CHARACTERIZATION OF THE CELLULAR RECEPTORS FOR CARDIOVIRULENT COXSACKIEVIRUS B3

by

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ABSTRACT

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This thesis examined interactions between group B coxsackieviruses (CVB) and cell surface receptors, and identified mechanisms by which virus-receptor interactions could be important in the development of CVB-induced heart disease. The first study examined interactions between cardiovirulent CVB3 variants and their receptor, the decay accelerating factor (DAF). DAF was found to serve as a receptor for select virus variants. A role for DAF in the triggering of T cell activation pathways characteristic of CVB-induced myocarditis was hypothesized. The second study examined interactions between CVB and the coxsackie-adenovirus receptor (CAR). CAR was shown to be used by all CVBs tested including reference serotypes, clinical isolates, and cardiovirulent CVB3 strains. CAR was also identified as a functional receptor for the related swine vesicular disease virus. From these findings, a model for the role of CAR in determining virus tissue tropism was developed. The third study identified sites on the CVB3 virion which may have a role in virus-receptor binding. A system was developed for rapidly and reliably cloning infectious virus genomes. In the course of this work the genome of CVB6 was sequenced, which led to the completion of a phylogenetic profile for group B coxsackieviruses.

EXPANDED ABSTRACT

Group B coxsackieviruses (CVB) are etiologically associated with many human diseases. Of primary interest to this setting is the role of CVB in the genesis of virus-induced heart diseases, namely myocarditis and dilated cardiomyopathy. Myocarditis is an inflammation of the myocardium in association with myocardial cell necrosis. It is the commonest cause of acquired heart failure in children, and is considered to have an important predisposing role in the development of dilated cardiomyopathy in adults. Dilated cardiomyopathy is one of the commonest conditions that requires heart transplantation. Yet despite the importance of these diseases with their high mortality in both pediatric and adult populations, no effective treatment has yet been developed. Over 50% of patients with biopsy proven myocarditis die within 4 years of diagnosis.

The pathogenesis of viral myocarditis arises by the interaction of three principal components. The first is an infecting virus, and CVB have been implicated in more than 50% of cases. The second is the host myocardial cell and its response to infection. The third component is the immune response in the host, with its potential pathogenic consequences.

A critical first event in the disease process involves virus binding and infection of host cells, which is determined by the presence of receptors for the virus. To date, two receptor molecules for CVBs have been identified, and they are termed the decay accelerating factor (DAF) receptor, and the coxsackie-adenovirus receptor (CAR). Since these receptor molecules have been only recently identified, the role that they play in CVB infections and in the genesis of CVB-induced heart disease is an important topic of investigation. The overall objective of this thesis was to characterize interactions between CVB and the DAF and CAR receptors, and to identify mechanisms in which virus-receptor binding may impact on the pathogenesis of viral heart disease.

In the first part of this study, interactions between cardiovirulent CVB3 and the DAF receptor are described. It was shown that only specific cardiovirulent CVB3

variants bind to the DAF receptor. These DAF binding variants were more cardiovirulent in murine models of CVB-induced heart disease than the virus strains which did not bind to DAF. It was further demonstrated that the virus variants bind to the third domain of the DAF molecule (SCR3), a site which may be physiologically relevant since it is also the domain important for DAF complement regulatory functions and for DAF-mediated activation of T cells. It was also shown that CVB binding to DAF positive cells leads to DAF downregulation on the cell surface, and thus may render the infected cells more susceptible to complement lysis. Finally, CVB3-DAF interactions were shown to potentially lead to the activation of signaling cascades involving protein tyrosine kinases, using transgenic $p56^{lck}$ knock-out mice.

In the second part of this study, interactions between CVB and the CAR receptor are characterized. CAR was shown to be a functional receptor for all CVBs tested, namely, CVB1-6 reference strains, clinical isolates, and cardiovirulent variants. Hamster CHO cells expressing CAR, but not control cells lacking the CAR receptor molecule, were permissive to these viruses. Conversely, virus infection of susceptible cells could be blocked by pretreating the cells with anti-CAR MAbs, or by pretreating virus with soluble CAR receptor. Using these assay systems, it was shown that CAR and DAF are also receptor molecules for the related swine vesicular disease virus (SVDV). Based on these findings, it was postulated that CAR is a determinant of virus tropism *in vivo*, and several new directions for examining CAR-CVB interactions and their role in the genesis of viral heart disease were formulated.

In the third part of this study, sites on the CVB3 virion which may be important for virus-receptor binding were identified. This was accomplished by comparing the capsid protein sequences of the CVB3 variants used in the above studies. A total of 18 amino acid differences between the virus sequences were found, and they were scattered among the 4 viral coat proteins. Since the crystal structure of the CVB3 virion has been solved, the amino acid differences could also be mapped onto the CVB3 virion. Of these, six sites were found on the virion surface. Several sites were located at or near

the receptor binding canyon depressions, and others were located on immunogenic domains protruding from the virion surface. The remaining amino acid differences mapped on the internal portions of the virion, and were often found within regions of secondary structure. The viruses segregated into two groups which were consistent with their DAF receptor binding phenotypes. Future studies involving cloning and site-directed mutagenesis are likely required to confirm the importance of the identified amino acid differences for virus-receptor binding. To facilitate this process, a model system for rapidly and reliably cloning infectious virus genomes was developed. As an example the genome of CVB6 was sequenced and cloned, which allowed for the successful completion of a phylogenetic tree for group B coxsackieviruses.

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LIST OF ABBREVIATIONS

A particle	enterovirus virion altered by virus-receptor binding
Ad	adenovirus
C3	complement factor 3
canyon	large depression on enterovirion, receptor binding site
CAR	coxsackie-adenovirus receptor
CAV	coxsackieviruses, group A
CD55	decay accelerating factor (DAF)
cDNA	complementary DNA
CHO	chinese hamster ovary cell line
CHOP	polyoma virus transformed CHO cells
CNS	central nervous system
CPE	virus induced cytopathic effect in cell monolayers
CSF	cerebral spinal fluid
CVB	coxsackieviruses, group B
DAF	decay accelerating factor
DCM	dilated cardiomyopathy
EL-4	murine T lymphoma cell line
EMCV	encephalomyocarditis virus
EV	echovirus
GPI anchor	glycophosphoinositol linkage to cell membrane
HeLa	human cervical carcinoma cell line
HLA	human leukocyte antigens
HRV	human rhinovirus
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRES	internal ribosomal entry site
MAb	monoclonal antibody
MAb 914	monoclonal antibody to DAF SCR3
MHC	major histocompatiblity complex
MOI	multiplicity of infection
NK cells	natural killer cells
NO	nitric oxide
NTR	nontranslated region of the enterovirus genome
p56lck	protein tyrosine kinase

PCR	polymerase chain reaction
PFU	plaque forming unit
PIPLC	phosphatidylinositol phopholipase C enzyme
PK cells	porcine kidney cells
pol	polymerase
pro	protease
protomer unit	array of enterovirus VP1-3 surface residues
PV	poliovirus
PVR	poliovirus receptor
RD cells	human rhabdomyosarcoma cells
RGD motif	arginine-glycine-aspartic acid tripeptide
RmcB	monoclonal antibody to CAR
RNA	ribonucleic acid
Rp-a	receptor protein a (CAR)
RT	reverse transcription
sCAR	soluble CAR
SCID	severe combined immunodeficiency mice
SCR	short consensus repeat motif
ST cells	swine testis cells
SVDV	swine vesicular disease virus
T-cells	lymphocytes
TCID50	amount of virus causing CPE in 50% of cell monolayer
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
Vero	african green monkey kidney cell line
VLA-2	very late antigen-2
VP	viral coat protein
VP2 puff	VP2 protrusion from enterovirion capsid, immunogenic site
VP3 knob	VP3 protrusion from enterovirion capsid, immunogenic site
VRC	virus-receptor complex
YAC-1	murine T lymphoma cell line

LIST OF PUBLICATIONS

Work presented in this thesis is based on the following publications

Introductory sections on the pathogenesis of CVB heart disease

Liu, P., T. Martino, A. Opavsky, and J. Penninger. (1996). Viral myocarditis: Balance between viral infection and immune response. *Can. J. Cardiol.* 12, 935-943.

Martino, T. A., P. Liu, M. Petric, and M. J. Sole. (1995). Enteroviral myocarditis and dilated cardiomyopathy: A review of clinical and experimental studies, p. 291-351. In H. A. Rotbart (ed.) *Human Enterovirus infections*, ASM Press, Washington D.C.

Martino, T. A., P. Liu, and M. J. Sole. (1994). Viral infection and the pathogenesis of dilated cardiomyopathy. *Circulation Research* 74, 182-188.

Martino, T. A., P. Liu, and M. J. Sole. (1994). Viral infection and the pathogenesis of dilated cardiomyopathy: Time to revisit the virus. *Heart Failure* 9, 218-226.

Study #1 - The DAF receptor

Martino, T. A., M. Petric, M. Brown, K. Aitken, C. J. Gauntt, C. D. Richardson, L. H. Chow, and P. P. Liu. (1998). Cardiovirulent coxsackieviruses and the decay accelerating factor (CD55) receptor. *Virology* 244, 302-314.

