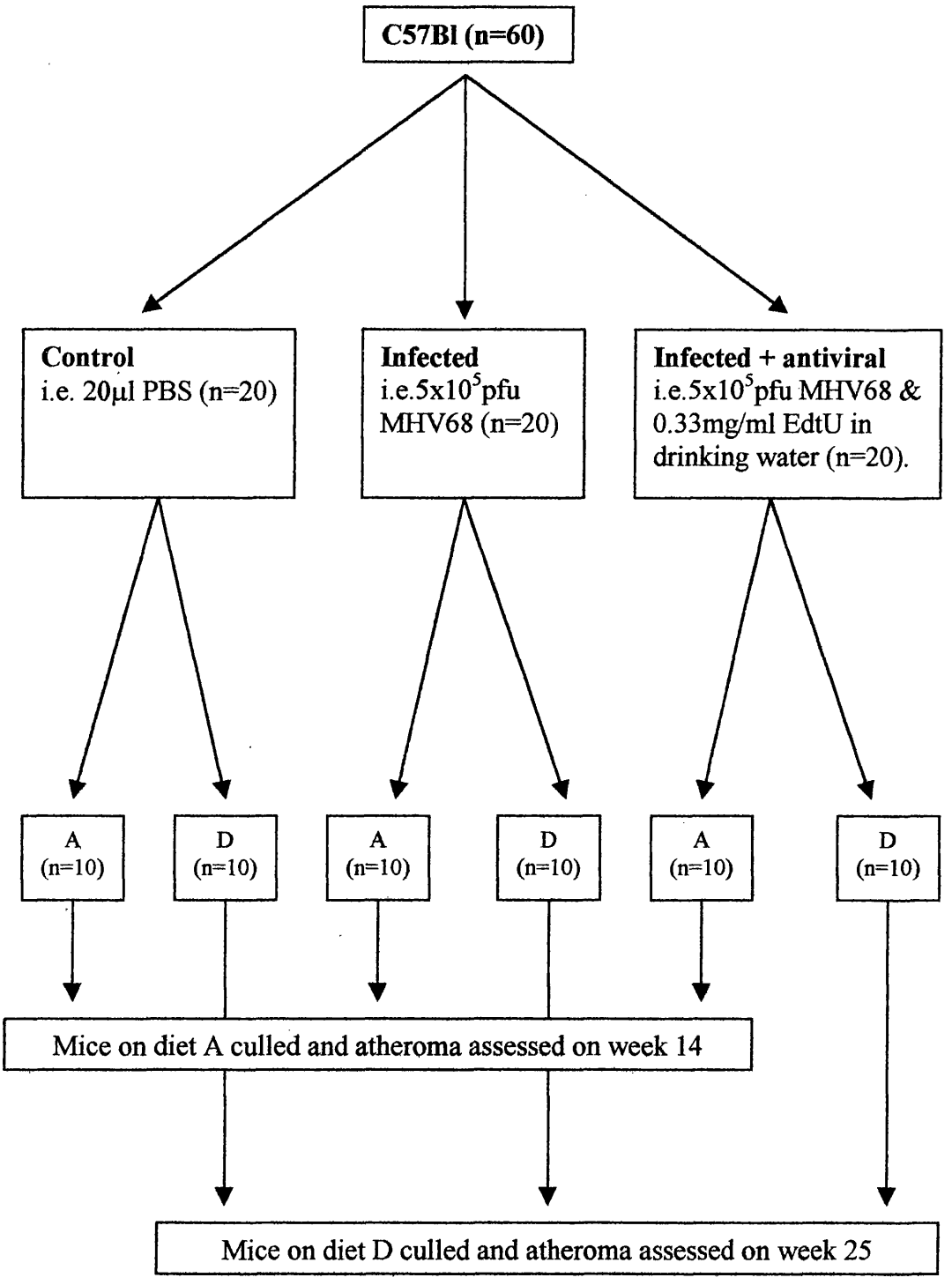
Random, non-fasting cholesterol and glucose measurements were carried out as described in Methods 2.5.4.

6.4 Experimental protocol for Chapter 6

Sixty female C57Bl mice aged 6-8 weeks were divided into three groups as outlined in Figure 6.1. Twenty mice received $5x10^5$pfu MHV-68 intranasally; another twenty mice received $5x10^5$pfu MHV-68 intranasally and at the same time were started on 0.33mg/ml of 2'-deoxy-5-ethyl-b-4'-thioridine (EdtU) in their drinking water; the remaining twenty mice received an equal volume of sterile PBS, also via the intranasal route. After 6 days half the mice in each group were started on either an atherogenic or a diabetogenic diet (see Methods 2.3.2.). Animals were then maintained on one or other diet (+/- antiviral drug), for 14 weeks. At 14 weeks all mice on the atherogenic diet (diet A) were culled. In the case of those mice the diabetogenic diet (diet D) only n=2 from the control and infected group were culled at 14 weeks. These 4 mice had developed hardly any atheroma therefore the

remaining mice on diet D were kept for a further 11 weeks (total 25 weeks) before being culled. All mice were weighed and then culled by cardiac puncture under terminal anaesthesia. Blood was spun and serum stored at –20°C for total cholesterol and random glucose analysis. Lung, liver, spleen, kidney and heart were collected, snap frozen in liquid nitrogen and stored at –80°C.

Figure 6.1. Outline of experimental protocol. Where A = atherogenic diet i.e. RM3 plus 20% fat, 1.25% cholesterol and 0.5% cholate, and D = diabetogenic diet i.e. Bio-Serve 1850, 35% fat, 31% carbohydrate (sucrose), 5% vitamin/mineral premix.



6.5 RESULTS

6.5.1 Animals on the atherogenic diet

Animals on the atherogenic diet appeared underweight and with ruffled fur from week 10 of the diet. Three animals were found dead in the infected group, one each in week 8, 9 and 13 of the diet. One mouse was found unwell and was culled in the control group on week 10. All mice on the antiviral drug survived. One aorta from both the infected and infected plus antiviral group was damaged during dissection, and therefore these animals were excluded from the atheroma and biochemical analysis.

6.5.2 Animals on the diabetogenic diet

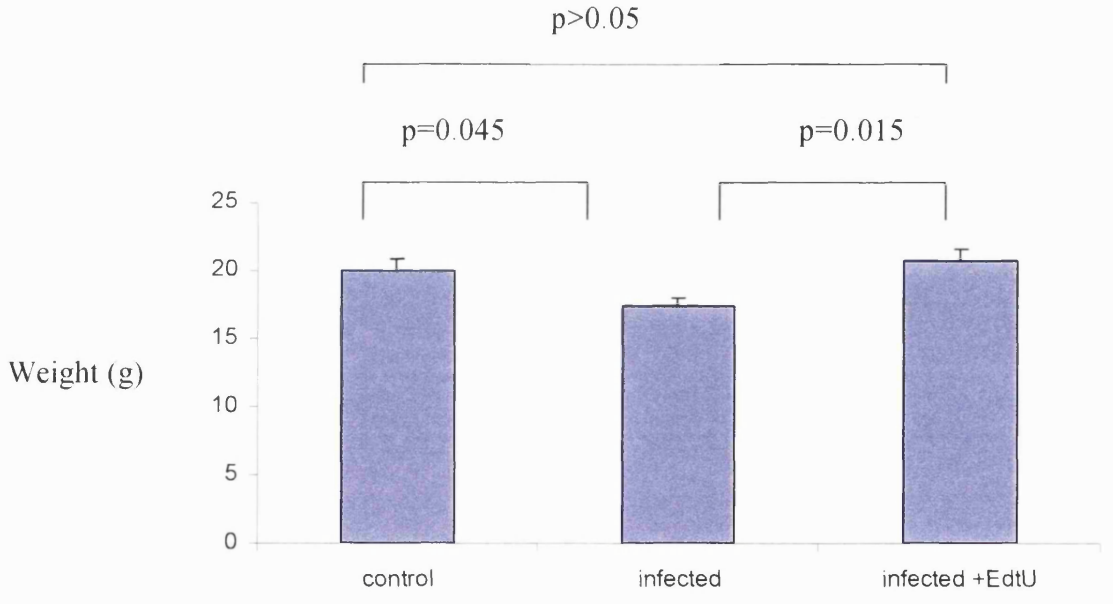
The diabetogenic diet was far better tolerated than the atherogenic diet and no mice appeared unwell or died during the course of the study. Mice (n=2) were taken from the control and infected group at week 14. These mice had very little atheroma and therefore the remaining mice on diet D were kept for a further 11 weeks. Two aortas from the antiviral group were found to be damaged during dissection, and were not therefore included in the atheroma and biochemical analysis.

6.5.3 Body weights of mice on the atherogenic diet

Infected animals were significantly lighter than control animals that received PBS ($p=0.046$) and infected animals that received EdtU ($p=0.015$). There was no significant difference between the weights of the control mice that received PBS or the infected mice that also received EdtU ($p>0.05$) (figure 6.2).

When all mice on the atherogenic diet were considered together there was no correlation between weight and the percentage of atheroma (correlation coefficient, r=0.14, p=0.07).

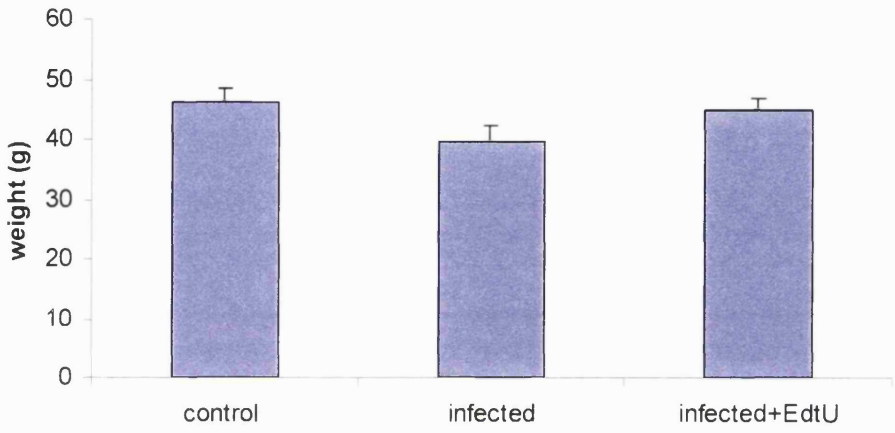
Figure 6.2. Decrease in weight with infection with MHV-68. Weights after 14 weeks on an atherogenic diet. Control mice (n=9), infected (n=6), infected plus EdtU (n=9). Significant results ($p<0.05$) by t-Test are shown.



6.5.4 Body weights of mice on the diabetogenic diet

At 14 weeks the control (n=2) and infected (n=2) mice on the diabetogenic diet were heavier than the mice on the atherogenic diet (mean=35.4g). At 25 weeks the mice had put on more weight, but there was no significant difference in the weights between the control, infected and infected plus EdtU groups (figure 6.3).

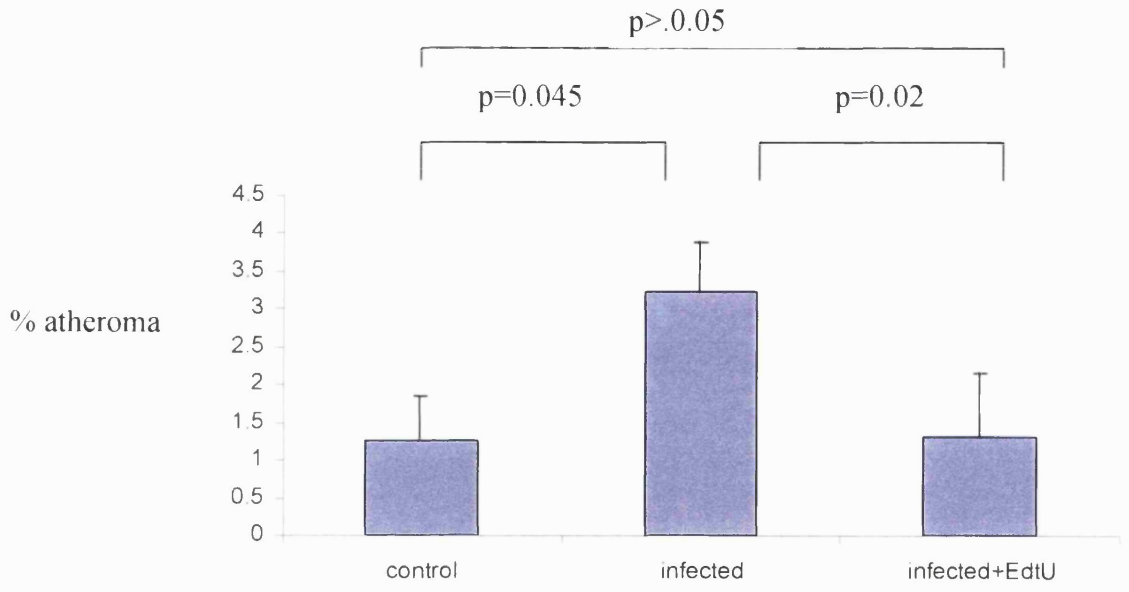
Figure 6.3. Body weights for the mice on the diabetogenic diet. Weights after 25 weeks on the diabetogenic diet; n=8 in each group. Non-significant ($p>0.05$) by Students t-Test.



6.5.5 Quantification of atheroma in C57Bl mice on the atherogenic diet

C57Bl mice infected with MHV-68 (n=6) showed a significant increase in atheroma compared to control (n=9) mice inoculated with PBS ($p=0.045$ by Students t test). This enhancement was reduced by the addition of 2'-deoxy-5-ethyl-b-4'-thioridine in the drinking water from the day of infection (n=9) ($p=0.02$ by Students t test). There was no significant difference between the amount of atheroma in the control group and the group that received MHV-68 infection together with the antiviral drug ($p>0.05$). Figure 6.4.

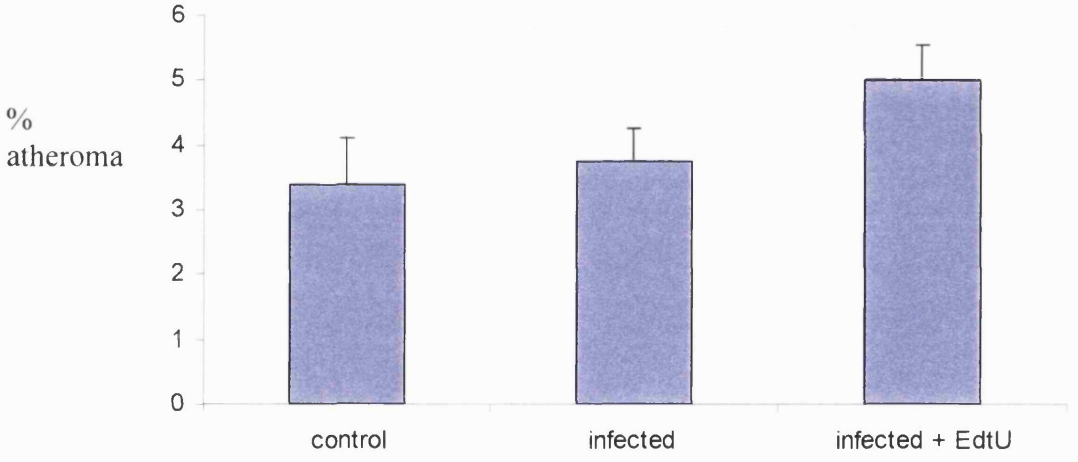
Figure 6.4. Increase in atheroma with MHV-68 infection. C57Bl mice on an atherogenic diet for 14 weeks. Control (n=9) mice, infected (n=6), infected plus EdtU (n=9). Significant results ($p<0.05$) by t-Test are shown.



6.5.6 Quantification of atheroma in C57Bl mice on the diabetogenic diet

At 14 weeks the amount of atheroma in the mice on the diabetogenic diet was very small (control (n=2) mean=0.22%, infected (n=2) mean=0.42%). At 25 weeks there was no significant difference in the amount of atheroma between control (n=8), infected (n=8) or infected mice receiving the antiviral drug (n=8) ($p>0.05$) (figure 6.5).

Figure 6.5. Percentage of atheroma in mice on the diabetogenic diet. Control (n=8) mice, infected (n=8), infected plus EdtU (n=8). Non-significant ($p>0.05$) by Students t-Test.