Liu, P., K. Aitken, Y. Y. Kong, M. A. Opavsky, T. Martino, F. Dawood, W. H. Wen, I. Kozieradzki, K. Bachmaier, D. Straus, T. W. Mak, and J. M. Penninger. (2000). The tyrosine kinase $p56^{lck}$ is essential in coxsackievirus B3-mediated heart disease. *Nature Medicine* 6, 429-434

Martino, T. A., K. Aitken, J. Penninger, T. Mak, M. J. Sole, F. Dawood, W. H. Wen, and P. Liu. (1995). Mice with p56lck T-lymphocyte tyrosine kinase knocked out are resistant to coxsackieviral myocarditis. *Can. J. Cardiol.* 11(Suppl. E): 78E (student award presentation).

Study #2 - The CAR receptor

Martino, T. A., M. Petric, H. Weingartl, J. M. Bergelson, M. A. Opavsky, C. D. Richardson, J. F. Modlin, R. W. Finberg, K. C. Kain, N. Willis, C. J. Gauntt, and P. P. Liu. (2000). The coxsackievirus-adenovirus receptor (CAR) is used by reference strains and clinical isolates representing all six serotypes of coxsackievirus group B and by swine vesicular disease virus. *Virology*, 271, 99-108

Study #3 - The CVB3 virion receptor binding sites

Martino, T. A., R. Tellier, M. Petric, D. M. Irwin, A. Afshar, and P. P. Liu. (1999). The complete consensus sequence of coxsackievirus B6 and generation of infectious clones by long RT-PCR. *Virus Research*, 64, 77-86.

Martino, T., K. Aitken, L. Chow, C. Gauntt, L. Bartoski, A. Bugeja, L. Weinrib, J. Ko, J. Martino, M. Shin, M. Sole, M. Petric, and P. Liu. (1997). Identification of capsid mutations in common myocarditic strains of coxsackievirus B3 by nucleotide sequencing: Implications for virus-receptor, -immune, and -autoimmune interactions. *Keystone Symposia on Immunologic Aspects of Cardiovascular Disease.* Keystone Colorado.

A. INTRODUCTION TO ENTEROVIRUSES

A.1. Classification of enteroviruses

Enteroviruses are classified into the family *Picornaviridae* (reviewed in Melnick 1996). All enteroviruses display similar physical and chemical properties, including their ability to infect via the alimentary tract, their small size (20-30nm), and resistance to ether. Enteroviruses were further classified by their ability to cause disease in mice, or to grow in cell cultures primarily of human or primate origin. Enteroviruses classified by these methods include the polioviruses (serotypes 1-3), group A coxsackieviruses (serotypes 1-22,24), group B coxsackieviruses (serotypes 1-6), and echoviruses (serotypes 1-7, 9, 11-33). However, some enteroviruses, after further characterization by serologic or pathogenic or phylogenetic analysis, have been reclassified. For example, coxsackievirus A23 is the same as echovirus 9, echovirus 8 is a strain of echovirus 1, echovirus 10 is reovirus type 1, echovirus 28 is human rhinovirus 1A, and echovirus 34 is a strain of coxsackievirus A24 (Harris et al., 1973; Huttunen et al., 1996; Melnick et al., 1961; Oberste et al., 1998, 1999; Pöyry et al., 1996, 1999; Schmidt and Lennette, 1970). To simplify classification, new prototypes are now designated as an enterovirus of a specific number. This group currently contains enteroviruses 68-71, while enterovirus 72 has been reclassified as human hepatitis A virus (Purcell, 1993). Vilyuisk virus is also considered a human enterovirus, and nucleotide sequencing indicates that it may have diverged from Theiler's murine encephalomyelitis virus (Pritchard et al., 1992). There are also enteroviruses that cause disease in non-human species (reviewed in Rueckert, 1990), and include Theiler's murine encephalomyelitis virus, simian enteroviruses (serotypes 1-18), bovine enteroviruses (serotypes 1,2), porcine enteroviruses (serotypes 1-11), and swine vesicular disease virus (SVDV). SVDV is similar antigenically and phylogenetically to coxsackievirus B5 (Graves, 19973; Zhang et al., 1993, 1999).

A.2. History of human enteroviruses.

Polioviruses were the first members of the enterovirus group to be discovered (reviewed in Melnick, 1990, 1996). They are the causative agents of poliomyelitis, an acute illness characterized by aseptic meningitis and weakness or paralysis of one or more extremities. Poliomyelitis may have affected mankind since antiquity, although recorded references to the disease can be traced back only to the 18th century, and clusters of poliomyelitis cases were first reported in the 19th century. Polioviruses were discovered to be the etiologic cause of poliomyelitis in 1908, when Landsteiner and Popper demonstrated that a "filterable agent" from the neuronal tissue of a human patient who died of poliomyelitis could be injected intraperitoneally into a *Cynocephalus* monkey and cause the characteristic spinal cord lesions (Landsteiner and Levaditi, 1909; Landsteiner and Popper, 1908). Investigative efforts on polioviruses also led to other important discoveries. The development of cell culture monolayers for the propagation of viruses and characterization of virus-induced CPE stems from work done by Enders, Weller, and Robbins with polioviruses in 1949 (Enders et al., 1949), for which they were recognized with a Nobel Prize. Also, the development of poliovirus vaccines leading to the anticipated eradication of poliomyelitis is a landmark development in the field of medical science.

The coxsackieviruses were discovered by Dalldorf and Sickles in 1948, while investigating an outbreak of poliomyelitis in the village of Coxsackie in New York (reviewed in Melnick, 1996). The investigators discovered that inoculating the virus intracerebrally into newborn mice produced paralysis and lesions in skeletal muscle (Dalldorf and Sickles, 1948). Coxsackieviruses were subsequently divided into two groups based on the type of paralysis produced in mice. Group A coxsackieviruses cause generalized myositis with flaccid paralysis, and affect mostly the striated muscles (Dalldorf and Sickles, 1948). The group B coxsackieviruses cause local myositis and spastic paralysis, as well as degeneration of the brain, pancreas, heart muscle, and embryonic fat pads under the skin (Godman et al., 1952). In humans, coxsackieviruses

are associated with a number of clinical conditions, including myocarditis and dilated cardiomyopathy. Over the past five decades, extensive investigative efforts spanning the disciplines of virology, cardiology, and immunology have provided considerable insight into the pathogenesis of these cardiac diseases. This is discussed in detail in section B below.

Echoviruses were also discovered in the course of investigations on poliomyelitis (reviewed in Fenner and White, 1976; Melnick, 1990, 1996). They do not generally cause disease in mice, and were isolated instead when monkey kidney tissue cultures first came into use. Echoviruses were initially isolated from the feces of apparently normal individuals, and thus were designated 'orphan' viruses without a parent disease. Indeed, the name ECHO is derived from the words *enteric* *c*ytopathogenic *h*uman *o*rphan virus. Enteroviruses have since been etiologically associated with clinical diseases, most commonly meningitis, encephalitis, and exanthemas (reviewed in Grist et al., 1978; Melnick, 1990)

A.3. The enterovirus genome.

The genomic sequences of all prototype strains of human enteroviruses and many phenotypic variants have been reported in whole or in part (Martino et al., 1999; Oberste et al., 1998, 1999; Polacek et al., 1999; Pöyry et al., 1996; and references within). The enterovirus genome is single stranded positive sense RNA, approximately 7.5 kb in length. It contains a 5' noncoding region, a open reading frame encoding a single large polyprotein, and a 3' nontranslated region. Organization of the enterovirus genome is shown in Fig. 1, and described below.

I). The 5'-non-translated region. The first approximately 10% of the enterovirus genome has no coding capacity. For polioviruses, the first approximately 88 nucleotides in this region form a cloverleaf structure (Fig. 1) (reviewed in Hellen and Wimmer, 1995; Wimmer et al., 1993). The cloverleaf binds a ribonucleoprotein complex containing viral protein 3CD (viral-encoded protease $3C^{pro}$ and viral encoded RNA-

dependent RNA polymerase 3D^{Pol}) and a host cell factor, which may serve to stabilize a partially melted double-stranded RNA for the initiation of positive strand RNA synthesis (Andino et al., 1990a,b, 1993; Wimmer et al., 1993). A similar structure has not been predicted to occur in the 5'NTR of coxsackievirus B3 (CVB3) (Fig. 1, inset), although nucleotides within this region of the CVB3 genome are believed to be important for initiation of CVB3 protein translation (Yang et al., 1997).

A second structure found in the 5'NTR is the internal ribosomal entry site (IRES) (Fig. 1). For polioviruses, the core structure of the IRES is found between nucleotides 134-585 (Nicholson et al., 1991). For CVB3, the core sequence of the IRES is located between nucleotides 432-639, and thus it is shorter and found closer to the end of the 5' NTR than the poliovirus IRES (Fig. 1, inset) (Liu et al., 1999; Yang et al., 1997). IRES structures are also predicted to occur in the 5'NTR of other enteroviruses and rhinoviruses (termed type 1 IRES), and cardioviruses and aphthoviruses (type 2 IRES), and in the genome of hepatitis C virus, the pestivirus swine fever virus, and in some cellular mRNA's such as eukaryotic initiation factor-4G (eIF-4G), and fibroblast growth factor 2 protein (reviewed in Flint et al., 2000). In infected cells, viral protein synthesis is initiated by ribosome's and other cellular and viral factors binding to the IRES (Hambidge and Sarnow, 1992; Hellen et al., 1994; Pilipenko et al., 1992a; Wimmer et al., 1993). Cell protein synthesis is inhibited because viral protein 2A^{pro} cleaves cellular factors such as eIF-4G (Krausslich et al., 1987; Sommergruber et al., 1994) and poly(A)-binding protein (Joachims et al., 1999; Ketekatte et al., 1999) which are required for cellular mRNA 5' 7-methylguanosine (m^7G) cap dependant protein synthesis.

A third feature of the 5'-noncoding region of enterovirus genomes is the presence of multiple AUG codons. Studies on poliovirus indicate that the AUG proximal to the polyprotein coding region is used for translation initiation (Pelletier et al., 1988). Other AUG codons are not essential for virus translation, although those that are found downstream of the 3'-end of the IRES may also be used under some conditions (Hellen et al., 1994b).

Figure 1

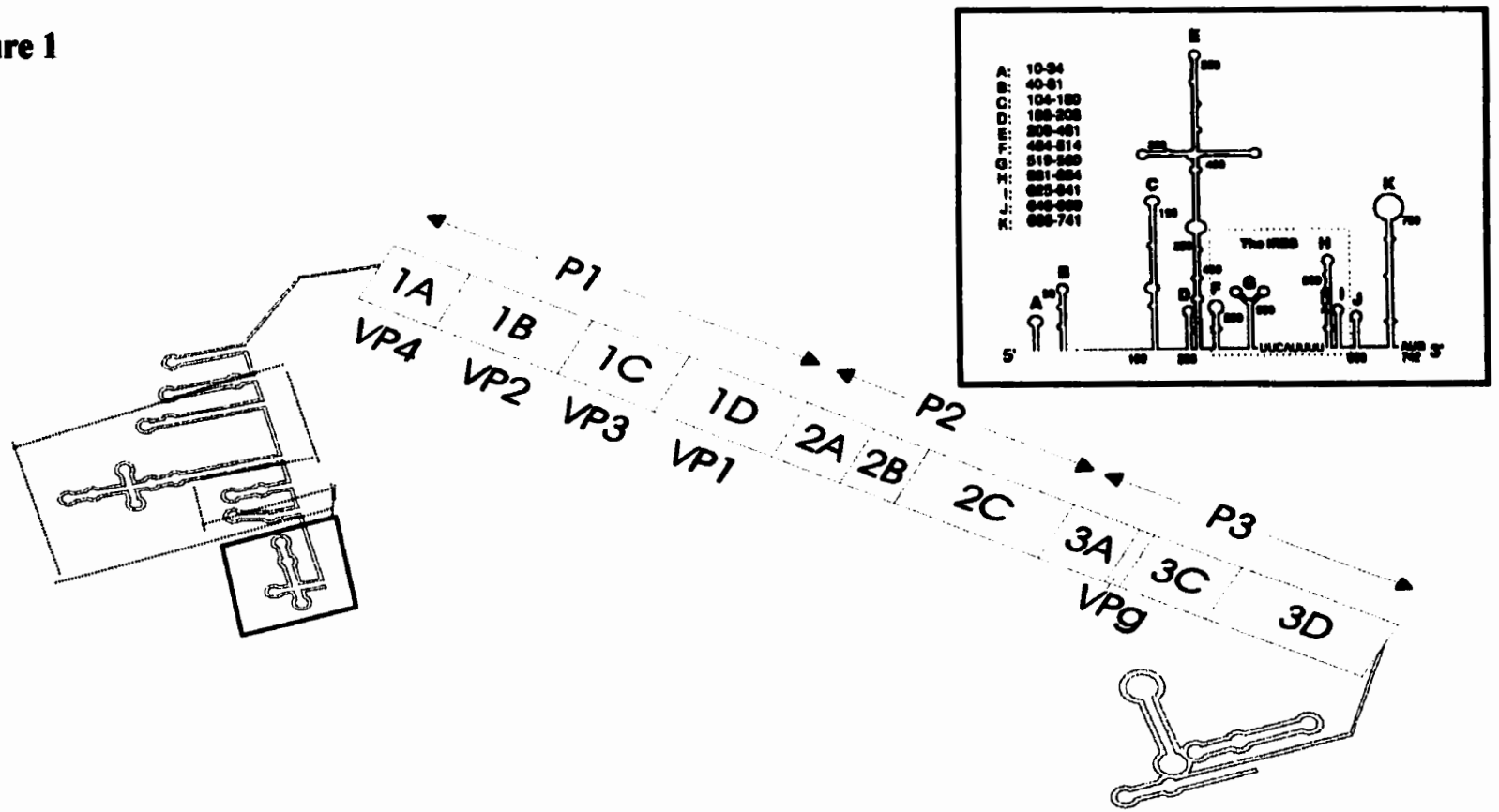


Figure 1. The enterovirus genome. The genome is single stranded positive sense RNA, approximately 7.5 kb in length (see Rueckert, 1996). The first 10% of the virus genome is the 5' nontranslated region. For polioviruses, this region contains the cloverleaf structure (in small black box) and the IRES (boundaries indicated by dotted line) (see Hellen and Wimmer, 1995). The 5'NTR of coxsackievirus B3 (CVB3) (see large box inset) contains an IRES which is shorter and closer to the initiation codon (see Liu et al., 1999; Yang et al., 1997). The enterovirus coding region is one long open reading frame encoding a 250 kDa polyprotein which can be cleaved into viral products (Rueckert, 1996). The precursor P1 region contains the capsid proteins 1A, 1B, 1C, 1D, also referred to as VP0 (VP4, VP2), VP3, and VP1. The P2 unit contains nonstructural proteins 2Apro, 2B, 2C. The P3 region codes for proteins 3A, 3B (VPg), 3Cpro, 3Dpol, and the stable intermediates 3AB and 3CDpro. The last approximately 1% of the genome is the 3' nontranslated region. Genetic organization of the enterovirus genome illustrated by David Hou.

Mutations affecting the highly ordered structure of the 5' noncoding region of enteroviruses can result in viruses with attenuated virulence (Pilipenko et al., 1989; Skinner et al., 1989; Tu et al., 1995). However, mutations affecting viral phenotype are not limited to this region. For example, attenuation of the Sabin vaccine strain PV1(S) neurovirulence is affected by a mutation in the 5' noncoding region at nt 480 (A→G) (Kawamura et al., 1989) along with other mutations scattered throughout the genome (Omata et al., 1986). The vaccine strain PV2(S) has two attenuation markers when compared to a neurovirulent revertent, one in the 5' noncoding region (481 G→A) and the other in VP1 (1143 Val→Ile) (Ren et al., 1991). Also, the vaccine strain PV3(S) has three mutations, one in the 5' noncoding region (472 C→U) (Evans et al., 1985; Svitkin et al., 1990; Westrop et al., 1989), a temperature sensitive mutation in VP3 (3091Ser→Phe) (Stanway et al., 1984; Westrop et al., 1989), and a third mutation in VP1 (1006 Ile→Thr) (Tatem et al., 1992). Mutations in the 5' noncoding region have also been implicated in controlling cardiovirulence of CVB3 (Tu et al., 1995), along with other regions of the CVB genome (Lee et. al., 1997). The 5' noncoding region may also play a role in determining the host range of the virus. For example, poliovirus mRNA translation is normally restricted in rabbit reticulocyte (Dorner et al., 1994; Nicklin et al., 1987) and in murine TgSVA cells that transgenically express the poliovirus receptor (Shiroki et al., 1997), but mutations within the 5' noncoding region of the poliovirus genome can alleviate this restriction.

ii). The polyprotein. The enterovirus genome contains one large open reading frame encoding a 250 kDa polyprotein which is specifically cleaved into numerous protein products (Fig. 1). The enterovirus gene products were determined by analyzing the amino and carboxy-terminus of poliovirus proteins, and mapping them onto the viral genome (reviewed in Hellen and Wimmer, 1995; Wimmer et al., 1993). The protein precursor unit P1 codes for the capsid proteins 1A, 1B, 1C, and 1D. These are more commonly referred to as VP4, VP2, VP3, and VP1. The P2 and P3 regions encode

nonstructural proteins including 2A (proteinase $2A^{pro}$); 2BC which is subsequently cleaved into 2B and 2C; and 3AB which yields 3A and 3B (5'-linked VPg protein); and 3CD (proteinase $3CD^{pro}$) which gives rise to 3C (proteinase $3C^{pro}$) and 3D (polymerase $3D^{pol}$). Some of the biological functions of these proteins are discussed below.

III). The P1 region and the virion capsid. The P1 region codes for enterovirus structural proteins VP1, VP3, and VP0 which is a precursor protein for VP2 and VP4 (Fig. 1). The capsid proteins form into virions as follows (reviewed in Hogle et al., 1990; Rueckert, 1990; Wimmer et al., 1993). 5S promoters are formed when the P1 capsid precursor protein is co-translationally myristoylated and then cleaved by $3CD^{pro}$ to yield the capsid proteins VP0, VP3, and VP1. The 5S promoters assemble into 14S pentamers stabilized by the N-terminal myristate moiety. These units then assemble into 75S empty capsids, then into 160S provirions containing 60 copies of each coat protein and a single copy of genomic RNA which has VPg covalently linked to the 5' end. Mature 160S virions are formed following the autocatalytic cleavage of VP0 to VP4 and VP2.

The tertiary structure of the picornavirus capsid has been determined by x-ray crystallography for all genera except hepatoviruses (reviewed in Muckelbauer and Rossman, 1997; Rueckert 1990). Overall, the picornaviruses have a relative molecular mass (Mr) of approximately 8.5×10^6, and contain approximately 30% by weight RNA. Their external diameter is roughly 300 Å. The capsid of the picornavirion is composed of 60 protomer units, each one containing a copy of the VP1, VP2, VP3 and VP4 proteins. These are arranged into an icosahedral lattice. Within the structure, VP1, VP2, and VP3 form into eight-stranded antiparallel β-sheets, while VP4 is completely internal and less well defined. The coxsackievirus B3 (CVB3) virion was first crystallized by Muckelbauer and colleagues (Muckelbauer et al., 1995) and is illustrated in Fig. 2.