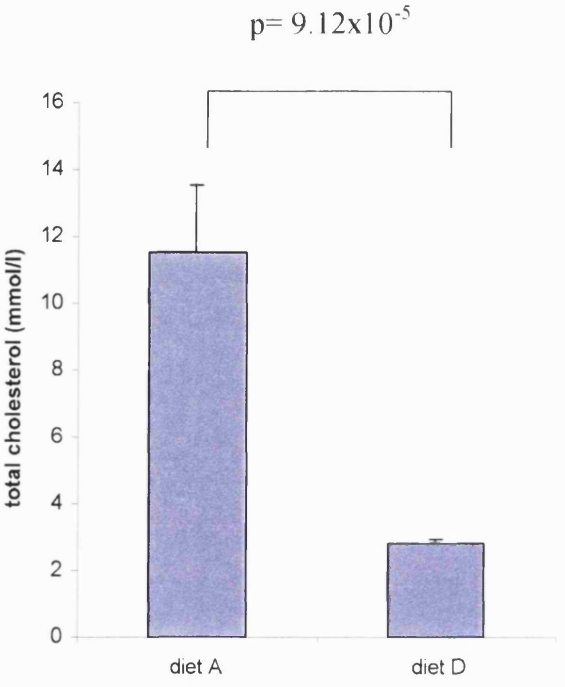


6.5.7 Total serum cholesterol

Mice receiving the atherogenic diet had significantly higher total cholesterol levels than mice on the diabetogenic diet ($p= 9.12x10^{-5}$ by Students t-Test, figure 6.6).

Total serum cholesterol levels were not statistically different between control, infected or infected-plus-antiviral groups of mice either on the atherogenic diet or on the diabetogenic diet (Diet A (n=24): control=11.37 +-4.09 , infected=14.57+/- 2.21, infected+EdtU=9.62+/- 3.47 mmol/l +/- s.e.m., $p>0.05$ by Students t-Test. Diet D (n=23): control=2.89+/-0.16, infected=2.73+/-0.16, infected+EdtU=2.80+/-0.21, $p>0.05$ by Students t-Test; one sample in the infected group haemolysed so it was not included in the analysis).

Figure 6.6. Total serum cholesterol levels on different diets. C57Bl mice on the atherogenic diet (diet A, n=24) and the diabetogenic diet (diet D, n=23). Significant ($p= 9.12x10^{-5}$) by t-Test.

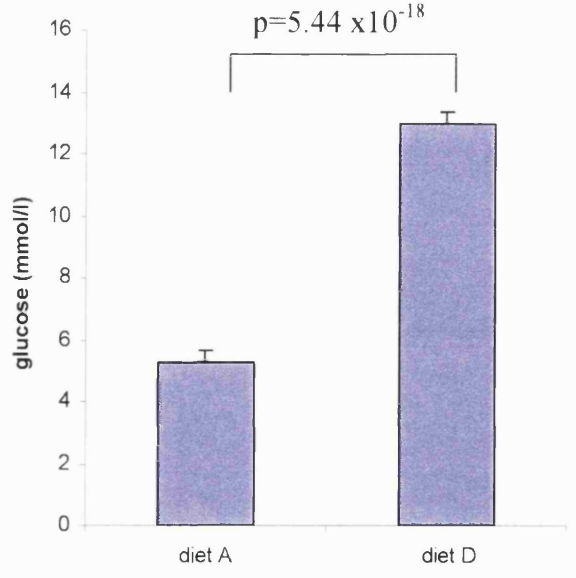


6.5.8 Serum glucose measurements

Mice receiving the diabetogenic diet had significantly higher glucose levels than mice on the atherogenic diet ($p=5.44 \times 10^{-18}$ by Students t-Test, figure 6.7).

Serum glucose levels were not statistically different between control, infected or infected + antiviral groups of mice either on the atherogenic diet or on the diabetogenic diet (Diet A (n=24): control=5.56+/-0.57, infected=4.45+/-0.38, infected+EdtU=5.59+/-0.70, $p>0.05$ by Students t-Test. Diet D (n=23): control=13.50+/-0.81, infected=12.37+/-0.52, infected+EtdU=12.93+/-0.73, $p>0.05$ by Students t-Test; one specimen was haemolysed).

Figure 6.7. Serum glucose levels on different diets. C57Bl mice on the atherogenic diet (diet A, n=24) and the diabetogenic diet (diet D, n=23). Significant ($p=5.44 \times 10^{-18}$) by t-Test.



6.6 DISCUSSION

The results presented in this Chapter show that infection with MHV-68 can accelerate atherogenesis in a diet-induced model, namely the C57Bl mouse on an atherogenic diet, and that this acceleration is prevented by the addition of the antiviral drug 2'-deoxy-5-ethyl-b-4'-thioridine. MHV-68 did not enhance atherogenesis in the identical murine strain on a diabetogenic diet. The crucial difference between the two groups of mice would appear to be the total cholesterol levels induced by the diets. This fact, in combination with the results in Chapter 4 where MHV-68 infection accelerated atheroma formation in the ApoE-/- mouse with spontaneous hypercholesterolaemia but not in the C57Bl mouse on normal chow, might suggest that a high serum cholesterol is a necessary prerequisite for developing MHV-68-enhanced atheroma.

6.6.1 Interaction between serum cholesterol and infection

Previous studies in animal models with pro-atherogenic organisms have not clearly shown cholesterol to be a pre-requisite for atheroma development. In experiments with recombinant murine CMV and bacterial β-galactosidase in Balb/c mice, virus was detected within the aortic wall and was associated with lipid accumulation in mice on an atherogenic diet. No vascular lesions were found in similarly infected mice on a normal diet, suggesting that cholesterol was necessary for lesion development (Berencsi et al., 1998). However these lesions were extremely early and animals had to be irradiated to allow viral replication. Further studies with suckling C57Bl and Balb/c mice (where irradiation was not necessary for viral replication) showed that dietary cholesterol supplementation increased the number of lesions, but was not a prerequisite for atherogenesis (Dangler et al., 1995).

Similarly, with Marek's disease in chickens and Bovine herpesvirus-4 in rabbits, cholesterol supplementation increased atherosclerosis although atheroma is still seen in infected animals on a low cholesterol diet (Fabricant and Fabricant, 1999; Lin et al., 2000; Njenga and Dangler, 1996). *Chlamydia pneumoniae* has been found by PCR within the aorta and increases atherosclerosis in the ApoE-/- mouse (Moazed et al., 1999). The same organism has been detected within the aorta of cholesterol-fed C57Bl mice, but no examination for atheroma was carried out in these mice (Moazed et al., 1997). Thus the results in this Chapter demonstrate for the first time that, in contrast to the situation in other animal models, a high serum cholesterol level appears to be a prerequisite for infection-enhanced atherogenesis in the mouse.

6.6.2 Underlying mechanism behind MHV-68 enhanced atherogenesis

Although a high serum cholesterol seems to be a prerequisite for acceleration of atheroma formation by MHV-68 infection, this is not on its own a full explanation of the underlying mechanisms behind the effect. In this respect it is noteworthy that the total cholesterol levels did not differ between the control, infected and infected-plus-antiviral groups of mice on the atherogenic diet. Rather, the mean body weights in these three groups were statistically significantly different. The relevance of this is that, as discussed in Chapter 4, the effect of MHV-68 infection on aortic atherogenesis could either be a **direct** interaction at the level of the endothelium (as in Ross' 'response to injury' theory), or alternatively an **indirect** effect. The finding that the weights were significantly reduced in the infected animals, compared to the controls and/or the infected mice who received the antiviral drug, would suggest that the latter mechanism is in operation. MHV-68 infection could, for example, be

inducing a systemic response that causes the animal to lose weight and separately promotes atherogenesis. The exact nature of such a systemic response is unclear, but possible candidates include the production of inflammatory cytokines and/or the induction of a response to chronic stress. Injections of IL-6 (a pro-inflammatory cytokine - see section 1.2) promote atherosclerosis in the C57Bl mouse on a high fat diet, and in the ApoE-/- mouse on both a low and high fat diet, but have no effect on an atherosclerosis-resistant murine strain despite similar total cholesterol levels (Huber et al., 1999).

6.6.3 Interaction between diabetes and MHV-68 infection

MHV-68 infection did not significantly increase the amount of atheroma in the mice on the diabetogenic diet. The diabetogenic diet appeared to be a weaker stimulus to atherogenesis than the atherogenic diet, as it induced a much smaller amount of atheroma after 14 weeks than the atherogenic diet. This has previously been reported (Schreyer et al., 1998). After 25 weeks the amount of atheroma had increased, and there was a significant difference in serum glucose levels. However, the total cholesterol levels were still significantly lower than in mice on the atherogenic diet.

The body weights of the mice were not significantly different after 25 weeks on the diabetogenic diet, although it appeared that a similar pattern was emerging of the infected group having lower average weights than the control and infected-plus-antiviral groups. It may be argued that had the mice been left for even longer, then a significant result in terms of the amount of atheroma in the infected group would have been found. However, the obesity of the animals may have become a problem.

Indeed, the degree of obesity hindered dissection and may have decreased the sensitivity of the atheroma quantification by increasing the amount of adventitial fat (although every effort was made to remove it). Against the argument that the mice should have been left longer is the finding that the amount of atheroma in mice on the diabetogenic diet after 25 weeks was of a similar order of magnitude as those on the atherogenic diet after 14 weeks. Therefore to leave the mice longer would not necessarily have resulted in a significant difference between infected and control groups.

6.6.4 Conclusion

The results presented in this Chapter show that MHV-68 infection interacts with an atherogenic diet to accelerate atherogenesis in the mouse, but that the same is not true for a diabetogenic diet. The exact mechanisms behind this effect remain unclear, but the preliminary findings here suggest a systemic response in the animal rather than a local effect on the aortic wall.

Chapter 7: Association between Kaposi's sarcoma and atherosclerosis; implications for γ–herpesviruses and vascular disease

7.1 INTRODUCTION

7.1.1 Human herpes virus 8 (HHV-8)

As discussed in section 1.5, human herpesviruses have been implicated in the aetiology of atherogenesis but no link has yet been firmly established. In the murine model described in Chapter 4, infection with murine γ–herpesvirus-68 (MHV-68) accelerates atherosclerosis in the ApoE-/- mouse (Alber et al., 2000). MHV-68 is closely related to, and shares sequence homology with, the most recently discovered of the human herpesviruses, human herpesvirus-8 (HHV-8, figure 1.3). HHV-8 has several biological properties which are potentially pro-atherogenic (see section 1.5.2), and the question therefore arises as to whether HHV-8 could be influencing atherogenesis in man.

HHV-8 does not appear to be a common infection in the general population, the seroprevalence being between <1% and 20% in populations studied to date (see (Schulz, 1998) for review). Moreover, Ye et al found no evidence of HHV-8 sequences by nested PCR in 38 atherectomy specimens from individuals apparently free of either HHV-8 or HIV infection (Ye et al., 1997). Nevertheless, studies in the MHV-68/ApoE-/- model have shown that even this virus, which potently enhances atherosclerosis, is not evident in the aorta after 10 days post infection (see section

4.5.7). Therefore the findings from Ye et al do not necessarily exclude the possibility of HHV-8 playing a role either in the general population or in certain sub-groups where HHV-8 infection is endemic.

7.1.2 HHV-8 in HIV positive individuals

HHV-8 was first identified in HIV-related Kaposi's Sarcoma, and hence was initially referred to as Kaposi's Sarcoma Virus (Chang, 1994). HHV-8 has since been identified in all cases of Kaposi's Sarcoma (KS), and it is now widely accepted that the virus plays a major role in its pathogenesis ((Boshoff, 1999; Chang, 1994; Ganem, 1997) and see section 1.4.3.1). The macroscopic appearance of KS can therefore be taken as a surrogate for HHV-8 infection. However, the converse is not the case, as the lag period between infection and tumour growth means that the absence of the skin tumour does not necessarily mean that an individual does not harbour HHV-8. Immunofluoresence assays form the basis of serology testing for HHV-8, but until recently these have proved unreliable (Boshoff and Weiss, 1998). Therefore in the absence of a reliable serological test for HHV-8 the macroscopic skin lesions of KS provide a usable surrogate for HHV-8 infection. KS is 20,000 times more common in HIV positive individuals than in the general population and, importantly, KS is 10 times more common in bisexual or homosexual men with AIDS than other HIV-infected groups (Whitby et al., 1998). Therefore it is in this group that any association between HHV-8 infection and atherosclerosis has the potential to be most clearly seen.

7.1.3 Evidence for increased atheroma in HIV positive individuals

As described in section 1.8, there is some evidence for individuals who are HIV positive having a greater incidence of atherosclerotic disease. Two small studies have shown an increase in atheroma in HIV positive individuals (Constans et al., 1995; Paton et al., 1993), and isolated case reports have implicated highly active anti-retroviral therapy (HAART) as causing a dyslipidaemia and so promoting atherogenesis (Behrens et al., 1998; Gallet et al., 1998; Henry et al., 1998; Vittecoq et al., 1998). Doubt exists whether such dyslipidaemic effects alone can account for any increase in atherosclerosis in this population. What is more likely is that any increase in atheroma is multifactorial in origin, and it can be postulated that an increased infectious burden over a prolonged time period secondary to a degree of immunosuppression may play a part.

7.1.4 Evidence for herpesvirus infection playing a role in the atherogenesis in HIV positive individuals

The strongest evidence for infection playing a role in atherogenesis in those with HIV infection comes from post-mortem studies. Tabib et al found that the histology of atheroma in young patients infected with HIV is intermediate between that of conventional atherosclerosis and the accelerated form of the disease found in cardiac transplant recipients (Tabib et al., 2000). As it is known that the risk of accelerated atheroma in transplants is greatest in those seropositive for HCMV (see section 1.5.1.), they postulated that a common herpesvirus infection in this population could be promoting atheroma formation.

7.2 HYPOTHESIS

As HIV infected individuals are more prone to infections, including HHV-8, it might be predicted that such individuals would be susceptible to advanced atherosclerosis if the infection/atheroma hypothesis were correct. Therefore this Chapter sets out to test the hypothesis that infection with HHV-8 is asscociated with an increase in atheroma in the HIV positive population.

7.3 METHODS

241 post-mortem reports of consecutive HIV positive patients autopsied at Middlesex/University College and St Guy's and Thomas' Hospitals were reviewed. All patients died between 1987 and 2000. All post-mortems were performed by the same group of pathologists following a standard protocol (Lucas et al., 1993).

The presence of Kaposi's sarcoma was taken to be a surrogate for HHV-8 infection. Post-mortem reports were divided into two groups on the basis of the presence or absence of Kaposi's sarcoma. The number of patients in each group who had macroscopic atheroma in their aorta or great vessels, including the coronary arteries, was then noted.

Table 7.1. Patient characteristics for the 241 HIV seropositive individuals who underwent a standard postmortem procedure.

One man in the KS- group was both homosexual and an intravenous drug user and was counted twice, Ψ. HAART=those patients receiving highly active anti-retroviral therapy. *** p<0.0001, ** p=0.001 by Chi Square test.

	Patients			*HIV risk factor*$^{\Psi}$			
	Age (mean yrs +/-s.e.m.)	**Male (%)**	**HAART (%)**	**Homosexual sex (%)**	**Heterosexual Sex (%)**	**Intravenous drug user (%)**	**Haemophiliac (%)**
KS+ (n=63)	39.5+-0.9	100 (n=63)	4.8 (n=3)	95.2 (n=60)	4.8 (n=3)	0	0
KS- (n=178)	38.6+-0.6	85.4 (n=152)	11.8 (n=21)	70.8 (n=126)	24.2 (n=43)	4.5 (n=8)	1.7 (n=3)

	Atheroma	
	Atheroma for all patients (%) * **	**Atheroma male patients (%)** **
KS+ (n=63)	47.6 (n=30)	47.6 (n=30)
KS- (n=178)	21.4 (n=38)	24.3 (n=37)

7.4 RESULTS

The relevant patient characteristics are shown in table 7.1. The two groups were of similar ages (Kaposi's sarcoma positive: 39.5 years +/-0.9 s.e.m.; Kaposi's sarcoma negative: 38.6 years +/- 0.6 s.e.m.). In the group with Kaposi's sarcoma (n=63) 30 (48%) had macroscopic evidence of atheroma, whilst in the group without Kaposi's (n= 178) 38 (21%) had atheroma. The difference was highly significant (Chi square test, χ^2=15.855, p<0.0001) and the odds ratio for having atheroma in the Kaposi's sarcoma group was 3.35 (1.85-6.06, 95% confidence limits). If only male patients were considered, the odds ratio was 2.83 (1.53-5.23, 95% CI; Chi square test, χ^2=11.250, p=0.001).

Protease inhibitors were being taken as part of highly active anti-retroviral therapy (HAART) by only 24 patients. Twenty-one of these has no evidence of KS, whilst the remaining 3 did have KS lesions. Twenty has no evidence of atheroma and 4 did have macroscopic atheroma.

7.5 DISCUSSION

7.5.1 Description of study and limitations

This study is a retrospective analysis of consecutive post-mortem reports using the macroscopic appearance of Kaposi's Sarcoma as a surrogate for HHV-8 serology. Because of the nature of the study any conclusions drawn must be interpreted with caution. Nevertheless, the fact that a strong association was found between the presence of the tumour and the presence of atheroma merits further comment.

7.5.2 Using the presence of Kaposi's Sarcoma as a surrogate for HHV-8 infection.

Kaposi's sarcoma is a good surrogate marker for HHV-8 infection, HHV-8 being found in all cases of the tumour so far examined (Schulz, 1998). However, there is a lag period between infection and appearance of the tumour, and so some of the KS negative patients studied here may indeed have been infected with HHV-8. However, this would be expected to bias the results in favour of a negative outcome. The proportion of individuals with Kaposi's sarcoma in this study (27%) is similar to the expected prevalence of HHV-8 seropositivity in an HIV positive group (Schulz, 1998; Whitby et al., 1998). Even if some of the Kaposi's sarcoma negative group were also infected with HHV-8 this would not negate our finding, but would suggest that the duration of exposure, or some other host factor, was related to both atherogenesis and sarcoma formation.

Indeed this study cannot exclude the possibility that KS may be just a marker for a more depressed immune system accompanied by greater number of chronic infections. It has already been shown that certain individuals who harbour a so-called 'greater infectious burden' have a higher likelihood of developing atheroma (Zhu et al., 2000). Therefore, it is possible that the presence of KS merely reflects the larger number of chronic infections, and thence chronic inflammation, which thus promote atherogenesis without HHV-8 being involved per se.

7.5.3 Biological rationale from animal and *in vitro* studies

Despite the fact that this study does not prove an association between HHV-8 and atherosclerosis there is considerable supportive evidence that HHV-8 may play a causative role in enhancing this disease both from *in vitro* and *in vivo* animal studies.

Firstly, from *in vitro* studies HHV-8 can infect endothelial cells (Flore et al., 1998), as well as induce the upregulation of adhesion molecules known to be involved in monocyte recruitment in the early stages of atherogenesis (Yang et al., 1994). The HHV-8 genome contains several homologues of known cellular genes, one of which (viral ORF2) is 25% identical at the amino acid level to human IL-6. The potential significance of this is that raised serum levels of IL-6 have been found to be associated with increased atheroma in man (Ridker et al,2000a) More recently oncostatin M (OSM), a potent autocrine and paracrine growth factor for AIDS-related KS (produced by virus-infected cells), has been shown to stimulate human aortic smooth muscle cells (hASMCs) to secrete IL-6 and stimulate COX-2 production (Bernard et al., 1999). COX-2 activity is an early and sensitive response of hASMCs to inflammation, and influences many vascular functions such as vascular tone and thrombogenesis which are known to be involved in atherogenesis (Libby et al., 1988; Rimarachin et al., 1994). OSM has also been found to upregulate adhesion molecule expresssion in endothelial cells, and is found in aortic aneurysms in man (Modur et al., 1997).

The second body of evidence in support of HHV-8 playing a role in atherogenesis comes from two *in vivo* models. Thus, as previously shown in Chapter 4, MHV-68 causes enhanced atheroma in the ApoE-/- mouse (Alber et al., 2000). More recently

Lin et al have shown that Bovine Herpesvirus-4 (BH4) also causes increased amounts of atheroma in the cholesterol-fed rabbit (Lin et al., 2000). Both MHV-68 and BH4 are γ-2 herpesviruses, and share with HHV-8 not only sequence homology but also the ability to infect endothelial cells (Alber et al., 2000; Lin et al., 1997; Simas and Efstathiou, 1998; Sun et al., 1999).

There is thus good evidence, from both *in vivo* and *in vitro* studies, that γ-herpesviruses in general and HHV-8 in particular may to be able to enhance atherosclerosis in susceptible hosts.

7.5.4 Interaction of herpesvirus infection with dyslipidaemia

Interestingly, in the animal models, γ–herpesviruses only cause atherosclerosis in the presence of hyperlipidaemia, suggesting that the virus cannot trigger or accelerate the disease unless other risk factors are present. If the same were true in humans, we would predict that the hyperlipidaemia induced by protease inhibitors would cause particular problems in patients who are positive for HHV-8. The evidence from this study however shows no link between protease inhibitors (taken as part of HAART) and atheroma. The main reason for this is that as such therapy only became available towards the latter end of the time period over which these post-mortems were conducted, very few of those eligible for such treatment were actually receiving it. Therefore no firm conclusions about the interaction between protease inhibitors and HHV-8 infection can be made. To answer this question it would be necessary to set up a prospective study with serum cholesterol measurements before and after initiating therapy and with quantification of the amount of atheroma present after a set period of time.

7.5.5 Conclusion

This study set out to test the hypothesis that HHV-8 infection affects atherogenesis in HIV positive individuals. The results show that in this group there was indeed a strong association between the presence of Kaposi's Sarcoma, a macroscopic marker of HHV-8 infection, and atheroma in the aorta and great vessels. Whilst this association does not prove a link between HHV-8 infection and atherosclerosis, it is supportive of such a link. Moreover there is good biological rationale, from *in vitro* and animal studies, that such an aetiological link might exist. In such animal models it is the interaction between high serum cholesterol levels and herpesvirus infection that synergistically provokes the greatest acceleration in atherosclerosis, and the same may also hold true in humans. The link between high cholesterol levels induced by protease inhibitors and HHV-8 infection in man is not supported by data derived from this work, but this would be eminently testable in a prospective study.

Chapter 8: HCMV seropositivity is associated with impaired responses to bradykinin and glyceryl trinitrate in both Type 1 diabetic and non-diabetic subjects.

8.1 INTRODUCTION

There is increasing evidence that chronic infection or systemic inflammation is associated with atherosclerotic disease (Danesh et al., 1997; Ridker et al., 1998; Ridker et al., 1997). Although clinical data are not yet conclusive, studies in animals have shown that specific organisms including Marek's Disease virus and *Chlamydia pneumoniae* may enhance atherosclerosis (Fabricant et al., 1978; Liuba et al., 2000). As shown in Chapter 4 murine γ-herpesvirus-68 (MHV-68) infection accelerates atherogenesis in the apolipoprotein E deficient (ApoE-/-) mouse (Alber et al., 2000). This study demonstrated that the effect of MHV-68 was only pro-atherogenic in the presence of another risk factor for atherosclerosis, namely hypercholesterolaemia. Epidemiological studies in humans have also suggested an interaction between human cytomegalovirus (HCMV) infection and another classical risk factor, diabetes mellitus (Sorlie et al., 2000; Visseren et al., 1997).

The precise mechanisms by which infections or inflammation might affect atheroma are unclear, but there is increasing evidence that endothelial dysfunction is important (Bhagat and Vallance, 1997; Fichtlscherer et al., 2000; Hingorani et al., 2000). Furthermore, assessment of endothelial function is often used as a marker of early vascular effects of classical risk factors (Calver et al., 1992; Chowienczyk et al., 1992). Therefore endothelial dysfunction may be a useful surrogate to determine

whether or not there is indeed any relationship between individual infectious agents and the type of vascular dysfunction that may pre-dispose to atheroma.

8.2 HYPOTHESIS

The aim of this study was to test the hypothesis that serology for specific herpesviruses (Human Cytomegalovirus (HCMV) and Herpes Simplex Virus (HSV-1)) is associated with altered vascular reactivity (particularly endothelial dysfunction), and that this effect would be more marked in individuals already at increased risk of atherosclerosis from diabetes.

8.3 METHODS

8.3.1 Subjects

Subjects were recruited from a previous study as described in Methods section 2.8.1.

8.3.2 Serology

Serolgy testing for HSV-1 and HCMV was performed as described in methods section 2.8.2.

8.2.3 *In Vivo* Endothelial Function Test

In vivo endothelial function studies were carried out as described in Methods section 2.8.3..

8.3.4 Electron Beam Computed Tomography

Electron beam computerised tomography was carried out as described in Methods section 2.8.4..

8.3.5 Serum cholesterol measurement

Serum cholesterol cocncentrations were determined as described in Methods section 2.5.4.

8.3.6 Statistical analysis

Statistical analysis was carried out as described in Methods section 2.9.3.

8.4 RESULTS

8.4.1 HCMV prevalence by sex and diabetes

Overall, 37% of those studied (n=142, total=381) were HCMV seropositive. Those who were HCMV seropositive had significantly different values for body mass index (1kg/m^2 higher, p=0.03, adjusting for age, sex and diabetes), systolic blood pressure (3mmHg higher, p=0.03, adjusting for age, sex and diabetes) and LDL cholesterol (0.2 mmol/l lower, p=0.03, adjusting for age, sex and diabetes) than seronegative subjects. The difference in systolic blood pressure was more apparent in women than men (table 8.1). There was no difference in smoking pack years and HCMV seropositivity for men or women. There was no difference in the seroprevalence for HCMV between diabetic and non-diabetic subjects of either sex, or for both sexes combined (odds ratio=0.82 for HCMV positivity in diabetic versus non-diabetic subjects, 95% CI 0.5-1.3, p=0.35 adjusted for age and sex). Among all subjects combined, women had a two-fold odds ratio of being seropositive for HCMV (odds ratio=1.97, 95% CI=1.3-3.0, p=0.002 adjusted for age and diabetes). This sex difference was of similar magnitude in both diabetic and non-diabetic groups (table 8.2).

8.4.2 HSV-1 prevalence by sex and diabetes

Overall, 58% of those studied (n=220, total=379) were HSV-1 positive. Those who were HSV-1 seropositive had significantly different values for body mass index ($2kg/m^2$ higher, $p<0.001$, adjusting for age, sex and diabetes) and systolic blood pressure (4mmHg higher, $p=0.004$, adjusting for age, sex and diabetes) than seronegative subjects (table 8.1). There was no difference in smoking pack years and HSV-1 seropositivity for men or women. The prevalence of HSV-1 seropositivity was slightly lower in diabetic compared to non-diabetic subjects of either sex, or for both sexes combined (odds ratio=0.67 for HSV-1 positivity in diabetic versus non-diabetic subjects, 95% CI 0.4-1.0, $p=0.06$, adjusted for age and sex). Among all subjects combined, women were twice as likely to be seropositive for HSV-1 (odds ratio=2.06 95% CI 1.3-3.1, $p=0.0008$, adjusted for age and diabetes). This sex difference was of similar magnitude in both diabetics and non-diabetics (table 8.2).

	Men		*Women*	
	HCMV+	**HCMV-**	**HCMV+**	**HCMV-**
BMI (kg/m²)	25 (4)	25 (3)	26 (5)	25 (4)*
LDL-c (mmol/l)	3.0 (0.9)	3.2 (1.1)	2.8 (0.8)	3.0 (0.9)
HDL-c (mmol/l)	1.6 (0.3)	1.7 (0.4)	1.9 (0.5)	1.9 (0.5)
TGs (mmol/l) $^{\Psi}$	1.3 (0.9)	1.1 (0.7)	0.9 (0.6)	1.0 (0.5)
SBP (mmHg)	128 (11)	126 (13)	118 (14)	113 (14)*
% now smoking	27 (6)	29 (4)	26 (5)	19 (4)
	HSV-1+	**HSV-1-**	**HSV-1+**	**HSV-1-**
BMI (kg/m²)	26 (4)	25 (3)*	26 (5)	24 (3)**
LDL-c (mmol/l)	3.2 (1.1)	3.1 (1.0)	2.9 (0.9)	2.9 (0.8)
HDL-c (mmol/l)	1.6 (0.4)	1.7 (0.4)	1.8 (0.5)	2.0 (0.4)
TGs (mmol/l) $^{\Psi}$	1.3 (0.9)	1.1 (0.6)	1.0 (0.6)	0.9 (0.4)
SBP (mmHg)	129 (12)	124 (12)***	116 (14)	114 (14)
% now smoking	31	26	24	19

Table 8. 1. Characteristics of subjects divided by seropositivity. Where * $p<0.05$, ** $p<0.01$, *** $p< 0.001$ for the difference between seropositive and seronegative subjects adjusted for age and diabetes within each sex. BMI= mean body mass index, TG= serum triglycerides, SBP= systolic blood pressure. Values given are the mean (s.e.m) apart from Ψ, where the median (interquartile range) rather than the mean is shown as TGs show a skewed distribution.

	NDM male (n=88)	DM male (n=99)*	NDM female (n=102)	DM female (n=92)
	% (SE)	**% (SE)**	**% (SE)**	**% (SE)**
HCMV positive	30 (5)	29 (5)	49 (5)	40 (5)
HSV-1 positive	56 (5)	43 (5)	70 (5)	63 (5)

Table 8.2. Prevalence of HCMV and HSV-1 in diabetic (DM) versus non-diabetic subjects (NDM) and in men versus women. Values are shown as percentages of total individuals in that group (+/- standard error). * two samples in the diabetic male group had insufficient volume to test for HSV-1.

8.4.3 Relationship between HCMV and HSV-1 serology and social class

The prevalence of atherosclerosis is linked to social class (Colhoun et al., 2000). Social class may therefore be a confounder of any relationship between HCMV/HSV-1 and atherosclerosis or endothelial dysfunction. It is therefore important to examine whether the prevalence of HCMV/HSV-1 varies with social class in this group.

The majority of the group as a whole were from the non-manual classes (82%). Overall, in the non-manual group 37% were HCMV seropositive, as against 40% of manual workers (odds ratio 0.8, 95% CI=0.4-1.3, p=0.34). In comparison, there was a significantly lower prevalence of HSV-1 seropositivity in the non-manual (55%) than manual (69%) subjects (odds ratio 0.5, 95% CI=0.3-0.9, p=0.02).

8.4.4 Relationship between coronary artery calcification (CAC) and HCMV/HSV-1 serology

Overall, those who were HCMV positive had a 1.5 fold odds ratio of having any CAC compared to the seronegative group, but this was not a statistically significant association (odds ratio 1.5, 95% CI=0.9-2.3, p=0.09). This was reduced to an odds ratio of 1.3 on adjustment for body mass index and systolic blood pressure. The odds ratio for CAC associated with HCMV seropositivity was of a similar magnitude in the diabetic (odds ratio 1.6, 95% CI 0.8-3.0, p=0.13) and non-diabetic groups (odds ratio 1.3, 95% CI 0.7-2.6, p=0.4) adjusting for age and sex.

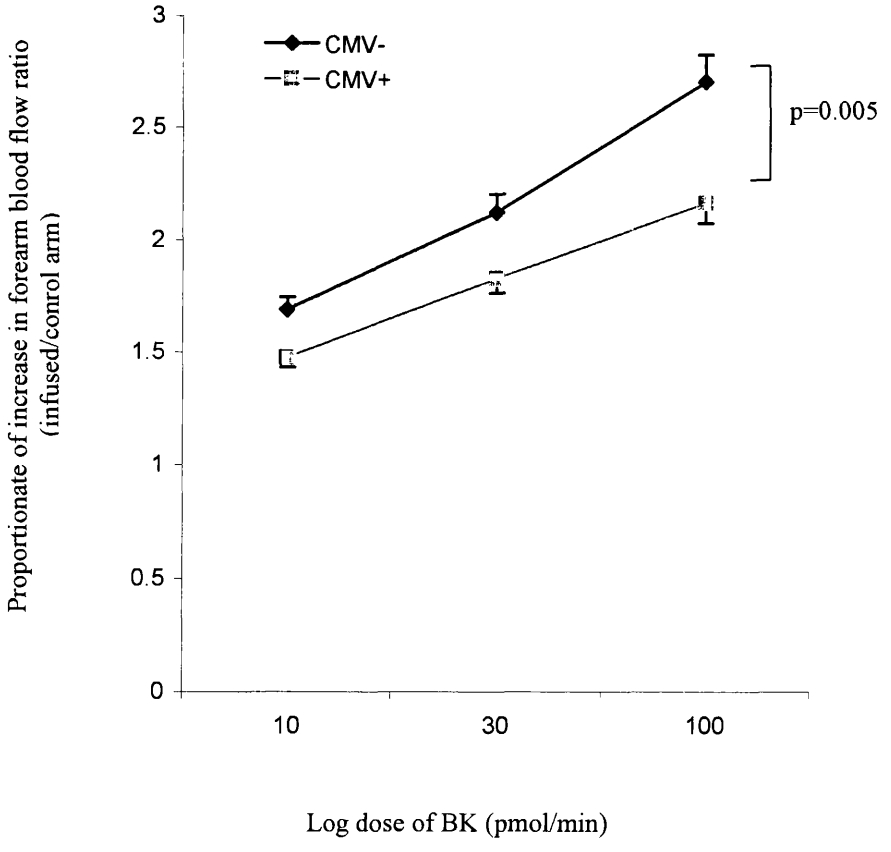
Similarly, there was no significant relationship between HSV-1 positive serology and CAC, with a 1.5-fold odds ratio overall of having any CAC compared to the seronegative group (odds ratio 1.5, 95%CI 0.9-2.3, p=0.09). This was reduced to an odds ratio of 0.99 on adjusting for body mass index and systolic blood pressure. Again the odds ratio for CAC associated with HSV-1 seropositivity was of a similar magnitude in the diabetic (odds ration 1.5, 95% CI 0.8-2.7, p=0.19) and non-diabetic groups (odds ratio 1.4, 95%CI 0.7-2.8, p=0.29) adjusting for age and sex.