FIGURE 2

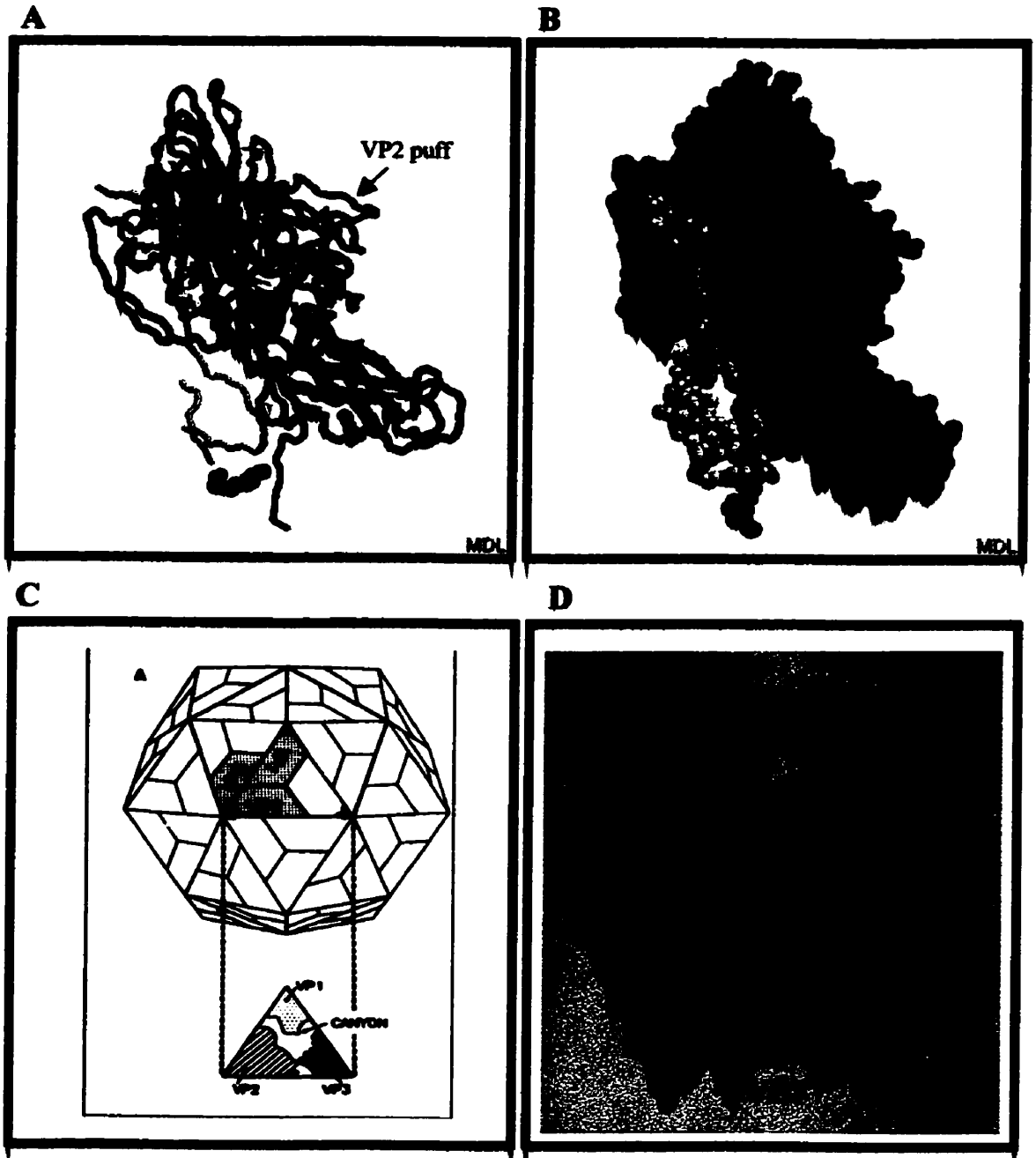


Figure 2. The coxsackievirus B3 virion. A) Ribbon model. VP1 (dark blue) with "pocket factor", VP2 (light blue), VP3 (green), VP4 (yellow) with N-terminal myristate moiety. Based on x-ray crystallography structure by Muckelbauer et al., 1995. Model made in the Protein Data Bank through the National Center for Biotechnology Information, Structure ID# 1COV. B) Filled model, generated as described above. C) Illustration of the picornavirion capsid, and arrangement of coat proteins VP1, VP2, VP3, and the receptor binding canyon. Adapted with permission from B.N. Fields et al., (ed)., *Fields Virology* (Lippincott-Raven Publishers, Philadelphia, Pa., 1996), with permission. D) The coxsackievirus B3 virion. Major and minor structural peaks and canyon depressions are visible. From Jean-Yves Sgro, University of Wisconson, with permission.

There are several characteristic features of the enterovirion surface. Loop structures connecting the β-strands of the VP proteins (which tend to occur in variable regions of the viral genome) form antigenic sites. For example, a large and variable surface region in VP2 termed the "VP-2 puff" (the EF loop) is an neutralizing immunogenic site, as is the major surface protrusion located in VP1 (the BC loop) and the VP3 knob (Minor et al., 1986; Page et al., 1988; Pulli et al., 1998; Reimann et al., 1991; Sherry et al., 1985, 1986). The enterovirion surface also has a prominent peak at each of the twelve 5-fold axes of symmetry, and a deep depression surrounding this peak. The depression is termed the "canyon", and is hypothesized to be the site of attachment for virus binding to its cell surface receptor. This hypothesis is based on analysis of the crystal structure of human rhinovirus 14 (Arnold and Rossmann, 1990; Rossmann et al., 1985), and human rhinovirus 16 interacting with its receptor intracellular adhesion molecule-1 (ICAM-1) (Olsen et al., 1993), and poliovirus complexed with its receptor termed PVR (Belnap et al., 2000; He et al., 2000), and on studies in which mutagenesis of amino acids within the canyon altered the binding phenotype of rhinoviruses (Colonno et al., 1988) and polioviruses (Colston and Racaniello, 1994, 1995; Harber et al., 1995). Additional receptor binding sites are likely used by the group B coxsackieviruses (CVB), since they can bind to more than one receptor molecule or to a complex of different molecules making up a receptor site (Agrez et al., 1997; Bergelson et al., 1995, 1997a, 1997b; Carson et al., 1997; Hsu et al., 1988; Martino et al., 1998; Pasch et al., 1999; Shafren et al., 1995, 1997; Tomko et al., 1997). A putative second receptor binding site for CVB is a large depression found uniquely at the twofold axes of the virion structure (Muckelbauer et al., 1995). Another characteristic of the enterovirion is a hydrophobic pocket which is found below the canyon floor within the β-strands of VP1, and which contains an unidentified "pocket factor". The pocket factor can be displaced by virus-receptor binding and by WIN antiviral compounds (reviewed in Muckelbauer and Rossman, 1997). Interestingly, the

antiviral affects of WIN compounds are ascribed to their ability to displace the pocket factor, which in turn leads to small changes in the capsid structure which effectively prevent the virus from uncoating and infecting cells. Finally, through mutagenesis studies, many individual amino acid residues in the coat proteins have been shown to be important for virus virulence, virion assembly and RNA encapsidation, or other phenotypic characteristics (Ansardi et al., 1993, 1994; Duncan et al., 1998; Lee et al., 1997; Pelletier et al., 1998; Ramsingh et al., 1992; Rezapkin et al., 1999; Wimmer et al., 1993 and references there in; Zhang et al., 1995).

iv). The P2 region. The P2 region of the enterovirus genome codes for the $2A^{pro}$, 2B, and 2C polypeptides, and for the stable intermediate 2BC (Fig. 1). The $2A^{pro}$ polypeptide has been assigned several biological functions. It is a proteinase, which can cleave the junction between P1 and P2 domains of the enterovirus genomes (Toyoda et al., 1986). $2A^{pro}$ is also hypothesized to play a role in the shut off of host protein synthesis. In support of this hypothesis, the $2A^{pro}$ proteinase can proteolytically cleave the eukaryotic translation initiation factor eIF-4G, leading to inhibition of capped cellular mRNA translation (Kräusslich et al., 1987; Sommergruber et al., 1994). It also cleaves poly(A)-binding protein (PABP), which is involved in stimulating translation initiation by its interaction with the poly(A) tail on host cell mRNAs, consistent with its role in shutting off host cell protein translation (Joachims et al., 1999; Kerekatte et al., 1999). Mutagenesis studies have further suggested roles for $2A^{pro}$ in initiation of IRES-dependant translation (Hambidge and Sarnow, 1992), and possibly viral RNA synthesis (Molla et al., 1993). Finally, $2A^{pro}$ may directly contribute to the pathogenesis of viral-induced dilated cardiomyopathy, through its ability to cleave dystrophin and dystrophin-associated glyocoproteins in infected cardiomyocyte cells (Badorff et al., 1999).

The 2B protein localizes in virus infected cells along the plasma membrane, and on membranous vesicles derived from the endoplasmic reticulum on which viral replication takes place (van Kuppeveld et al., 1997a,b). It has been hypothesized that protein 2B modifies the permeability of these cellular membranes, through the creation

of amphipathic pore structures. Changes to membrane permeability in turn lead to an increase in free cystolic Ca2+ concentrations. The membrane lesions that occur may help to facilitate viral progeny release from the infected cells.