8.4.5 Relationship between HCMV/HSV-1 serology and vascular response to bradykinin (BK)

In all subjects the vasodilator response to BK was significantly lower in those who were HCMV positive (p=0.005, adjusting for age, sex and diabetes - see table 8.3 and figure 8.1). This relationship was independent of systolic blood pressure, lipids,

smoking or social class (p=0.005 on adjustment for these factors). The magnitude of the difference in area-under-the-curve for BK, between seropositive and seronegative subjects, was greater in the non-diabetic than the diabetic subjects, although not significantly so (p=0.29 for the diabetic and HCMV interaction - table 8. 4). There was no difference in the basal flow between those seropositive or seronegative, for diabetic or non-diabetic subjects (p=0.7). The response to BK was slightly but not significantly lower in those who were seropositive for HSV-1 (p=0.3, table 8. 3).

Figure 8.1. Area under the curve for vascular response to BK. Vascular response by HCMV seropositivity (mean +/- s.e.m.).



8.4.6 Relationship between HCMV/HSV-1 serology and vascular response to GTN

Overall, the response to GTN was lower in those subjects who were HCMV seropositive (p=0.006, adjusted for age, sex and diabetes - table 8.3 and figure 8.2). This relationship was still significant after adjusting for systolic blood pressure, lipids and smoking (p=0.001), and for social class (p=0.003). The magnitude of this difference in area under the curve for GTN by HCMV status was similar in non-diabetic and diabetic subjects (p=0.5 for the diabetes and HCMV interaction, table 8.4). In contrast, there was no significant difference between the response to GTN in those who were HSV-1 seropositive (p=0.6).

Figure 8.2. Area under the curve for vascular response to GTN. Vascular response by HCMV seropositivity (mean +- s.e.m.).

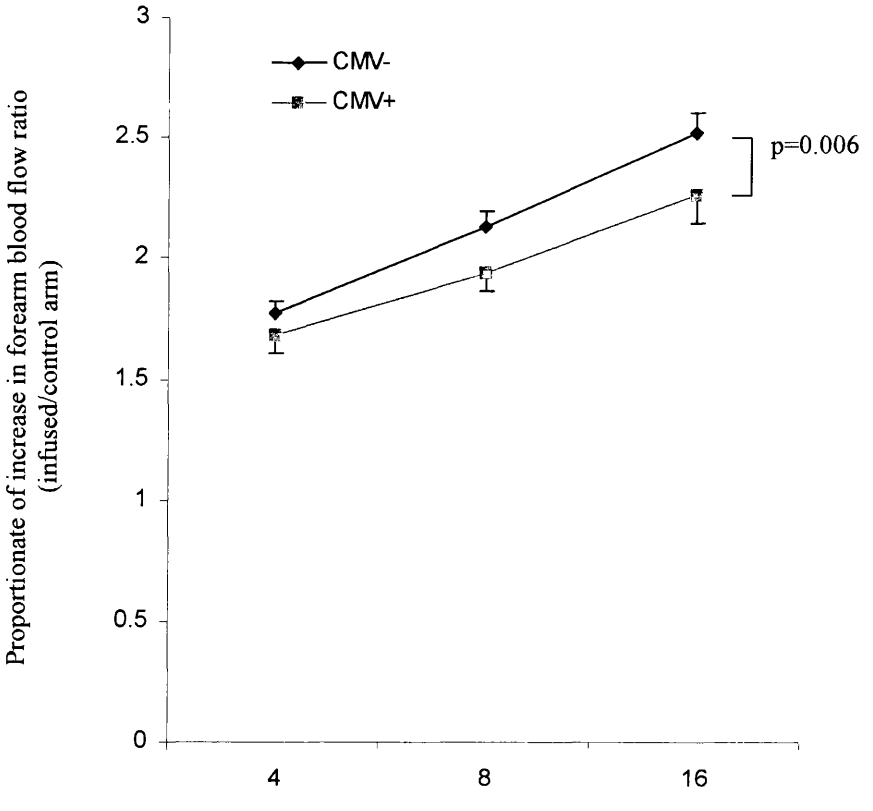


Table 8.3. Area under the curve for drug response by seropositivity status. Where ** $p<0.01$ for the difference in area under the curve by seropositivity status adjusted for age, sex and diabetes.

	HCMV+	*HCMV-*
ACh	3.32	3.16
BK	4.21	4.99**
GTN	2.71	2.96**
NE	2.10	2.12
L-NMMA	2.05	2.01

	HSV-1+	*HSV-1-*
ACh	3.23	3.32
BK	4.62	4.82
GTN	2.84	2.89
NE	2.20	1.97
L-NMMA	2.07	1.96

Table 8.4. Area under the curve for drug response by seropositivity status in nondiabetic (NDM) and diabetic subjects (DM). Where *$p<0.05$ and **$p<0.01$ for the difference in area under the curve by seropositivity status adjusted for age and sex.

		HCMV+	*HCMV-*	*HSV-1+*	*HSV-1-*
BK	**NDM**	4.23	5.56**	4.94	5.27
	DM	4.23	4.59	4.40	4.49
GTN	**NDM**	3.02	3.36*	3.13	3.33
	DM	2.48	2.68	2.63	2.57

8.4.7 Relationship between vascular response to BK and HCMV status, adjusting for vascular response to GTN

GTN was used in this study to assess vascular smooth muscle response to NO. Since the response to GTN was impaired in subjects seropositive for HCMV, the response to BK (which stimulates NO release) would be expected to be also reduced for this reason alone. To assess whether all the relationship between HCMV seropositivity and BK response was attributable to the impaired GTN response, we examined the association of HCMV seropositivity whilst adjusting for the GTN response. The difference in the BK response in HCMV positive compared to HCMV negative subjects was decreased by about a third and remained significant ($p=0.045$) on adjusting for the GTN response.

8.4.8 Relationship between the vascular response to acetylcholine (ACh), norepinephrine (NE), N^G-monomethyl -L-arginine (L-NMMA) and HCMV/HSV-1 seropositivity

There was no significant difference between the response to ACh, NE or L-NMMA in those who were seropositive for either virus, compared to those who were seronegative (table 8.3 and appendix A6).

8.5 DISCUSSION

This is the first study to show that individuals who are seropositive for HCMV, and who have therefore been previously infected with HCMV, have abnormal vascular reactivity consistent with either endothelial dysfunction and/or reduced responsiveness to NO. There was no significant association between HCMV-positive serology and coronary artery calcification.

This relationship appears to be independent of other established risk factors for coronary artery disease. These findings in humans extend work in animal models in which herpesviruses and other organisms have been shown to cause endothelial dysfunction and accelerate atheroma formation (Eerdmans et al., 1996; Liuba et al., 2000). Together with animal and *in vitro* data, this study provides a plausible mechanistic link between chronic herpesvirus infection and enhanced atherogenesis. Diabetic subjects did not have a higher prevalence of seropositivity for HCMV, and therefore previous HCMV infection did not appear to contribute to the increased rate of coronary artery disease in diabetic as compared to non-diabetic subjects.

8.5.1 Discrepancy between findings for bradykinin and acetylcholine responses

Reduced responses to endothelium-dependent agonists are often taken as indicative of endothelial dysfunction (Calver et al., 1992; Chowienczyk et al., 1992). In the present study, whilst responses to bradykinin were highly significantly reduced in seropositive individuals, those to acetylcholine were unaffected. Both BK and ACh cause vasodilation in part by stimulating endothelial NO synthesis via the B2 receptors and muscarinic receptors respectively. The finding of a selective defect

with BK but not ACh suggests that the B2 receptor mediated transduction mechanism is selectively impaired in HCMV seropositive subjects. This differential impairment has been found in previous studies. Thus in one study comparing hypertensive patients to normotensive controls, the hypertensives had a greater reduction in response to bradykinin compared to acetylcholine (Kelm et al., 1996). Conversely, it has also been reported that acetylcholine responses were reduced in hypercholesterolaemic patients compared to controls, whilst the response to bradykinin was unaffected (Gilligan et al., 1994). A reduced BK response has previously been noted in the context of inflammation, and indeed would seem to be a particularly good marker of inflammation-induced vascular dysfunction. Thus acute experimental inflammation induced by *S.typhi* vaccination resulted in more marked impairment of forearm blood flow responses to BK than ACh (Hingorani et al., 2000). The mechanisms are unclear, but might indicate changes in receptors or in signal transduction pathways linking responses to activation of NO synthesis. Alternatively, the subcellular localisation of receptors and NO synthases (e.g. in caveloae) may be particularly important in determining which responses are affected first or to the greatest degree.

Neither bradykinin nor acetylcholine work exclusively through NO. Bradykinin in the forearm has been shown to operate in part through NO (O'Kane et al., 1994) and in part through activation of potassium channels (Honing et al., 2000). In hypertensive patients bradykinin-induced vasodilation has been shown to be mediated by compensatory pathways rather than being NO-mediated (Taddei et al., 1999). In the endothelial NO synthase knock-out (eNOS-/-) mice, bradykinin produces vasodilation which is unaffected by NO synthase inhibitors (Brandes et al.,

2000). Therefore inflammation could be affecting one effector mechanism more than another. Alternatively, compensatory mechanisms may allow the response to acetylcholine but not bradykinin to normalise. Finally, whereas acetylcholine may be considered a useful experimental tool, bradykinin responses are thought to be more important in terms of physiological control of vascular tone (Bhoola et al., 1992; Colman and Schmaier, 1997), and therefore defects in bradykinin responses may be more relevant to atherogenesis.

8.5.2 Impaired endothelium independent vasodilation

The GTN response was also highly significantly diminished in those seropositive for HCMV. This might be due to reduced vascular smooth muscle responsiveness to NO, some specific effect on GTN metabolism, or some non-specific effect on vasodilation. Increasingly, studies of vascular responses in the presence of cardiovascular risk factors have shown some impairment in GTN responses, suggesting that not all the defects described as 'endothelial' are confined to this cell type and that more widespread effects on vascular smooth muscle responses may occur. For example, in one study hypertensives showed a diminished response to sodium nitroprusside as well as to acetylcholine and bradykinin (Kelm et al., 1996), whilst in diabetic subjects a reduced response to GTN/NO has been seen to accompany the endothelial defect (N.Chan *et al,* unpublished observations). This may possibly be due to decreased NO bioavailability due to oxidative stress, or to changes in the structural/contractile mechanisms within the arterial smooth muscle. It is unclear how oxidative stress may preferentially affect NO-related responses to bradykinin and exogenous NO (GTN), but not to acetylcholine, and further studies

would be needed to explore this directly. From the data in this study it is clear that the diminished response to GTN does not fully account for the reduced bradykinin response in HCMV seropositive subjects.

8.5.3 No reduction in basal NO mediated vasodilation with seropositivity

Whereas the relaxation secondary to BK and ACh is often referred to as the stimulated release of NO, the contraction seen secondary to L-NMMA is taken as a reflection of the amount of basal release of NO. The finding that responses to L-NMMA are unchanged signifies that, despite an altered vascular relaxation to stimulated NO, the basal NO is unaffected by past HCMV or HSV-1 infection. Similar differences between basal and agonist-stimulated responses have been seen in the presence of classical risk factors such as hypercholesterolaemia (John et al., 1998; Stroes et al., 1995).

In summary HCMV seropositivity was associated with a very significantly reduced vasodilatory response to bradykinin and GTN. Such changes have also been reported in the presence of conventional risk factors for cardiovascular disease and may be considered as a marker of increased risk from atherosclerosis or its complications.

8.5.4 Herpesviruses and vascular disease

The finding that HCMV seropositivity is associated with impaired vascular dilator function needs to be considered in relation to the epidemiological and animal data. Although many human studies have shown an association between herpesvirus serology and atherosclerosis, the largest study to date refuted any association between herpesvirus infection and C-reactive protein levels or the risk of having a

myocardial infarction (Ridker et al., 1998). Animal studies, however, have shown that certain herpesviruses enhance atherosclerosis, and in one instance can cause endothelial dysfunction (Alber et al., 2000; Eerdmans et al., 1996). It is possible that the large epidemiological outcome studies are negative because they rely on a single serology measurement taken late in the disease, and therefore do not take into account when infection occurred or how often reactivation has taken place. Alternatively, it may be that the end-points of myocardial infarction or other events are determined largely by other factors that influence the atherosclerotic plaque, whilst viral infections still have a greater effect on atherogenesis itself. Whatever the explanation, our finding of clear association between HCMV seropositivity and vascular dysfunction suggests that specific herpesvirus infections can be associated with cardiovascular dysfunction of a type associated with a predisposition to atherosclerotic disease.

8.5.5 Herpesvirus infection and diabetes

One aim of this study was to explore the interaction between herpesvirus infection and Type 1 diabetes. The results show that there is no predilection for herpesvirus infection to alter vascular reactivity specifically in Type 1 diabetic compared to non-diabetic subjects. Other studies have shown an association between seropositivity for HCMV and atherosclerosis in diabetic subjects (Sorlie et al., 2000; Visseren et al., 1997). For example, Visseren *et al* found HCMV antibody titres in diabetics with atherosclerosis were almost twice as high as in diabetic subjects without atherosclerosis (Visseren et al., 1997). In a larger study Sorlie *et al* reported that those with the highest levels of antibody titres for HCMV had a greater relative risk of developing coronary heart disease, and that the risk was increased still further if

they also had diabetes (Sorlie et al., 2000). These two studies would lead one to speculate that diabetic individuals are more susceptible to the atherogenic consequences of HCMV infection. This would not negate the findings reported here, as HCMV may still be a risk factor for atherosclerosis in those with diabetes, but via a mechanism that does not involved altered vascular reactivity. It should also be noted however that both previous studies included Type 1 and 2 diabetics, whereas the current study has focused on Type 1 diabetics; direct comparisons must therefore be made with caution.

8.5.6 Limitations of this study

A high level of either false negatives or false positives for serology would have an effect on the power in a study of this kind. However, it would only influence the association if the results were non-randomly assigned to those with or without endothelial dysfunction. A false negative result is unlikely given the sensitivity of the assay. There may have been some individuals acutely infected with virus who would express an IgM rather than an IgG response. However given the incidence of infection rates, such numbers are likely to be small or non-existent. The chances of false positive results is heightened with repeated freeze/thawing in the specimens. Specimens had only been previously refrozen on a maximum of two previous occasions. Furthermore, the number of equivocal tests was very low (7 in total), suggesting that neither a false positive nor a false negative test was a common occurrence. The young age range of the subjects and the fact that the majority were from the non-manual social classes could have accounted for both the relatively low seroprevalence for both herpesviruses and the low calcification scores. This

however would have weakened rather than strengthened any association between herpesvirus infection and vascular dysfunction.

8.5.7 Gender difference in antibody levels

Women had a higher seroprevalence of IgG antibodies to both HSV-1 and HCMV than men, and therefore on a simplistic level may have been expected to have demonstrated more atheroma. However, the immunological defence against herpesviruses involves both antibody and cell-mediated immune responses and the presence of IgG antibodies therefore gives no indication of how quickly the virus has been cleared or of how much inflammation it provoked. Interestingly, HCMV IgG antibodies have found to be an independent risk factor for coronary artery disease in women as opposed to men, and susceptibility to HCMV-related coronary artery disease in women was found to be associated with a predominantly humoral rather than cell mediated response (Zhu et al., 2000). In any event, the results of the present study were adjusted for sex and the association between HCMV seropositivity and altered vascular reactivity remained extremely robust.

8.5.8 Conclusion

These results show a clear relationship between herpesvirus infection and impaired NO-mediated vascular relaxation which could provide a mechanistic link with atherosclerosis. Further studies will be needed to explore this in more detail. It will also be important to determine whether these effects are pathogen-specific or subject-specific, in that some individuals are more prone to harbouring a larger burden of infection and hence a source of chronic inflammation.

Chapter 9: Summary and future directions

As outlined in the introduction, although it is known that inflammation plays a major part in atherogenesis, the exact role of chronic infections in promoting this inflammation and thereby increasing atherosclerosis remains obscure. Herpesviruses, especially those belonging to the beta- and gamma-herpesvirus families, have biological characteristics that suggest that they have the potential to promote atherosclerosis. Many *in vitro* studies with cytomegalovirus (HCMV) and to a lesser extent with human herpesvirus-8 (HHV-8) also support such an hypothesis (section 1.5.2.). However unequivocal serological evidence for their involvement in human atherogenesis remains lacking.

An animal model could provide useful information in establishing a direct link between herpesvirus infection and atherosclerosis. Previous animal models such as Marek's disease in chickens, as well as CMV in rats and mice, have shown that herpesvirus infection can enhance certain forms of atherosclerosis; but all these models have certain drawbacks (section 1.9). What is needed is a murine model of atherosclerosis with a natural herpesvirus infection which behaves like most herpesvirus infections in man; thus in the absence of clinical signs of illness, periodic reactivation through the lifetime of the host could thereby promote atherogenesis. Murine gammaherpesvirus-68 (MHV-68) infection in the Apolipoprotein E deficient mouse (ApoE-/-) fulfills these requirements. The development of this model and the novel, albeit derivative, method of quantification of aortic atheroma provides the first example of a herpesvirus infection accelerating

atherosclerosis in a mammalian model. This model has thereby proved the first hypothesis under investigation in section 1.13.1.

The subsequent hypotheses were not proven. These are namely that infection with such a pro-atherogenic virus will cause impaired endothelium-dependent relaxation in aortic rings or an increase in the expression of adhesion molecules on a murine endothelial cell line. In the case of impaired endothelium-dependent relaxation, other studies have found similar difficulty in demonstrating impaired vasodilation in the mouse as compared to man and other animal models (Bonthu et al., 1997; Deckert et al., 1999). It may be that in the mouse reduced endothelium-dependent responses are either absent or compensated for by additional vasodilatory mechanisms. What has been shown is that infected ApoE-./- mice have increased constrictor responses to a thromboxane mimetic, U19. Whilst this has not yet been explored further, this finding may be due either to a reduced basal release of endothelial NO (Kauser et al., 2000), or to a change in the smooth muscle contractile function (Katsumata et al., 1997). Indeed enhanced contractile responses may be more representative of the changes that occur both in inflammatory porcine models of atherogenesis as well as in infection-induced atheroma in man (Henry, 1990). The hypothesis that hypercontractility underlies atherogenesis has been around for some considerable time, although it has recently received less attention than impaired vasodilation. Interestingly, it was nearly a century ago that William Osler commented: 'We have, I think, evidence that sclerotic arteries are specially prone to spasm' ((Osler, 1908), Figure 9.1). Clearly further studies still need to be performed to see if this enhanced response to vasoconstrictors is a consistent finding, and to define further its underlying mechanism.

The finding that MHV-768 infection did not upregulate mRNA for endothelial adhesion molecules in a murine cell line does not exclude this being an underlying mechanism in the mouse. Recently Eriksson et al reported an *in vivo* technique looking at the mouse aorta with an intravital microscope. They showed that leukocyte rolling and subsequent adherence was increased in response to inflammatory cytokines, depended upon the expression of P-selectin, and was modified by E-selectin and VCAM (Eriksson et al., 2000). It would be informative to use this technique in the mouse infected with MHV-68 compared to control mice.

This work described here has therefore established that a pro-atherogenic herpesvirus can accelerate atherosclerosis, although a direct effect on the aortic endothelium remains unproven. It has also shown that MHV-68 infection in the background strain C57Bl on a normal diet does not cause atherosclerosis. The question thus raised is, given that the genetic backgrounds were identical apart from the ApoE deletion, whether the hypercholesterolaemia was the crucial factor. To this end the effect of MHV-68 infection on the diet-induced C57Bl model was explored. This demonstrated that MHV-68 infection could enhance atherosclerosis in the C57Bl mouse when on an atherogenic diet, but not when on a diabetogenic diet. The crucial difference between the two diets appears to be the resulting serum cholesterol levels, therefore suggesting that hypercholesterolaemia is a necessary pre-requisite for infection-induced atherosclerosis in this model. It would be interesting to explore whether the C57Bl mouse on an atherogenic diet also gets an increased vasoconstrictor response to U19 with MHV-68 infection. Whilst a diabetogenic diet also produced atheroma, albeit over a longer time period, MHV-68 infection did not

appear to interact with this stimulus for atherogenesis as no acceleration in atherosclerosis was observed. Murine models for Type 1 diabetes exist, where an immune-mediated destruction of the β-islets is induced by injections of streptozotocin (STZ), and indeed ApoE-/- mice develop more atheroma and premature aortic calcification when injected with STZ. Therefore the use of STZ in both the ApoE-/- and C57Bl-diet-induced models of atherosclerosis, in conjunction with MHV-68 inoculation, would provide interesting information on the interaction between diabetes and infection-accelerated atherosclerosis. One further major risk factor for atherosclerosis is hypertension. Achieving hypertension through dietary means is difficult in mice, but various transgenic models of hypertension do exist, including the ApoE-/- eNOS double knockout (Knowles et al., 2000) and the CRH-2 knockout (Coste et al., 2000) (see (Cvetkovic and Sigmund, 2000) for review). Feeding such animals with either diet could provide valuable data on the interaction between cardiovascular risk factors in a murine system. Thus multiple avenues exist whereby the model established in this thesis could be used to explore the interaction between the various known and postulated risk factors for atherosclerosis.

One intriguing finding with the C57Bl diet-induced model of atherosclerosis was that the weights of the animals were significantly lower in the infected group as compared to the control and infected plus antiviral groups, thus mirroring the acceleration in atherogenesis. Weight loss is a 'stress response' in mice. The atherogenic diet appeared to produce less weight gain and and also to accelerate atherogenesis as compared to mice on the diabetogenic diet. The additional effect of MHV-68 infection was then to cause further weight loss and yet more atherosclerosis. Could infection and diet both be 'stressors' on the mouse and be

acting via some sort of 'stress mechanism' to promote the development of atheroma? One universal cellular response to stress, (be it temperature, hypoxia or infection) is the release of chaperonins or heat shock proteins that protect cells. Heat shock protein-68 (HSP65) is of bacterial origin but is antigenically cross-reactive with its mammalian counterpart, heat shock protein-60 (HSP-60), as these proteins have been highly conserved throughout evolution. HSP 60 has been identified within atherosclerotic plaques in man (Xu et al., 1993), and anti-HSP antibodies have been found in patients with carotid stenosis (Xu et al., 1993). Serum antibodies to HSP65 have been found to correlate with seropositivity to *Chlamydia pneumoniae* and peripheral vascular disease (Mayr et al., 2000). HSP 65 has been shown to induce an immune response in mice and so promote atherosclerosis (George et al., 1999). Moreover in LDL-receptor deficient mice fed an atherogenic diet and rendered diabetic through STZ, there was an enhancement of both antibody and cell-mediated response to HSP65 (Keren et al., 2000). Therefore one relatively simple way of looking at the stress response in mice in Chapter 6 would be to examine their serum antibody levels to HSP60/65, and to see if this correlates with the amount of aortic atheroma.

Although many serological studies in man have supported the hypothesis that herpesviruses, namely HSV-1 and HCMV, have a role in atherogenesis, the largest study to date did not find such an association (Ridker et al., 1998). From the work presented in this thesis it would seem that high serum cholesterol levels and γ–herpesvirus infection provide a potent combined stimulus for atherogenesis. Individuals with a similar combination of high cholesterol levels and high likelihood of γ-herpesvirus infection may be at particular risk of infection-induced

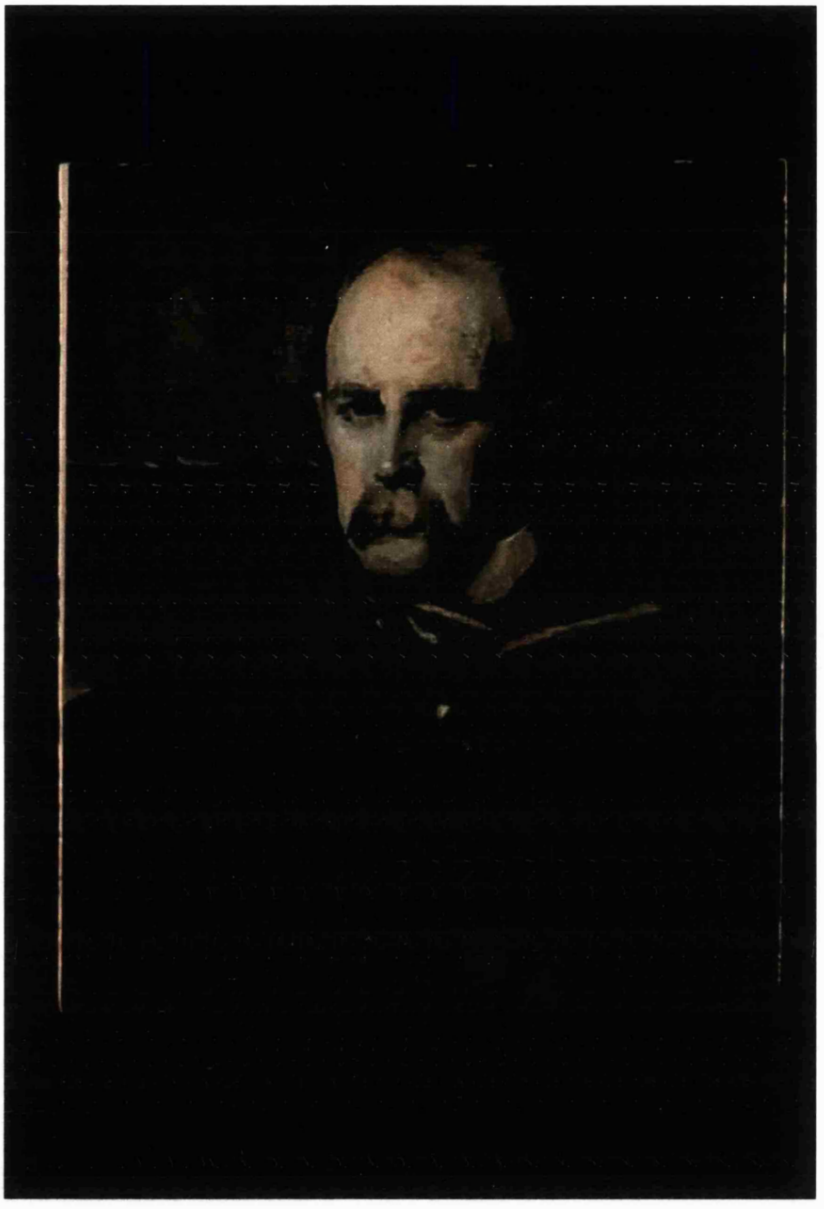
atherosclerosis. One such population is HIV positive individuals, who have drug-induced (protease inhibitors) or HIV-induced dyslipidaemia, and who have been infected with human-herpsvirus-8 (HHV-8). The results of the retrospective post-mortem survey presented in Chapter 7 would support the hypothesis that atheroma of the aorta, great vessels and coronary arteries is more common in those with Kaposi's sarcoma lesions which provided a surrogate for HHV-8 infection. Although this is an interesting finding, and is the first time HHV-8 infection has been implicated in atherosclerosis in man, the study has obvious flaws. What is needed is a large, prospective study with various serology tests for all the potentially pro-atherogenic organisms including that for HHV-8 (Dupin et al., 2000; Kellam et al., 1999).

Previous serological epidemiological studies on herpesviruses and human atherosclerosis have used either direct measures of atheroma (Zhou et al., 1996) or clinical endpoints such as myocardial infarction (Ridker et al., 1998). As endothelial dysfunction is such an established marker for atherosclerosis (section 1.7), this may prove a better endpoint than straightforward clinical indices in the test of an association with herpesvirus serology. As the data on HIV positive individuals has illustrated, certain human groups are more prone to infection-induced atherosclerosis than others, and previous evidence would also suggest that diabetics are another such group. Diabetics become dyslipidaemic and may also have reduced immunity to viruses (Diepersloot et al., 1987; Wismans et al., 1991). The data presented in Chapter 8 demonstrate for the first time that a significantly impaired vasodilation response to bradykinin is associated with previous infection with HCMV.

No interaction with diabetes was noted, which concurs with the findings in Chapter 6, that MHV-68 infection does not enhance atheroma induced by a diabetogenic diet. Gammaherpesvirus serology was not looked at in this study due to concerns raised over the consent obtained from those subjects taking part in the study. It would be important to address this in future studies although the prevalence of HHV-8 in the general London population is thought to be low. Finally, a further alternative that should not be forgotten is that a new and as yet unidentified γ-herpesvirus could be present in the population and be influencing atherogenesis in the presence of high cholesterol levels. The use of nested PCR with degenerate primers at conserved DNA segments has uncovered new herpesviruses in elephants and monkeys (Lacoste et al., 2000; Richman et al., 1999); similar studies on human atheromatous specimens might uncover new herpesviruses which have been promoting atherosclerosis in man but have only become relevant now our diet is higher in cholesterol.

This thesis explores the role of herpesvirus infection in atherosclerosis both in the context of an animal model and in specific groups of humans who may be especially at risk from herpesvirus-accelerated atherogenesis. Indeed the results in the murine model have directed and refined the design of the human studies. Although further experiments are evidently needed the work presented here provides valuable insights into the role of herpesviruses in atherosclerosis.

Figure 9.1.



'We have I think, evidence that sclerotic arteries are specially prone to spasm'.

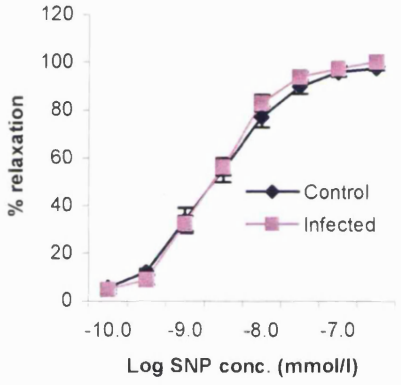
William Osler (1908), in Modern Medicine

With thanks to the Wellcome Library, London.

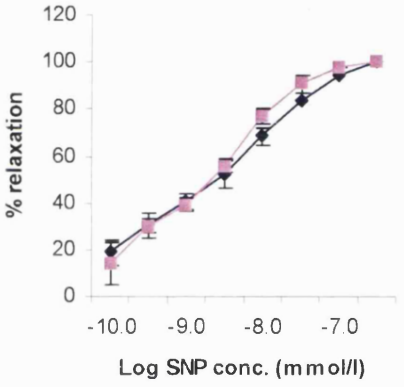
Appendix

A1 : Concentration response curves to endothelium independent vasodilators for Experiments 5.1-5.2. Non-significant ($p>0.05$) by 2 way ANOVA, and by t-Test on EC50s and on maximum relaxation achieved for all experiments.

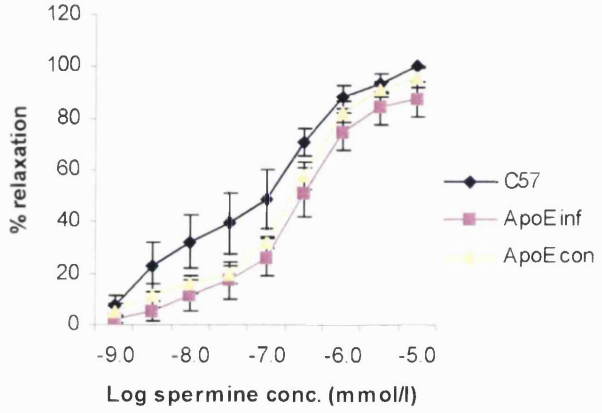
i) SNP responses in n=9 infected and n=10 control C57Bl mice at day 5 p.i..