A role for the 2C protein in RNA replication has been postulated in studies using polioviruses. This is based on the observation that poliovirus 2C contains 3 structural motifs; an amino-terminal amphipathic helix (Paul et al., 1994), a nucleoside triphosphate (NTP)-binding site (Dever et al., 1987), and a putative zinc finger (Hellen and Wimmer, 1995). A role for 2C in viral replication is also indicated since sensitivity to guanidine hydrochloride (which can inhibit enterovirus replication by blocking single stranded RNA synthesis) maps to this region (Pincus and Wimmer, 1986). The poliovirus 2C polypeptide may also play a role in RNA encapsidation (Li and Baltimore, 1990). Interestingly, in studies using coxsackieviruses, a role for 2C in the development of type 1 diabetes mellitus is also indicated. Sequence homology between the 2C protein of some CVBs and the major diabetes autoantigen flutamic acid decarboxylase (GAD(65)) provides a basis for the hypothesis of molecular mimicry underlying the pathogenesis of this disease (Kaufman et al., 1992).

v). The P3 region. The P3 region of the enterovirus genome codes for the polypeptides 3A, 3B (VPg), and the stable intermediate 3AB, as well as $3C^{pro}$, $3D^{pol}$, and the stable intermediate $3CD^{pro}$ (Fig. 1). The 3A protein and its precursor 3AB protein are postulated to play a role in RNA replication. This was initially based on the observation that a single amino acid mutation within the poliovirus 3A protein led to severe viral replication defects (Berstein and Baltimore, 1988). It was subsequently demonstrated that the precursor 3AB protein (which also contains the sequence of the VPg protein - a 22 amino acid uridylylated oligopeptide that is linked to the 5' terminus of the viral RNA) localizes to membranous replication complexes where active viral replication takes place (Bienz et al., 1983; Takegami et al., 1983). At this site, it is postulated to serve as a cofactor or primer to initiate the polymerase activity of the viral protein $3D^{pol}$ (Lama et al., 1994, 1995).

The $3CD^{pro}$ polypeptide is a proteinase, and for poliovirus it has been shown to cleave between Gln/Gly pairs in the P1 precursor polyprotein to yield the VP0, VP3, and VP1 core proteins (Jore et al., 1988; Ypma-Wong et al., 1988). The $3C^{pro}$ protein contains the core proteolytic component (Hanecak et al., 1982; Nicklin et al., 1987, 1988). The $3C^{pro}$ proteinase has also been implicated in the shut down of host protein synthesis, along with the polypeptide $2A^{pro}$ (Das and Dasgupta, 1993; Joachims et al., 1999). Moreover, $3C^{pro}$ may also play a role in viral RNA replication, since mutations to $3C^{pro}$ can suppress defects in RNA replication caused by mutations in the 5' noncoding cloverleaf region (Andino et al., 1990b). Interestingly, nitric oxide has been shown to inactivate the coxsackieviral protease $3C^{pro}$, indicating that this polypeptide could be a target for anti-enteroviral host defenses (Saura et al., 1999).

Finally, the $3D^{pol}$ protein is a viral RNA-dependent RNA polymerase. It is activated following its cleavage from the 3CD precursor polypeptide (Van Dyke and Flanegan, 1980). In structural studies, the $3D^{pol}$ protein has been shown to contain four highly conserved motifs which are believed to be important for the polymerase activity (Poch et al., 1989).

vi). The 3' nontranslated region. Approximately 1% of the enterovirus genome is termed the 3' nontranslated region (3'NTR) (Figure 1). The tertiary structure of the 3'NTR contains two (polio-like viruses) or three (CVB-like viruses) hairpin loops, which can interact with each other via an intramolecular "kissing" interaction (Iizuka et al., 1987; Pilipenko et al., 1992b; Wang et al., 1999). This forms the structure of the origin of replication (*oriR*) for the initiation of negative strand RNA synthesis (Jacobson et al., 1993; Melchers et al., 1997; Pilipenko et al., 1996; Wang et al., 1999). 3'NTR interactions with viral proteins 3AB, 3CD, and a host cell factor(s) may also be important for viral RNA replication (Harris et al., 1994; Mellits et al., 1998; Todd et al., 1995).

A.4. Replication and the quasi-species nature of enterovirus RNA

An overview of the enterovirus replication cycle is as follows (reviewed in Flint, 2000; Rueckert, 1990; Wimmer et al., 1993). The enterovirus genome is released into the cell cytoplasm, and serves as a template for translation of the viral polyprotein. The viral RNA genome also serves as a template for viral RNA replication, which takes place on membranous replication complexes. The (+) strand RNA is transcribed to a complementary (-) strand RNA, giving rise to a double stranded RNA termed a replicative intermediate. Additional (+) RNA strands are then generated, which can serve further as templates for viral transcription or translation, or be encapsidated within the virions.

It is interesting to note that the RNA of enteroviruses does not exist as a uniform genetic sequence, but rather as a pool of genetic variants or a "quasi species" group (Nichol, 1996; Wimmer et al., 1993). This genetic variability is due in part to the enterovirus RNA polymerase, which has relatively low fidelity thus allowing for frequent base substitutions in the genome. Indeed, the error frequency rate of introduced mutations is so high that it is said to be near the threshold of error catastrophe - approximately 10^{-3} to 10^{-4} substitutions per base. Since the genome consists of approximately 7.5×10^3 bases, it is reasonable to expect one error for each transcription event. The lack of genetic fidelity found in enterovirus genomes may be biologically advantageous, as it could confer on the virus an ability to adapt to changing host environments.

It is also interesting to note, however, that despite the opportunity for frequent genetic change, the genotype of enteroviruses remains relatively stable over time. There are still only 3 poliovirus serotypes, and overall there are less than 70 enterovirus members that commonly infect humans. This constancy is achieved in part by the maintenance of a majority "consensus sequence" in the viral genome (Wimmer et al., 1993). The consensus sequence remains stable over time if the virus is grown under similar conditions. Also, errors introduced into the genome may be removed through

genetic recombination during viral RNA synthesis. The consensus sequence is also likely maintained because of an intolerance for mutations which affect tertiary RNA structures in the genome (i.e. those which are critical for viral RNA replication such as the cloverleaf or IRES), or critical protein structures (i.e. sites which are important for virion assembly, receptor binding), or mutations which affect the large polyprotein open reading frame.

B. ENTEROVIRAL HEART DISEASE

B.1. Myocarditis

The term "myocarditis" was initially designated to mean inflammation of the myocardium (Sobernheim, 1837). Today, myocarditis is operationally defined in pathological terms, as a "cardiac disease in which there is inflammation of the myocardium with necrosis and/or degeneration of adjacent myocytes, that is not typical of ischaemic damage associated with coronary artery disease" (Aretz et al., 1987a,b).

i) Epidemiology. The "true" incidence of myocarditis is difficult to determine because many cases of myocarditis may be clinically silent or subclinical. However, there have been some studies which estimated the incidence of myocarditis using pathologic evaluations. Woodruff predicted the overall incidence of myocarditis to be between 2.3% to 5.0% (Woodruff, 1980), based on findings from unselected autopsy material submitted to the United States Army Institute of Pathology during World War II (Gore and Saphir, 1947), and on autopsy findings of adult males (Stevens and Ground, 1970) and children (Bandt et al., 1979) who died a sudden violent or accidental death. A later study from Malmö Sweden predicted a lower overall incidence of myocarditis of 1.06%, using stricter histopathologic criteria (Gravanis and Sternby, 1991). As shown in Table 1, the incidence of myocarditis also varies with age. Myocarditis occurs in a bimodal distribution, with a predilection for the very young, and then in mature adults. Several studies have also reported that myocarditis is more prevalent among males than females, and gender ratios range from 1.1:1 to 2:1 (Helin et al., 1968; Sainani et al., 1968, 1975; Smith, 1970; Woodruff, 1980).

Table 1. Age distribution of patients with myocarditis or DCM.

Age group (years)[c]	[a]% of myocarditis patients in each age bracket	[b]% of DCM patients in each age bracket
3 - 20	14	6
21 - 30	30	16
31 - 40	27	12
41 - 50	12	23
50 - 60	13	31
61 - 70	4	12

[a]Summarized from four studies with a total of 100 patients (Helin et al., 1968; Sainani et al., 1968, 1975; Smith 1970).

[b]Summarized from four studies with a total of 77 patients (Bowles et al., 1989; Kitaura 1981; Werner et al., 1993; Wesslén et al., 1993).

[c]Young children including those less than 3 years of age can also develop myocarditis (see Baboonian et al., 1997a; Cherry, 1990; Hosier and Newton, 1958; Klein and Remington, 1990; Modlin et al., 1997a,b; Woodruff, 1980) and cardiomyopathy (see Heikkinen 1993; Matitiau et al.,1994).

ii). Clinical presentation and diagnosis. The clinical and diagnostic features of acute myocarditis have been extensively reviewed (Abelmann, 1971; Goodwin, 1987; Heikkilä, 1982; Karjalainen, 1993; Kawai et al., 1978; See and Tilles, 1991; Sekiguchi et al., 1988). Briefly, the most common presentation is that of chest pains and palpitations. Major physical findings include a pericardial friction rub, a ventricular gallop, and possibly tachyarrhythmias. Sinus tachycardia disproportionate to the febrile response is often seen in children. Patients may present with heart failure, and occasionally the disease mimics a myocardial infarction.