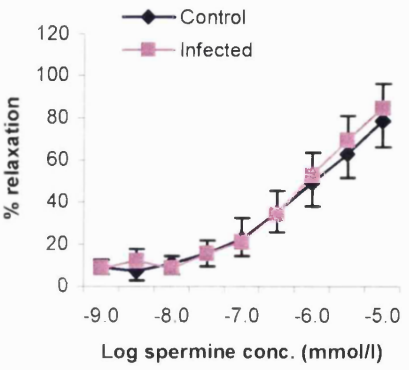
ii) SNP responses for n=5 C57Bl and n=7 ApoE-/-. infected older mice



iii) Spermine NONOate responses in n=4 C57Bl, n=6 ApoE-/- control & n=7 ApoE-/- infected older mice.



iv) Spermine NONOate responses in ApoE-/- control and ApoE-/- infected mice (n=5) at 5 days p.i.



A2: Phenylephrine responses in Experiment 5.1-5.3.

(i) Experiment 5.1: EC90 ($x10^{-7}M$) and force generated by that concentration (in grammes) in control and infected C57Bl mice 5 days post infection with MHV-68. Mean value (s.e.m.).

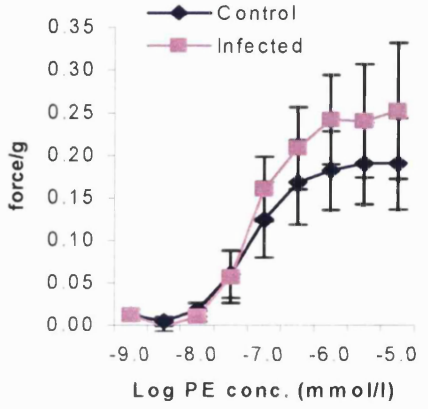
	Control	Infected	t Test p=
EC90	1.78 (0.64)	1.57 (0.29)	0.78
Force generated by EC90	0.40 (0.04)	0.73 (0.34)	0.31

(ii) Experiment 5.2: EC90 values (x10-7M) and the force generated by that concentration (in grammes) in C57Bl mice and ApoE-/- controls and infected mice. Mean value (s.e.m.).

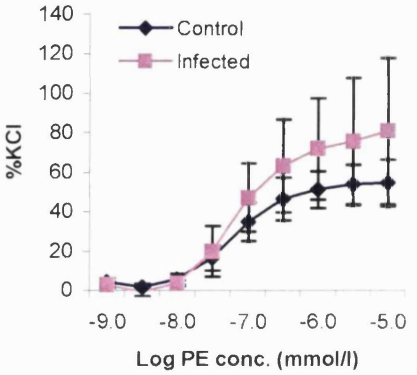
	C57Bl	ApoE control	ApoE infected	t Test p=	t Test p=
				C57Bl/ApoE control	C57Bl /ApoE infected
EC90	0.80 (0.35)	0.56 (0.21)	0.60 (0.16)	0.60	0.64
Force	0.22 (0.03)	0.25 (0.03)	0.29 (0.04)	0.57	0.20

(iii) Experiment 5.3: Concentration response curves to PE in n=5 ApoE-/- mice 5 days post infection with MHV-68 compared to n=5 control C57 mice aged 6-8 weeks, expressed as (a) absolute force generated and (b) as a percentage of the response to 50mM KCl. . Non-significant ($p>0.05$) by both 2 way ANOVA, and by t-Test on EC50s and maximum response achieved.

a)

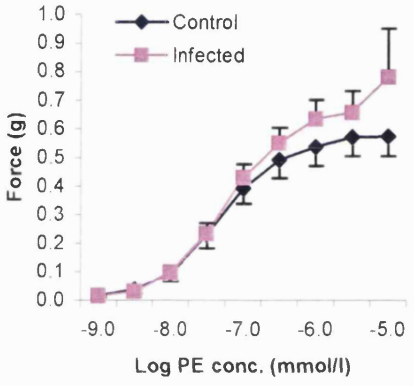


b)

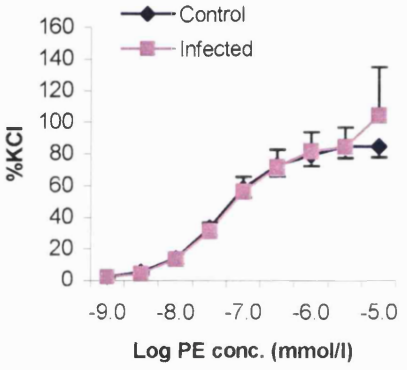


(iv) Experiment 5.4: Concentration response curves to PE in n=6 C57 mice 5 days post infection with MHV-68 compared to n=6 control C57 mice aged 6-8 weeks, expressed as (a) absolute force generated and (b) as a percentage of the response to 50mM KCl. Non-significant ($p>0.05$) by 2 way ANOVA, and by t Test on EC50s and maximum response achieved.

a)



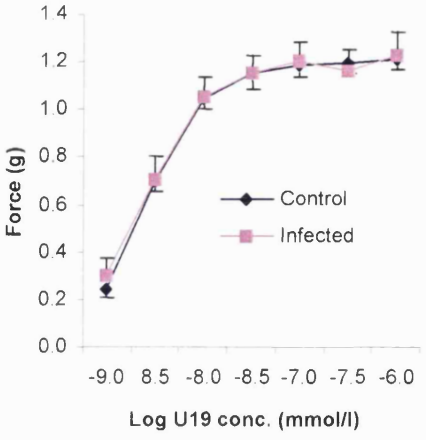
b)



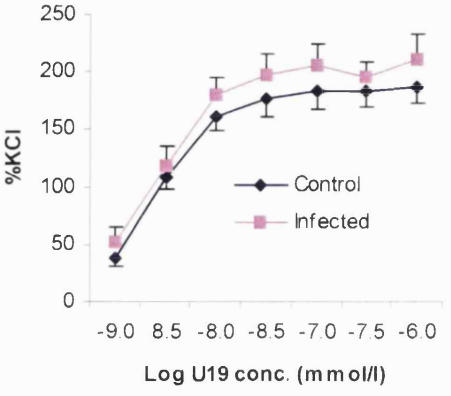
A3: Experiment 5.4.

Concentration response curves for U19 in the presence of $3x10^{-4}$M L-NAME for n=5 control and n=6 infected C57 mice 5 days post infection aged 6-8 weeks. $P>0.05$ by 2 way ANOVA, T test on EC50s and maximum achieved, expressed as (i) absolute force and (ii) as a percentage of the response to KCl.

(i)



(ii)



(iii) Precontracted aortic rings from n=3 C57 control compared to n=3 C57 infected with MHV-68 mice, aged 6-8 weeks had no significant difference ($p>0.05$ by t Test) in their response to a single dose of 10^{-4}M L-NAME (i.e. control 0.85g+-0.3, and infected 0.64g+-0.16,mean +- s.e.m.).

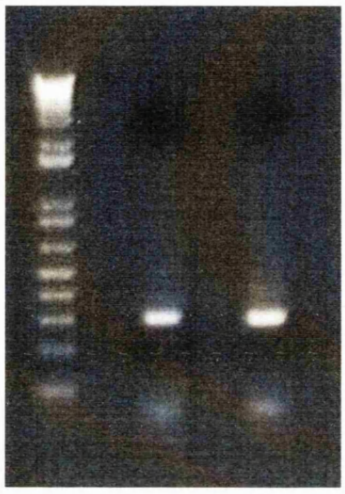
A4: Miscellaneous data on experiments in Chapter 5

Experiment 5.2. Relative sexes and ages.

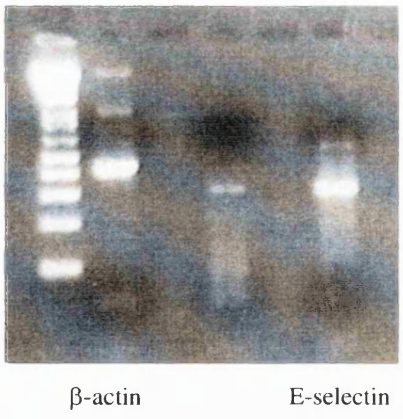
Mouse strain	Sex	Age(months)
C57Bl	4 female,5 male	7.8
ApoE-/- control	7 male	7.9
ApoE-/- infected	6 female, 5 male	8.5

Appendix 5. Distinct bands were obtained by RT-PCR for the adhesion molecules under investigation. All gels show a DNA ladder in the first left-hand lane, and the adhesion molecule in the adjacent lane (indistinct band in (b) represents E-selectin at a different magnesium concentration).

(a) VCAM and ICAM



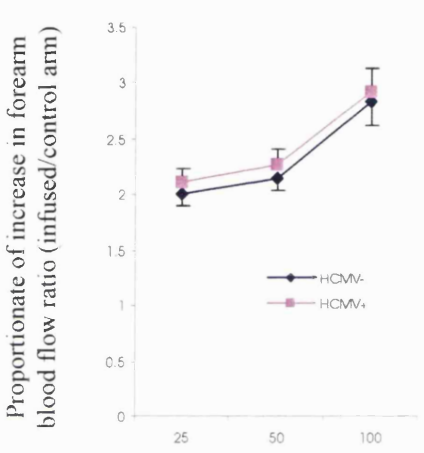
(b) E-selectin



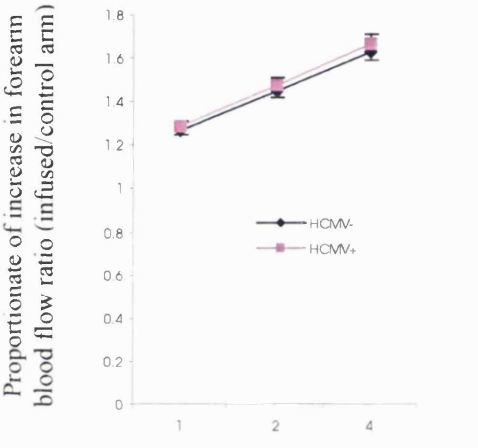
(c) P-selectin

A6: Chapter 8- Graphs showing area under the curve for vascular responses to vasoactive drugs. All non-significant ($p>0.05$) differences by statistics described in section 2.9.3.

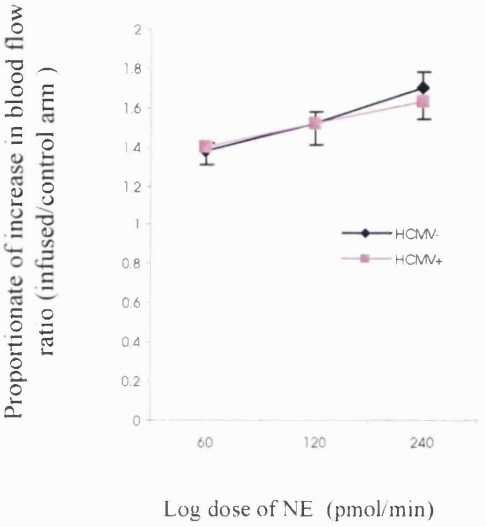
i) Response to ACh by HCMV seropositivity.



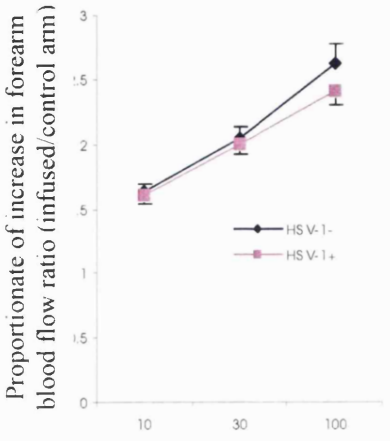
ii) Response to L-NMMA by HCMV seropositivity.



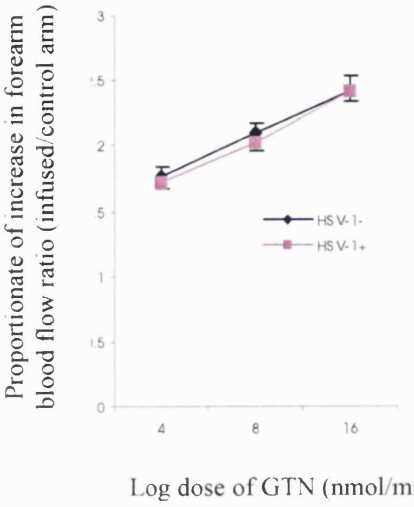
iii) Response to NE by HCMV seropositivity.



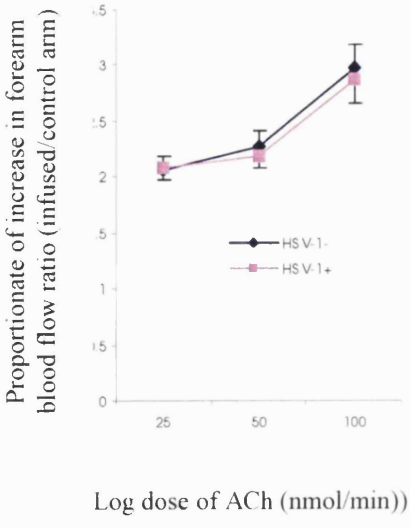
iv) Response to BK by HSV-1 seropositivity.



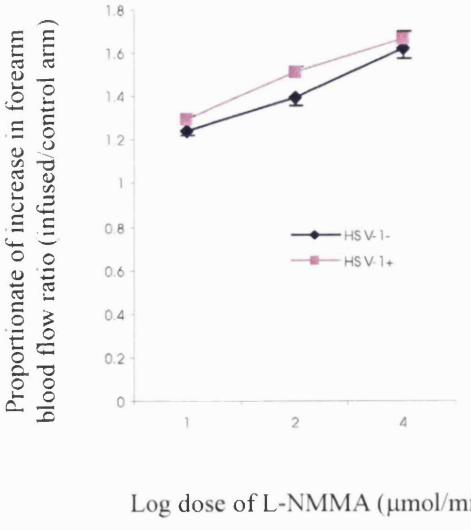
v) Response to GTN by HSV-1 seropositivity



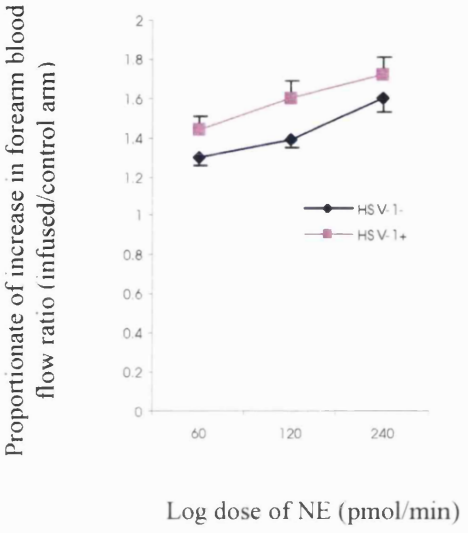
vi) Response to ACh by HSV-1 seropositivity.



vii) Respones to L-NMMA by HSV-1 seropositivity.



viii) Response to NE by HSV-1 seropositivity.



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Herpesvirus Infection Accelerates Atherosclerosis in the Apolipoprotein E–Deficient Mouse

Dagmar G. Alber, PhD; Kenneth L. Powell, PhD; Patrick Vallance, FRCP; David A. Goodwin, BSc; Cairistine Grahame-Clarke, MRCP

Background—Human herpesviruses have been implicated but not proven to be involved in the etiology of atherosclerosis. To determine whether there is a causal relationship, the effect of herpesvirus infection on the development of atherosclerosis was assessed in the apolipoprotein E–deficient (apoE−/−) mouse.

Methods and Results—In the present study, 3- to 4-week-old apoE−/− mice were infected with murine γ-herpesvirus-68 (MHV-68). Atheroma formation was accelerated over a 24-week period in infected apoE−/− mice compared with control uninfected apoE−/− mice. Acceleration of atherosclerosis was reduced by antiviral drug administration. Histological analysis of the atheromatous plaques showed no difference between lesions of infected and control mice. Viral mRNA was present in the aortas of infected mice before lesion development on day 5 after infection. This suggests that the virus may initiate endothelial injury, which is believed to be an early event in the development of atherosclerosis. Therefore, the virus may play a direct role in atherosclerosis rather than be an "innocent bystander."

Conclusions—These data demonstrate that a γ-herpesvirus can accelerate atherosclerosis in the apoE−/− mouse. This study provides the first report of a murine model in which to study the causative role of herpesvirus infection in the development of atherosclerosis. (***Circulation.* 2000;102:779-785.**)

Key Words: infection ■ atherosclerosis ■ viruses ■ apolipoproteins ■ pathology

The classic risk factors for atherosclerosis, namely, cigarette smoking, hypercholesterolemia, hypertension, and diabetes, account for only 50% of its incidence.[1] Infectious agents have been suggested as additional risk factors[2–5]; 2 strong candidates are *Chlamydia pneumoniae*[6,7] and herpesviruses. Herpesviruses have been proposed as potential initiators of arterial injury,[8–10] endothelial dysfunction, and local inflammation, which might trigger or exacerbate atherosclerosis.[11] Current evidence for a role of herpesviruses in vascular disease is conflicting, with data for and against causation.[12–14]

In the present study, apolipoprotein E (apoE)-deficient (apoE−/−) mice were infected with murine γ-herpesvirus-68 (MHV-68). ApoE−/− animals on a normal diet have high cholesterol levels and spontaneously develop atheroma,[15,16] resembling the human disease.[17] MHV-68 virus is a naturally occurring mouse pathogen[18–21] that causes arteritis in immune-deficient animals.[22] It is homologous to both human herpesvirus 8 (HHV-8, also known as Kaposi's sarcoma herpesvirus) and Epstein-Barr virus.[23,24] We show that infection of apoE−/− mice with MHV-68 significantly increases the amount of atherosclerosis in these animals. This suggests a direct correlation between virus infection and atherosclerosis. Possible mechanisms are discussed.

Methods

Experimental Mice

ApoE−/− mice (Jackson Laboratory, Bar Harbor, Me) were bred at the Royal Veterinary College, London, UK, under specific pathogen-free conditions. Mice were screened regularly for the presence of common adventitious mouse pathogens, and specifically, no evidence of murine cytomegalovirus and herpes simplex virus (HSV) type 1 was found. Experiments were carried out according to the guidelines of the Animals (Scientific Procedures) Act, 1986.

Virus Propagation and Infection of Mice

BHK-21 cells were infected at a multiplicity of infection of 0.001 with MHV-68 as previously described.[25] Extracellular virus was concentrated by centrifugation, resuspended in PBS, and titrated on NIH 3T3 cells.[19] For the preparation of purified virus, subconfluent BHK-21 cell layers were infected at a multiplicity of infection of 0.1, and the virus was separated from cell debris and media by velocity gradient sedimentation.[25] Three- to 4-week-old age- and sex-matched mice were inoculated intranasally under light anesthesia with 20 μL PBS containing of 5×10^5 plaque-forming units (pfu) of MHV-68. Control mice received an equivalent volume of PBS. Mice were culled 5, 11, 16, 20, and 24 weeks after inoculation. In a separate study, mice were inoculated intranasally or intraperitoneally with 5×10^5 pfu of MHV-68 or PBS (control). Some mice received the antiviral drug 2′-deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU), which was added to the drinking water (0.33 mg/mL) immediately after infection and throughout the experiment.

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From the Wolfson Institute for Biomedical Research (D.G.A., K.L.P., D.A.G., C.G.-C.) and the Centre for Clinical Pharmacology and Therapeutics (D.G.A., P.V., C.G.-C.), University College London, London, UK.

Correspondence to Dr Dagmar G. Alber, Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London, WC1E 6AU, UK. E-mail rmgzdga@ucl.ac.uk

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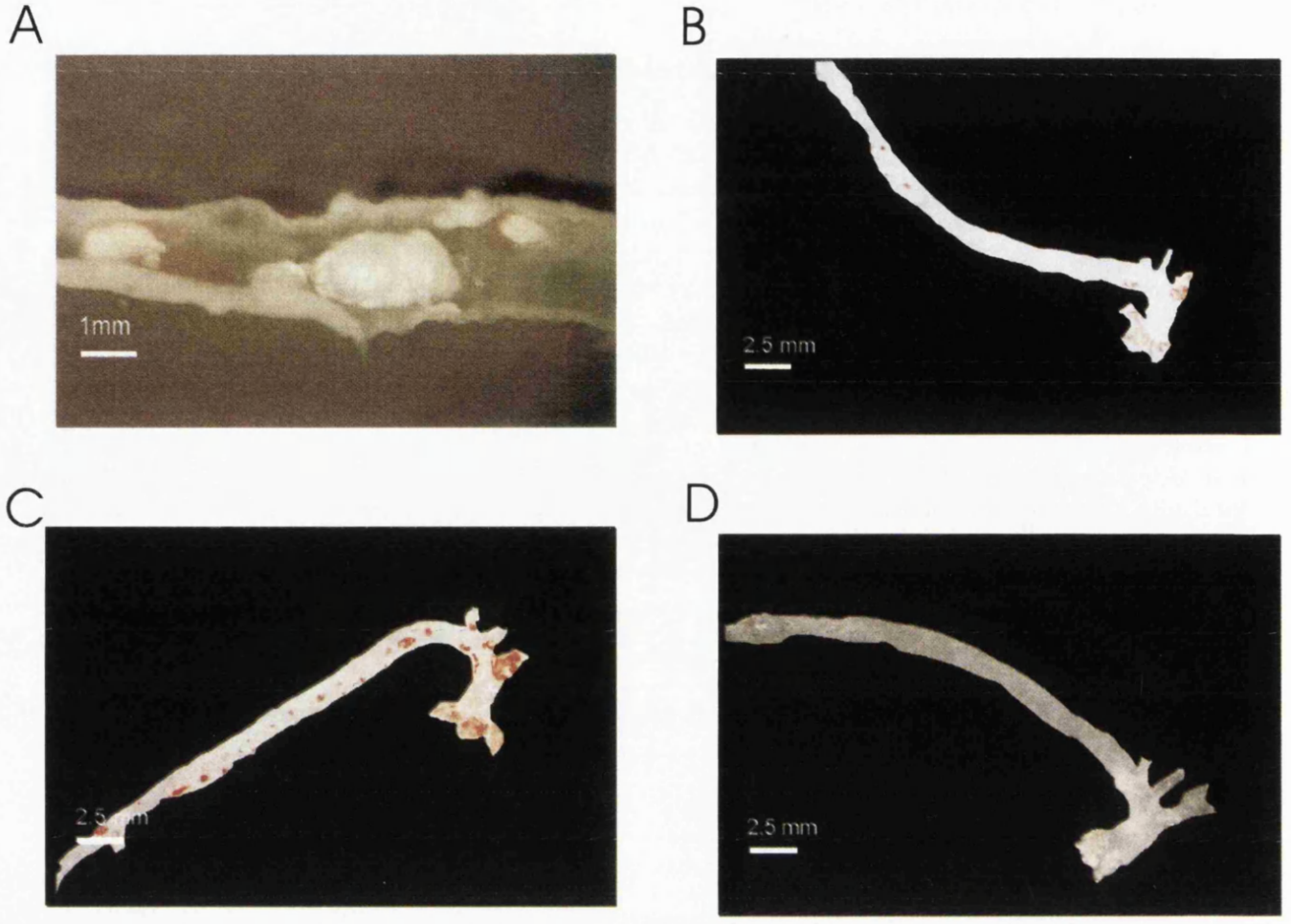

Figure 1. Atherosclerotic lesions in aortas of apoE−/− mice. A, Unstained aorta of infected apoE−/− mouse culled at 24 weeks, showing yellowish white raised atherosclerotic lesion in the lumen. B, Aorta of 28-week-old control apoE−/− mouse stained with oil red O with 6.4% atheroma. C and D, Stained aortas of infected apoE−/− mouse (22% atheroma, C) and C57BL/6J mouse (no atheroma, D) culled 24 weeks after infection.

Histology

Aortas were fixed overnight in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4), washed in PB, and postfixed in 1% osmium tetroxide in PB for 90 minutes. Tissue was washed in PB and dehydrated through a graded series of ethanol with a final change in propylene oxide. This procedure was followed by infiltration and embedding in epoxy resin. Sections (1 μm) were cut and stained with toluidine blue.

Quantification of Atherosclerotic Lesions

Aortas were excised and placed in cold PBS. Adventitial fat was carefully removed, and the aorta was opened up longitudinally from the cusps to the iliac bifurcation and divided into 2 strips. These were then placed sequentially in 70% ethanol (5 minutes), oil red O (90 minutes, Sigma Chemical Co), 70% ethanol (5 minutes), and water (5 minutes) and then mounted en face in glycerol-gelatin mounting medium (Sigma). Images were analyzed by use of the computer program ImageStat 1.0 (http://www.ucl.ac.uk/~ccaamrg/imagestat.html). The amount of atheroma was expressed as a percentage of the total area of the aorta.

Serology

An indirect ELISA was used as previously described,[26] with the exception that 96-well plates were coated with purified MHV-68.

Cell-Mediated Immunity

T-cell proliferation assays were carried out by culturing lymphocytes isolated from the spleen or the para-aortic lymph nodes of infected or control apoE−/− mice in the presence of UV-inactivated MHV-68 or influenza antigen (control antigen). Assays were set up as previously described.[26] The stimulation index (SI) was calculated as SI=mean cpm (test)/mean cpm (control).

Detection of Viral Message by RT-PCR

Total RNA was isolated from tissue by using TRIZOL reagent (Sigma). Reverse transcription (RT)–polymerase chain reaction (PCR) reactions were set up according to the manufacturer's instructions (Perkin-Elmer). For the RT reaction, an anchored oligo(dT) (17-mer) was used. As a negative control and to assess for possible DNA contamination, the RT reaction was set up without the reverse transcriptase enzyme for each sample tested. β-Actin primers (GACATGGAGAAGATCTGGCA and GCTCGAAGTCTAGAGCAACA) were used as a positive control for the PCR reaction (436-bp PCR product). Specific primers against viral genes were designed to correspond with fragments of the genes encoding the major capsid protein (AACGTCAGCTCTCCAGTTTG and AGCAGTCACAACATTCCCTC) and the DNA binding protein (AGAGCTACTACACCAACGTG and TCACGTACAGGACAGAGTTG) of MHV-68 (472 and 387 bp, respectively). PCR conditions were 94°C for 4 minutes and 30 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. The final cycle was 72°C for 7 minutes. Samples were run on a 2% agarose gel containing ethidium bromide, and bands were made visible by UV transillumination.

Localization of Virus by Immunohistochemistry

Frozen sections (10 μm thick) were cut from aortas of C57BL/6J mice, air-dried, and fixed in ethanol. Slides were washed with

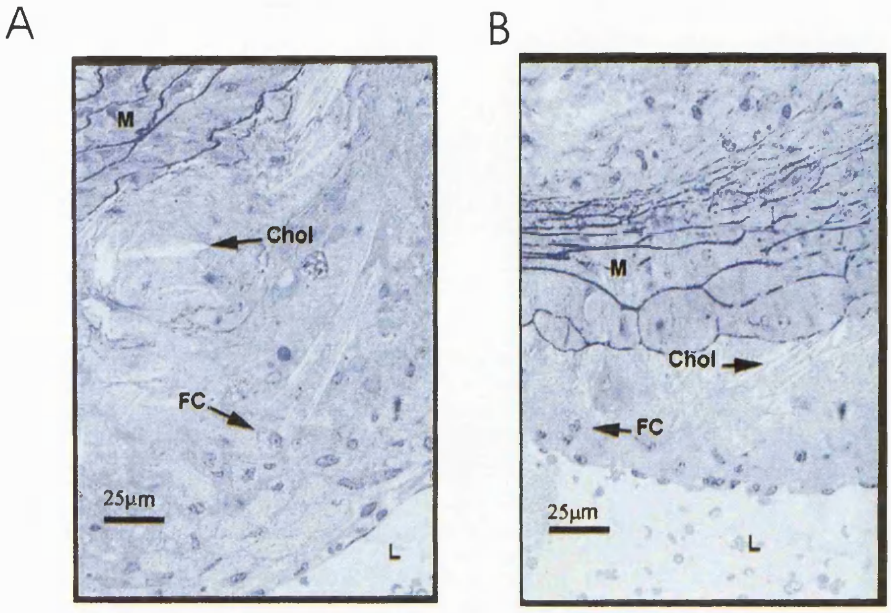

Figure 2. Histological analysis of atherosclerotic lesion in apoE−/− mice. Mice were inoculated intranasally with PBS (A) or MHV-68 (B) and culled 24 weeks after infection. Aortas were embedded in resin, as described in Methods, and 1-μm sections were cut. L indicates lumen; M, media; FC, foam cell; and Chol, cholesterol crystal.

Tris-buffered saline and 0.3% Tween (TBST, pH 7.4). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were washed 3 times with TBST and blocked with 10% serum in TBST for 10 minutes, and primary antibody (anti–MHV-68 polyclonal rabbit serum) was added at a 1/250 dilution in TBST. Sections were incubated at room temperature for 1 hour in a humidified chamber and washed 3 times with TBST. Horseradish peroxidase anti-rabbit antibody (1/100 dilution, DAKO) was used as the secondary antibody, and sections were incubated for 30 minutes before they were washed 5 times with TBST. Horseradish peroxidase activity was detected with use of a DAB solution (DAKO). The reaction was stopped, and sections were counterstained with Mayer's hemalum, dehydrated, and mounted with DPX (BDH). Viral antigen stained brown; nuclei stained purple.

Results

Virus Isolation From Lungs of ApoE−/− Mice Infected With MHV-68

Intranasal infection of apoE−/− mice (n=5) with MHV-68 caused a subclinical infection. Virus titers isolated from the lungs 5 days after infection were similar to those measured in infected C57BL/6J mice, the parental strain, indicating that apoE−/− mice were equally susceptible to MHV-68 infection (data not shown).

Lesion Development in Mice Infected With MHV-68

Typical macroscopically visible atherosclerotic lesions were seen in the aorta 24 weeks after infection. These plaques were yellowish white in appearance and projected into the lumen of the aorta (Figure 1A). To quantify the amount of atheroma, aortas were stained with oil red O (Figure 1B and 1C). The extent of atheroma was considerably greater in the infected animals than in the control animals 24 weeks after infection (Figure 1B and 1C). Aortas taken from C57BL/6J mice infected with MHV-68 for 24 weeks did not stain with oil red O, and no macroscopic lesions were detected (Figure 1D).

Histological analysis of aortas of apoE−/− mice taken 24 weeks after infection showed typical atherosclerotic lesions (Figure 2). These lesions showed thickening of the vessel wall with disruption of the elastic fibers and deposition of cholesterol crystals. The increase in smooth muscle cells (SMCs), the large number of foam cells, and the presence of inflammatory cells were also typical of atherosclerotic lesions in these animals.

Time-Dependent Virus-Accelerated Atherogenesis in ApoE−/− Mice Infected With MHV-68

The increase in atheroma was time dependent in infected and control apoE−/− mice. Compared with the control condition, MHV-68 infection led to a progressive increase in the amount of atheroma in the aorta, and this increase was most significant 24 weeks after infection (Figure 3A).

Effect of Antiviral Treatment of Infected Mice on the Development of Atherogenesis

The induction of atheroma by virus was similar when mice were infected either intranasally (group 1) or intraperitoneally (group 2) and examined 20 weeks after infection (Figure 3B). Compared with infected control mice (group 2, Figure 3B), mice that received antiviral treatment with 4′-S-EtdU (group 3) showed a mean 67% reduction in atherosclerosis.

Possible Mechanisms of Virus-Induced Atherogenesis

Cholesterol Levels in ApoE−/− Mice Infected With MHV-68

To determine whether differences in lipid metabolism might have contributed to the virus-induced accelerated atherosclerosis, we examined cholesterol levels. Total serum cholesterol levels were not significantly different at any time point in any group of mice and were in the range expected for apoE−/− mice (data not shown).