Electrocardiographic changes support the diagnosis of acute myocarditis (Goodwin, 1987; Karjalainen et al., 1983, 1986, 1993; Kawai, 1978). Serial electrocardiograph recordings indicate ST-segment elevation in the first few days, and early T wave inversion. The electrocardiogram more or less normalizes in the intermediate stage, although T-wave inversions may occur again in the late stage. The degree of ST-segment elevation and the degree and duration of the late T-wave inversions correlates with myocardial enzyme release, and thus with the amount of cell necrosis. Abnormal serum levels of the cardiac enzymes creatine kinase, aspartate aminotransferase, and troponin T may be found (Karjalainen et al., 1983, 1986, 1993).

Imaging has proven to be a valuable tool (Goodwin, 1987; Karjalainen, 1993). Chest x-rays may display transient cardiac enlargement in some patients. Ventricular dilation, thickening of the myocardium, and lowered ejection fraction responses may be shown by echocardiography. In addition to global left ventricular dysfunction, regional wall motion abnormalities may occur. A pericardial effusion may also be found. In acute myocarditis abnormal echocardiography changes are common, even though some may normalize in a few days.

Non-invasive nuclear imaging has proven useful in the clinical diagnosis of myocarditis. Myocardial scintigrams with technetium-99m pyrophosphate are diagnostic

in severe forms of myocarditis (Olsen et al., 1980). Indium-111 antimyosin antibody imaging can document the extent of myocardial necrosis (Carrio et al., 1988; Casans et al., 1989; Dec et al., 1990; Matsumori et al., 1993; Yasuda et al., 1987). Gallium-67 uptake has been shown to occur in a limited number of cases making these scans of more limited clinical value (O'Connell et al., 1984). Preliminary studies indicate that magnetic resonance imaging detects tissue alterations, including those caused by myocardial inflammation during the acute stage of the disease, and thus it may serve as a useful noninvasive diagnostic tool (Chandraranta et al., 1987; Friedrich et al., 1998; Gagliardi et al., 1991).

The pathological diagnosis of myocarditis requires endomyocardial biopsy analysis. The procedure, which is associated with very low morbidity or mortality (<1%) (Sakakibara and Konno, 1962), is performed through the right ventricle, with samples taken from the interventricular septum. Due to the multifocal nature of myocarditis, a minimum of 3 biopsies or more are recommended (Chow et al., 1989; Hauck et al., 1989; McManus et al., 1991a,b; Olsen, 1993). In 1984, a standardized set of pathological criteria for evaluating myocarditis by biopsy was established, and termed the "Dallas Criteria" (Aretz et al., 1987a,b). Pathologic diagnosis of myocarditis using the Dallas Criteria was employed in the NIH myocarditis treatment trials. However, the criteria are very conservative, and it has been reported that cardiac dysfunction ranges widely even in patients that meet the criteria of biopsy positive myocarditis, thus pathologic evaluations likely serve best only as an adjunct to the clinical diagnosis of myocarditis (Billingham, 1989; Burke 1990; Lieberman et al., 1991, 1993; McManus et al., 1991a; O'Connell, 1987).

iii). Etiologic role of enteroviruses in myocarditis. The pathologic definition of myocarditis does not take into account its etiology. Perhaps this is because many disease processes may involve the heart, and thus there is an extensive list of causal associations. These include numerous infectious agents (i.e. viruses, bacteria, fungi,

and parasites), metabolic disorders, connective tissue diseases, hematological disorders, neoplasm's, neuromuscular diseases, endomyocardial diseases, toxins, and drugs (reviewed in Abelmann, 1971, 1984; Caforio and McKenna, 1996; Fairley et al., 1996; Friman and Fohlman, 1993; Opavsky et al., 1998). Among the viruses, the cardiotropic group B coxsackieviruses are deemed to be the primary etiologic agents of acute viral myocarditis (Tables 2,3,4 and Baboonian et al., 1997a; Baboonian and Treasure, 1997b; Gelfand, 1961; Grist et al., 1972, 1978; Kawai et al., 1978; Martino et al., 1995b; Why, 1995; Woodruff, 1980). The association of enterovirus infections and myocarditis is based on serological, virological, and molecular studies.

Serology. Serological studies have established a preliminary association of enterovirus infections with myocarditis (Table 3). IgM antibodies to enteroviruses (primarily CVB) are present on average in approximately 36% of reported cases, although some variation is noted among individual reports. Elevated neutralizing antibody titers of at least 1:160 (the majority were significantly higher) are present in about 37% of tested patients. A fourfold or greater change in paired sera is noted in approximately 46% of patients. By contrast, control patients show minimal reactivity to enteroviruses in comparative tests.

Virus isolation. The demonstration of replicating virus in myocardial cells would help to establish the viral etiology in human heart disease. However, myocardial specimens are seldom available, and when they are, isolation of virus has been rare except in sporadic cases. Infectious CVBs have been isolated from the myocardium, pericardium or pericardial fluid in fatal cases of myocarditis at autopsy (Hosier and Newton, 1958; Longson et al., 1969; Mandin and Mandin, 1963; Sanyal et al., 1965; Sutton et al., 1963, 1967). In one particular case, it was also demonstrated that virus isolated from the heart was cardiotropic when used to infect mice (Sutton et al., 1967). CVB antigens have also been detected in heart tissues at autopsy by indirect immunofluorescence (Burch et al., 1967; Foulis et al., 1990).

Molecular detection of virus RNA. Enterovirus RNA detection in endomyocardial biopsy samples provides strong supportive evidence of a causative role for enteroviruses in acute myocarditis (reviewed in Baboonian and Treasure 1997; Martino et al., 1995b; Rotbart, 1991). Viral RNA in the myocardium was first detected by slot blot hybridization (Bowles et al., 1986). Overall, viral RNA is found in 45% of patient heart samples by this technique (Table 4). It is important to note, however, that slot blot hybridization lacks specificity, and thus the true incidence of viral RNA in the heart is likely overestimated by this technique. In contrast, PCR of viral RNA from heart biopsy samples is considered more sensitive and specific than slot blot hybridization (Jin et al., 1990). Overall, viral RNA is detected in 27% of patient heart samples by PCR analysis (Table 4), although the incidence varies from study to study, indicating a need for qualitative or quantitative PCR controls (Martino et al., 1993). In situ hybridization is also considered a sensitive and specific technique for detecting viral RNA in the myocardium, and it offers the advantage of visualizing the specific cells infected with enteroviral RNA. Overall, enteroviral RNA has been detected in 25% of patient heart samples by this technique (Table 4). Molecular techniques can be used to detect both plus-strand and minus-strand RNA in heart biopsy samples, thus indicating whether the viral RNA retains replicative capacity (Pauschinger et al., 1998). The pattern of cell-to-cell spread of viral RNA as visualized by in situ hybridization may also indicate whether actively replicating virus is present in the infected heart tissue (Kandolf et al., 1991). Antisera against nonstructural viral proteins may also help to elucidate whether the detected viral RNA is capable of replicating in the infected tissue (Hohenadl et al., 1994).

iv). Prognosis. The recent Myocarditis Treatment Trial indicated that 20% of patients with biopsy-proven myocarditis died 1 year after diagnosis (Mason et al., 1995). Moreover, 56% of patients died within 4.3 years of the initial diagnosis (Mason et al., 1995), presumably from persistent or new cardiac abnormalities that occur. Patients with evidence of enterovirus infection exhibit a significantly poorer prognostic outcome than those without a defined virus diagnosis. In a 15-year follow-up study of myocarditis patients with serologic evidence of enterovirus infection, 10/42 patients exhibiting a fourfold or greater rise in antibody titer to CVB died from subsequent chronic myocarditis or cardiomyopathy, as compared to 0/26 patients with negative viral serology (Levi et al., 1988). Also, in a follow-up study of patients with myocarditis, DCM, or other specific heart muscle disease, 26% of patients positive for enterovirus RNA in myocardial biopsy samples died, compared with only 3% of patients whose biopsies were enterovirus-negative (Archard et al., 1991). An explanation for why enterovirus positivity conveys a higher risk of subsequent death is not known, but it is tempting to speculate that virus infection or virus-activated immune responses are important pathogenic factors.

Table 2. Role of cardiotropic viruses in myocarditis

	[a]Causative agents in viral myo(peri)carditis (%)	[b,c]Rate of infections with viruses in cardiac disease
Coxsackie group B (CVB)	73	35
Influenza A	9	12
Influenza B	6	17
Enterovirus untyped	5	-
Picornavirus (unspecified)	-	10
Coxsackie group A (CAV)	-	9
Poliovirus	-	5
Picornavirus/CAV/Polio	-	8
Echovirus	2	6
Adenovirus	1	3
Cytomegalovirus	1	8
Other viruses	3	1

[a]In England and Wales 1990-1994, modified from Fairley et. al., 1996.

[b]World 1975-1985 cardiovascular disease, modified from Grist and Reid 1997.