Immune Response of Infected ApoE−/− Mice

Induction of an inflammatory immune response is a potential mechanism in the development of atherosclerosis in mice.[27–29] We examined whether mice showed an altered humoral or cell-mediated immune response against MHV-68

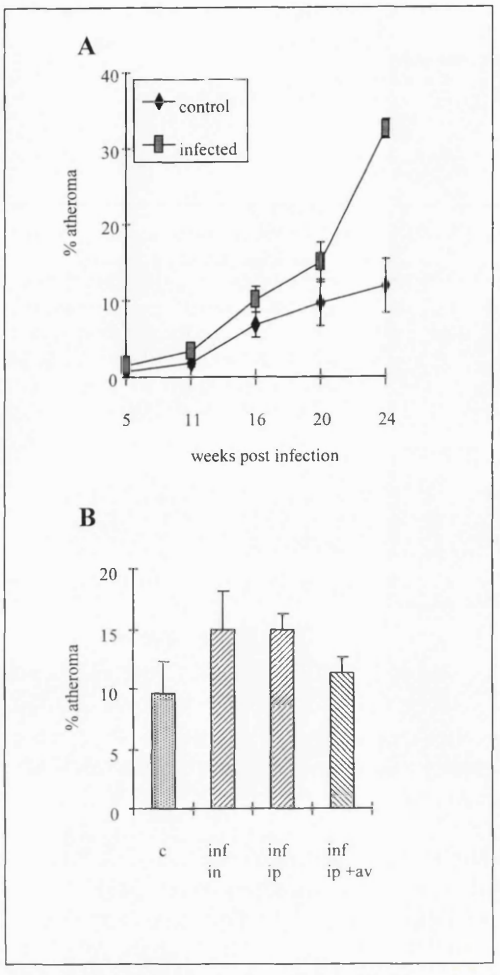

Figure 3. Acceleration of atherosclerosis in apoE−/− mice infected with MHV-68. A, Mice were infected intranasally with MHV-68 and culled at different time points after infection. Aortas were cut in half longitudinally, stained with oil red O, and mounted en face, and area of atheroma was quantified by computer image analysis. Area of stain deposition was expressed as percentage of whole area of aorta. Data represent mean±SEM (n=27 or 28). $P=0.0001$ for infection effect by 2-way ANOVA. B, Reduction of virus-induced atherosclerosis by treatment with antiviral drug is shown. Mice were mock-inoculated with PBS (group 4, c; n=6) or infected intranasally (group 1, inf in; n=7) or intraperitoneally (group 2, inf ip; n=7) or infected and treated with 4′-S-EtdU (group 3, inf ip+av; n=8). Mice were culled 20 weeks after infection. $P=0.055$ for infection vs infection plus antiviral treatment.

over a period of 24 weeks. Figure 4A shows that there was no significant difference in the antibody response measured by ELISA in serum samples of mice culled at any time after infection. The antibody response in serum samples was significantly lower in infected mice treated with 4′-S-EtdU than in infected control mice (Figure 4B).

Cell-mediated immune response was measured by in vitro T-cell proliferation assays in lymphocytes isolated from the spleen of infected mice. There was no significant difference between the proliferative response of splenocytes isolated from group 1 (infected intranasally, data not shown), group 2 (infected intraperitoneally), and group 3 (infected and treated with 4′-S-EtdU, Figure 4C). Uninfected mice (group 4) did not mount a MHV-68–specific immune response. Interestingly, lymphocytes isolated from the para-aortic lymph nodes

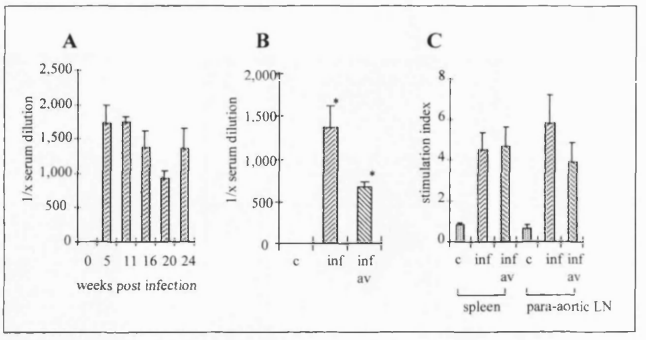

Figure 4. MHV-68–specific immune response in apoE−/− mice infected with MHV-68. In panels A and B, IgG anti–MHV-68 response was measured by ELISA. A, Mice were infected intranasally and culled at various time points after infection (n=4 to 7). B, Mice were inoculated with PBS (group 4, c) or intraperitoneally with MHV-68 (group 2, inf) or infected and treated with 4′-S-EtdU (group 3, inf av). The end-point ELISA antibody titer was calculated for each serum sample, and data represent mean±SEM values (n=6 to 9). *$P=0.02$ (by Student t test). C, T-cell proliferative response of lymphocytes isolated from infected or control mice is shown. LN indicates lymph nodes. Data represent mean±SEM values (n=3 to 5). $P<0.03$ (for infection vs control by Student t test). Mice were culled 20 weeks after infection.

of infected mice also proliferated when stimulated in vitro with MHV-68. Lymphocytes did not proliferate in the presence of a control influenza antigen (data not shown). These results suggest that viral antigen may be present in the aorta and that this maintains an MHV-68–specific T-cell response in the para-aortic lymph nodes.

Detection of Viral Message in Aortas of Infected ApoE−/− Mice and in ECs

To establish whether replicating MHV-68 was present in the aorta of infected mice, aortas were harvested 5 days after infection, and total RNA was isolated. Viral mRNA was detected by RT-PCR in the aortas. Two bands corresponding to the mRNA encoding the major capsid protein and the DNA binding protein were detected (Figure 5A). No viral message was detected in whole blood–derived RNA samples at this time point. This demonstrates that the aorta itself was infected with MHV-68. No MHV-68 RNA was detected in aortas from control mice.

To establish whether MHV-68 could directly infect endothelial cells (ECs), a murine endothelial cell line (sEND1) was infected with MHV-68 at a multiplicity of infection of 0.1 for 3 days. Viral mRNA was detected by RT-PCR in infected, but not in mock-infected, sEND1 cells (Figure 5A).

To determine whether MHV-68 could directly infect aortic tissue, an in vitro system was used. Dissected aortas were cultured for 24 hours and infected on day 2 with 1×10^3 pfu of MHV-68 or were mock-infected (control). Aortas were harvested 3 days after infection. Virus replicated within the aorta, as measured by RT-PCR (data not shown). Viral antigen was localized predominantly at the luminal side of the aorta (Figure 5B), which suggests that SMCs and ECs were both infected. No viral antigen was detected in mock-infected aortas (Figure 5C).

Discussion

The data in the present study demonstrate that infection of apoE−/− mice with MHV-68 accelerates atheroma forma-

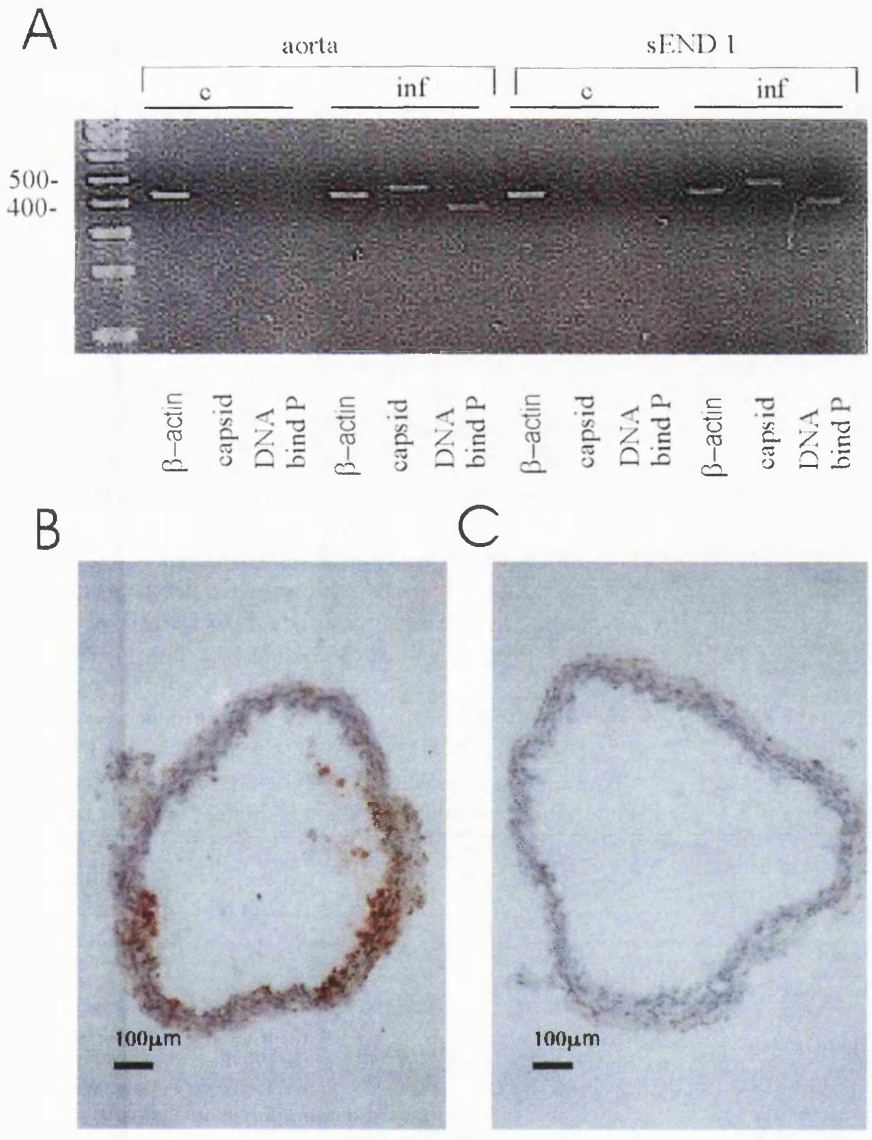

Figure 5. Detection of MHV-68 in aortas of infected mice and sEND1 cells. A, Aortic RNA was isolated from an apoE−/− mouse infected intranasally with MHV-68 for 5 days (inf) and from a control mouse (c). RNA was isolated from sEND1 cells infected with MHV-68 (inf) or mock-infected with PBS (c). MHV-68 RNA was detected by RT-PCR by use of primers specific for viral major capsid protein (capsid) and DNA binding protein (DNA bind P). β-Actin was used as a positive control. Size of relevant bands in base pairs is shown on 1-kb DNA plus ladder on the left. B and C, Localization of MHV-68 antigen by immunohistochemistry in aortas from C57BL/6J mice infected with MHV-68 in vitro (B) and mock-infected with PBS (C) is shown.

tion. Previous work in apoE−/− mice showed that repeated inoculation with *C pneumoniae* causes a slight increase in atheroma formation, and that *C pneumoniae* is found within plaques.[30,31] In the present study, we show that a single infection with MHV-68 is sufficient to markedly enhance atherosclerosis without altering lesion histology. Furthermore, MHV-68 was detected in the aorta before atheroma developed. Antiviral treatment reduced lesion formation. These findings are not consistent with the suggestion that infective organisms are merely "innocent bystanders" in mature atheromatous plaques[32] but suggest a direct role for MHV-68 in accelerating atherogenesis.

Evaluation of the ApoE−/− Mouse Infected With MHV-68 as a Model for Atherosclerosis

The quantification of atheroma with oil red O is a well-established method to assess atherosclerosis in a murine model. Histological analysis of lesions confirmed a typical appearance of atherosclerosis with no change in cellular composition between infected and control animals. This indicates that the virus accelerates rather than fundamentally changes atheroma. This acceleration was present early in the time course of atherogenesis and became even more marked at 20 to 24 weeks. Whether the amount of atheroma in infected animals increases further remains to be established. Although inhibition of virus-accelerated atheroma formation by antiviral treatment just failed to reach statistical significance, the effect of treatment with the antiviral drug 4′-S-EtdU would support the finding that the acceleration seen in infected animals was due to the virus. 4′-S-EtdU does not prevent the establishment of virus latency,[20] which may explain why the effect of virus infection on the development of atherosclerosis was not completely inhibited by drug treatment. It is possible that the antiviral treatment has some other effect on atherogenesis, and further studies would be required to test this hypothesis. Our findings extend and support the early work of Fabricant et al[9] but, for the first time, demonstrate that a herpesvirus can induce atherosclerosis in a mammalian model.

Antibody Response and Serology

The need for an appropriate mammalian model in the study of the role of herpesvirus infection in atherogenesis is para-

mount, because despite mounting evidence from clinical studies, no conclusive causative link has been demonstrated. Positive serology for human cytomegalovirus has been associated with the presence of atheroma,[33] restenosis,[34] accelerated atheroma, and subsequent graft rejection after cardiac transplantation.[35] Yet, recently, a large study showed no evidence of an association between human cytomegalovirus (HCMV) or HSV serology and systemic inflammation or C-reactive protein, both of which are known to be predictive for cardiovascular risk.[13] In the present study, the serum IgG response to MHV-68 was similar at 5 weeks after infection, when little atheroma was present, or later (at 24 weeks), when a marked increase in atheroma was seen. Furthermore, apoE–/– mice inoculated at 9 to 10 weeks seem to develop less atheroma than those infected at 4 to 5 weeks (authors' unpublished data, 2000) despite generating similar antibody responses. It may be that the age at which the initial infection is acquired and the frequency of viral reactivation are both crucially important in determining the effect on atherogenesis. If this is the case in humans, then studies based on serology might yield false-negative results.

Possible Mechanisms for Virus-Induced Accelerated Atherogenesis

How does the virus, either alone or in conjunction with other known risk factors, influence atherogenesis? In the model of atherogenesis examined by Ross,[11] various noxious stimuli induce inflammatory changes within the arterial wall to initiate a fatty streak, which then may develop into mature plaque. Viruses could act at any stage in this process either indirectly or directly.

Indirectly, viruses may act by increasing serum cholesterol levels and thus promoting atherosclerosis. In the present study, we found that the virus had no effect on total cholesterol levels. However, it would be important to examine cholesterol subfractions to determine whether infection alters the lipid profile in more subtle ways. In the Marek's disease model of atherosclerosis in chickens, changes in lipid metabolism have been detected,[36] but no increase in total cholesterol was seen. Previous studies in the apoE−/− mouse have suggested that the development of atherosclerosis is caused by hyperlipidemia,[15–17] although other yet-unknown mechanisms could contribute to this process. Infection of C57BL/6J mice, which have normal cholesterol levels, did not cause atherosclerosis; thus, virus infection alone is not sufficient to induce atherosclerosis. It would seem that a high level of total serum cholesterol is a necessary prerequisite for the virus to enhance atheroma.

A direct mechanism by which MHV-68 may accelerate atherogenesis is to target 1 of the 2 main cellular constituents of the plaque, either the EC or the SMC. In the present study, we have demonstrated that (1) MHV-68 can be detected in aortas of infected apoE−/− mice, (2) MHV-68 localizes predominantly to the luminal site of the aorta after infection of cultured aortas in vitro, and (3) MHV-68 can infect sEND1 cells. Human herpesviruses have similar properties. Both HCMV and HSV can infect ECs, initiating cellular responses similar to those in atherogenesis.[37,38] Furthermore, HCMV behaves differently in aortic ECs (a vessel susceptible to atheroma) than in brain microvascular ECs (in which atheroma is not found).[39] In aortic ECs, HCMV is nonlytic and is released persistently, whereas in small vessel ECs, it causes rapid lysis. The human homologue of MHV-68 is HHV-8, and this is known to transform ECs.[40] Thus, viral infection may alter EC function and thereby promote atherosclerosis.

Weck et al[22] showed that MHV-68 can also infect SMCs. Benditt et al[10] were the first to show that SMCs from a single plaque were monoclonal rather than polyclonal in origin. Thus, a single SMC may proliferate in a manner analogous to tumor development. It is plausible that MHV-68 is enhancing atherosclerosis via SMCs by as-yet-unexplored means.

Clinical Significance

The present study shows that a murine γ-herpesvirus can induce atherogenesis in a murine model of hyperlipidemia and atheroma. We selected MHV-68 because it is a naturally occurring infection in mice and establishes a latent infection. It is unknown whether the results of our experiments relate to general systemic inflammation and atherosclerosis, to herpesviruses as a family and atherosclerosis, or, more specifically, to γ-herpesviruses and atherosclerosis. It will be logical to test whether these findings can be reproduced with an α-herpesvirus (HSV-1) or a β-herpesvirus (MCMV).

The human homologue of MHV-68, HHV-8, infects immunosuppressed individuals and is thought to be the cause of AIDS-related Kaposi's sarcoma. These data might suggest a possible link between HHV-8 and AIDS-induced atherogenesis, in particular in those patients with high lipid levels. Such a link is eminently testable by epidemiological studies.

Acknowledgments

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