[c]Rates per 1000 infections for world data

Table 3. Serologic association of enterovirus infection with heart disease in humans[a]

Test[b]	Anti-enterovirus antibody detected (number of patients positive / number tested [%] in patients with							
	Myo(peri)carditis		Cardiomyopathy		Other cardiac conditions		Healthy controls	
IgM Ab to EV	38/102 (37)	El-Hagrassy 1980	2/28 (7)	El-Hagrassy 1980	4/69 (6)	El-Hagrassy 1980	1/38 (3)	Kawai 1971
	72/220 (33)	McCartney 1986	5/43 (12)	Kawai 1971	23/79 (29)	Muir 1996	5/100 (5)	Keeling 1994
	27/67 (40)	Muir 1989	38/114 (33)	Keeling 1994	6/8 (75)	Tracy 1990	11/41 (27)	Keeling 1994
	6/8 (75)	Tracy 1990	19/41 (80)	Keeling 1994	0/7 (0)	Weeslén 1993.	26/304 (9)	McCartney 1986
			28/86 (33)	Muir 1989			10/84 (12)	Muir 1996
			23/43 (53)	Muir 1996				
			6/8 (75)	Weeslén 1993				
Average	143/397 (36)		121/363 (33)		33/163 (20)		53/567 (9)	
N Ab ≥ 1:160	23/57 (40)	Grist and Bell 1974	14/50 (28)	Cambridge 1979	23/50 (46)	Cambridge 1979	7/72 (10)	Grist 1974
	23/83 (28)	Grist and Bell 1974	6/9 (67)	Heim 1997a	14/158 (9)	Grist 1974	34/87 (39)	McCartney 1986
	16/18 (89)	Helin 1988	21/64 (33)	Morgan-Capner 1984	2/14 (14)	Morgan-Capner 1984	16/60 (27)	Shingu 1989
	110/352 (31)	McCartney 1986			3/14 (21)	Shingu 1989		
	17/35 (49)	Morgan-Capner 1984			5/8 (63)	Tracy 1990		
	22/39 (56)	Shingu 1989						
	10/20 (50)	Smith 1970						
	4/8 (50)	Tracy 1990						
Average	225/612 (37)		41/123 (33)		47/244 (19)		57/219 (26)	
4-fold change	15/19 (79)	Sainani 1975	3/17 (18)	Cambridge 1979				
	3/20 (15)	Smith 1970	8/113 (7)	Kitaura 1981				
Average	18/39 (46)		11/130 (8)					

[a]In some cases select patient groups were used

[b]Abbreviations: Ab, antibody; EV, enteroviruses (mostly CVB); N Ab, neutralizing antibody; 4-fold change, change in antibody titer in paired sera.

Table 4. Molecular detection of enterovirus RNA in hearts of patients with myocarditis and DCM

Method[a]	Enterovirus detected (no. of patients positive/no. tested [%]) in patients with			References
	Myocarditis	DCM	Controls	
Slot blot	21/47 (45)	35/82 (43)	0/39 (0)	Archard 1987, 1991
				Bowles 1986, 1989
		1/6 (17)	0/8 (0)	Wiegand 1990
Average	21/47 (45)	36/88 (41)	0/47 (0)	
PCR	2/25 (8)	3/23 (13)	0/9 (0)	Jin 1990
	1/5 (20)	0/11 (0)	0/21 (0)	Weiss 1991
		5/11 (45)	9/24 (38)	Weiss 1992
	3/9 (33)	8/25 (32)	0/9 (0)	Koide 1992
		0/21 (0)	0/19 (0)	Grasso 1992
		1/5 (20)	0/8 (0)	Zoll 1992
	3/10 (30)	30/45 (67)	9/23 (39)	Petitjean 1992
		6/50 (12)	13/75 (17)	Keeling 1992
		0/35 (0)	0/23 (0)	Liljeqvist 1993
		6/19 (32)	0/21 (0)	Schwaiger 1993
	2/10 (20)[b]		0/10 (0)[b]	Hilton 1993
	1/3 (33)	4/53 (8)	1/28 (4)	Giacca 1994
	8/38 (21)		0/17 (0)	Martin 1994
	12/36 (33)		0/10 (0)	Satoh 1994
	4/5 (80)	7/42 (17)	3/27 (11)	Ueno 1995
	5/6 (83)		0/8 (0)	Nicholson 1995
		4/16 (25)	0/10 (0)	Marti 1996
		11/19 (58)	0/68 (0)	Androeletti 1996
		1/25 (4)	4/51 (8)	Muir 1996
		6/34 (18)	0/16 (0)	Heim 1997a
		4/31 (13)	1/24 (4)	Arbustini 1997
		9/21 (43)	1/14 (7)	Archard 1998
		0/38 (0)[f]	0/39 (0)	De Leeuw 1998
		7/23 (30)	0/5 (0)	Grumbach 1998
	2/10 (20)	4/12 (33)	0/8 (0)	Grumbach 1999
Average	43/157 (27)	116/559 (21)	41/567 (7)	
In situ	5/12 (42)		0/5 (0)	Easton 1988
	2/10 (20)[b]		0/10 (0)[b]	Hilton 1993
	2/9 (22)		1/8 (13)[c]	Tracy 1990
	23/95 (24)	10/33 (30)[d]	0/53 (0)	Kandolf 1988, 1989, 1990
		8/47 (17)[e]		Kandolf 1991, 1993
Average	32/126 (25)	18/80 (23)	1/76 (1)	
Average, all methods	96/330 (29)	170/727 (23)	42/690 (6)	

[a]Abbreviations: Slot blot, slot blot hybridization; In situ, in situ hybridization.

[b]These studies used two different hybridization probes with different sensitivities.

[c]Controls in this study had clinically suspected myocarditis but were negative by the Dallas Criteria.

[d]Recent onset.

[e]Chronic.

[f]End-stage DCM

B. 2. Dilated cardiomyopathy.

Cardiomyopathy was originally defined by the World Health Organization as "heart muscle disease of unknown origin" (WHO, 1980). Dilated cardiomyopathy (DCM) is characterized by dilation and impaired function of one or both cardiac ventricles (Abelmann, 1984, 1988; Goodwin, 1992; Richardson et al., 1996). Epidemiological, clinical, immunological, etiologic, and prognostic aspects of DCM are discussed below.

i). Epidemiology. In a US Hospital Discharge Survey of 400 institutions and 18 346 000 patients with diseases of the circulatory system, approximately 0.67% (126 000 cases) were classified as cardiomyopathy (Abelmann, 1985). In a survey of the professional activities of American cardiologists', it was reported that cardiomyopathy accounts for 1.2% - 1.9% of all cardiologists patient encounters (Swan and Gifford, 1974). In the United States, dilated cardiomyopathy reportedly comprises about 90% of all the cardiomyopathies (Abelmann, 1985). The incidence of dilated cardiomyopathy in the general population ranges from 0.73 to 10 cases per 100 000 inhabitants per year (Abelmann, 1985; Bagger et al., 1984; Codd et al., 1989; Torp, 1978, 1981; Williams and Olsen, 1985). DCM is more prevalent among males than females, with gender ratios ranging from 1.9:1 to 4.3:1 (Bagger et al., 1984; Codd et al., 1989; De Maria et al., 1993; Torp, 1978, 1981). Moreover, mortality rates from cardiomyopathy, as a percentage of cardiovascular deaths, were 0.94% in males and 0.48% in females (Abelmann, 1985). Of exception is the sudden onset of DCM in women during the final trimester of pregnancy or within 5 months of delivery, termed peripartum cardiomyopathy (Demarkis and Rahimtoola, 1971; Homans, 1985; Julian and Szekely, 1985; Veille, 1984). As shown in Table 1, 66% of DCM cases occur in adults over 40 years of age. In terms of race, the mortality rate from cardiomyopathy in the United States is higher in blacks (1.5%) then whites (0.63%) (Abelmann, 1985). Finally, cardiomyopathy is encountered more frequently in underdeveloped and tropical

countries (Abelmann, 1985; Akinkugbe et al., 1991; Correa et al., 1963; Köberle, 1957), due in part to the prevalence of endemic pathogens such as *Trypanosoma cruzi* and arboviruses which can cause a myocarditis/DCM-like disease (Abelmann, 1985; Factor et al., 1993; Köberle, 1957; Obeyesekere and Hermon, 1972).

ii). Clinical presentation and diagnosis. The clinical features of DCM have been extensively reviewed (Abelmann, 1984; Fuster et al., 1981; Karjalainen, 1993; Kawai et al., 1978; Kawai and Takatsu, 1975; Keren and Popp, 1992; O'Connell and Henkin, 1985a; Olsen, 1984a, 1991; Werner et al., 1993; Yamada et al., 1993). Briefly, the initial presentation of DCM varies in patients, ranging from asymptomatic status with an abnormal electrocardiogram, to chest pain, arrhythmia's, or symptoms of heart failure. Physical findings may indicate mitral insufficiency, ventricular dilation and/or cardiac failure. Chest x-ray often displays an enlarged heart. Echocardiograph abnormalities are frequent, and may demonstrate an enlarged left ventricle and poor systolic function. Ventricular thrombi are noted in up to one-third of patients. Many of these features are similar to those seen in patients with coronary-induced heart failure, thus imaging studies may be employed to help differentiate between the two disease etiologies. Endomyocardial biopsy is helpful in patients with recent symptoms to determine whether active myocarditis is also present.

Immunological abnormalities have also been identified in patients with DCM. Defective *in vitro* suppresser cell function and decreased natural killer (NK) cell activity are noted in a significant number of patients (Anderson et al., 1982; Eckstein et al., 1982; Fowles et al., 1979; McManus et al., 1989, 1991b). However, due to the nature of the NK assay systems, it remains unclear as to whether this reduction in cellular activity is directly related to the DCM condition. Quantitation of NK cell populations in these patients using serological markers or other bioassay systems may help to resolve the issue. It has also been observed that patients with DCM produce autoantibodies which may contribute to degenerative processes in the failing myocardium. Autoantibodies

have been identified against the cardiac cell ADP/ATP carrier (Schultheiss et al., 1983, 1984, 1985a,b, 1986, 1987, 1996), cardiac β-adrenoceptor (Limas et al., 1989a,b, 1990a,b, 1991a,b, 1993; Magnusson et al., 1990, 1996; Wallukat et al., 1991), and structural proteins such as myosin and laminin and other unidentified molecules (Cafario et al., 1990; Wolff et al., 1989). Heart tissues from patients with DCM also show significant nitric oxide synthase (NOS) activity (De Belder et al., 1993; Stein et al., 1998), and upregulation of HLA class I antigen and intracellular adhesion molecule (ICAM-1) (Seko et al., 1995). Finally, uneven HLA distributions have been noted in select groups of patients with DCM. HLA-DR4 or HLA-DQw4 or HLA-B27 or HLA-DQB1 antigen expression or a combined DR4-DQw4 haplotype are reportedly over represented in patients with DCM, and the HLA-DR6Y and HLA-DRw6 alleles are underrepresented, as compared to controls (Anderson et al., 1984; Carlquist et al., 1991; Hammond et al., 1993; Nishi et al., 1992; Wesslén et al., 1993). However, other studies fail to confirm these findings. For example, a molecular study of human leukocyte antigen associations undertaken on patients from the Myocarditis Treatment Trial found no association between the DR4 antigen and cardiomyopathy, but did indicate a relationship between the DQ locus and DCM (Lozano et al., 1997). A reason for these discrepancies is not known. It is possible that specific HLA subgroups may be more prone to disease of immune-mediated nature, but the strength of the associations may be overestimated because sample sizes are small.

iii). The etiology of DCM. DCM is believed to be a final common pathway of a heterogenous group of disorders which may be infectious or familial in origin (Abelmann, 1984; Mestroni et al., 1998, Towbin et al., 1999). These pathways may be commonly linked by their ability to affect the cytoskeletal matrix of the heart and cause the characteristic cardiovascular deterioration observed in DCM. Some mechanisms involved in the development of DCM are discussed in the pathogenesis sections, below.

It has been postulated that myocarditis can serve as a trigger for the development of DCM. This hypothesis is supported by long-term follow-up studies which show progressive cardiac disease in a proportion of patients with myocarditis, some of whom subsequently develop DCM, and retrospective studies on patients with DCM who had prior evidence of myocarditis (Abelmann, 1984; Bengtsson and Lamberger, 1966; Bergström et al., 1970; Levander-Lindgren, 1965; Levi et al., 1988; Quigley et al., 1987; Remes et al., 1990; Sainani et al., 1968; Smith, 1970). An overall estimate of myocarditis contributing to DCM appears to be in the range of 10-20%. This incidence is considerably greater by several orders of magnitude than the incidence of DCM in the general population, as noted above (0.73-10 cases/100,000 population).

Since enteroviruses have been shown to be causative agents of myocarditis, and since myocarditis may lead to DCM, it has also been proposed that enteroviruses are causally linked with DCM. Clinical and experimental studies are supportive of an association between CVB infection and the development of DCM (reviewed in Tables 2,3,4 and Baboonian et al., 1997a; Baboonian and Treasure 1997b; Kawai, 1971, 1978; MacArthur et al., 1984; Martino et al., 1994a,b, 1995b; Olsen, 1992; Richardson et al., 1992), although many other causes of DCM are also indicated (Abelmann, 1988; Mestroni et al., 1998). One potential mechanism by which enteroviruses could be involved in the pathogenesis of DCM is that they could exacerbate an underlying heart disease (such as myocarditis), leading to DCM. Alternatively, enteroviruses could be directly involved in the genesis of DCM. A number of studies examining enterovirus presence in the hearts of patients with DCM have been reported.

Serology. As shown in Table 3, IgM antibodies to enteroviruses and/or neutralizing antibodies titers of at least 1:160 were present in an average of 33% of cardiomyopathy patients. Also, a fourfold or greater change in paired sera was noted in an average of 8% of patients. However, these results are difficult to interpret, since some patients had high and/or rising serological titers, some had high and prolonged antibody titers, some had high and dropping titers, and others had low anti-viral antibody

titers. Moreover, the rate of anti-enterovirus antibody detection was not always significantly greater in patients with DCM than in the general population. Thus these findings may be suggestive of a causal relationship between enteroviruses and cardiomyopathy, or they may indicate that enterovirus infections are coincidental and unrelated to the cardiac condition. Based on these observations, serology provides only a presumptive association of enterovirus etiology in DCM.

Virus isolation. To date, virus isolation has not been a productive technique for establishing an enterovirus etiology in DCM. Enterovirus-like particles have been detected in the myocardium for only one cardiomyopathy patient, by electron microscopy at postmortem, but virus could not be isolated (Hibbs et al., 1965).

Molecular detection of virus RNA. Enterovirus RNA was detected in the myocardium of approximately 23% of patients with DCM and only 6% of controls (Table 4). On average, virus RNA was detected in 41% of patient samples by slot blot hybridization, 21% by PCR, and 23% by *in situ* hybridization. No specific serotype has been associated with DCM (Fujioka et al., 1996), although CVB are the most pursued virus group. The significance of enteroviral RNA in DCM is not clear. One study showed a link between enterovirus RNA in DCM and increased myocardial uptake of ^{111}In-antimyosin antibody, indicating a role in ongoing cardiac damage (Marti et al., 1996), but a follow-up study failed to support this hypothesis (Bengel et al., 1997). Animal models have also been used to examine a pathologic role for viral RNA in the myocardium, as described below. Alternatively, enterovirus RNA in the heart may be relic genetic material from previous infections, and its presence unrelated to the DCM.

iv). Prognosis. The prognosis for patients with DCM is poor. A 20 year follow-up study of 104 patients diagnosed with DCM at the Mayo Clinic, United States, found that "77% of patients had an accelerated course to death, with two thirds of the deaths occurring within the first two years" (Fuster et al., 1981). This is in agreement with the

recent clinical trials at the National Institute of Health, United States, which reported that approximately 50% of patients with DCM die within 2 years of diagnosis (Mason et al., 1995). An etiologic role for enteroviruses in DCM is not resolved, however, enterovirus RNA is detected in the myocardium in a higher proportion of DCM patients who undergo cardiac transplantation or died from the disease, as compared to control groups (Archard et al., 1991; Bowles et al., 1989).

B.3. Pathogenesis of CVB myocarditis and DCM.

Mice infected with CVB3 develop heart disease closely resembling myocarditis and DCM in humans and therefore comprise a valuable model to study viral pathogenesis (Martino et al., 1994a,b, 1995b and references therein). The pathogenesis of CVB3-induced murine heart disease involves two principal mechanisms. The first is viral pathogenicity, in which the virus plays a direct role in producing myocardial damage and triggering the immunologic responses of the host. The second is immune-mediated pathogenicity in which the heart disease is brought about by a host defense system gone awry. In this section, studies on both aspects of enterovirus mediated heart disease will be discussed to demonstrate the complementary relationship of the pathogenic mechanisms involved.

i). Overview of acute CVB3 myocarditis. When mice are inoculated intraperitoneally with cardiotropic CVB3, they develop heart disease strikingly similar to the human conditions of myocarditis and dilated cardiomyopathy. Noticeable changes first occur in the myocardium within 2 days of infection. The cardiocytes show evidence of cytopathology including coarse granularity, pallor of the cytoplasm, and multivesicular vacuolation (McManus et al., 1993; Wilson et al., 1969). From days 3 to 5, the infection spreads to large clusters of myocytes. At this time, there is also early calcification of single cells (McManus et al., 1993). Changes in gene expression in the myocardium can be appreciated by differential display analysis (Yang et al.,1999). By day 7, numerous foci of necrotic and calcified myocardial cells are evident in the heart tissue (McManus et al., 1993). At this time, a shift from fast to slow myosin isoforms in the infected ventricles has been observed using pyrophosphate gel analysis, indicating that remodeling processes in the myocardium are already occurring (Hamrell et al., 1994). Immunohistochemical staining of CVB3 infected murine heart tissue reveals enhanced expression on myocytes of the major histocompatibility complex (MHC) class I antigen,

intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and the costimulatory molecules CD40/B7-1/B7-2 (Seko et al., 1993a, 1996a, 1998a,b). Soon after virus infection, perforin-containing inflammatory cells enter the myocardium (Seko et al., 1992, 1993). By day 7 post infection characteristic lesions consisting of inflammatory infiltrate cells and necrotic myocytes are commonly found throughout the myocardium (Chow et al., 1991; Klingel et al., 1992; Lodge et al., 1987; Wilson et al., 1969 Woodruff, 1980, and others). Studies in several laboratories have also demonstrated extensive microvascular abnormalities in the myocardium of virus infected mice. Casting with Microfil (liquid silicone rubber) reveals spasm or constriction of the coronary microvasculature (Dong et al., 1992; Silver and Kowalczyk, 1989), similar to that seen in genetically myopathic hamsters (Factor et al., 1982; Sole and Liu, 1993). Treatment of virus infected mice with the calcium channel blocker verapamil (Dong et al., 1992), or the serine elastase inhibitor ZD0892 (Lee et al., 1998) reverses microvascular spasm and markedly ameliorates myocarditis.

ii). Host defense mechanisms. Approximately 4 days after inoculation with CVB, the hosts' immune responses become evident. Neutralizing antibody as well as infiltrating macrophages and natural killer cells contribute towards limiting viral dissemination in the heart, and help to clear the virus from the myocardium. There is also mounting evidence that interferon and nitric oxide enhance resistance to viral challenge.

Neutralizing antibody. The protective role of antibodies against CVB3 infection has been extensively reviewed (Gauntt 1997). Serum antibodies neutralize the homologous strain of CVB *in vitro*. Passive administration of very high doses of normal immunoglobulin before, along with, or very shortly after CVB3 inoculation protects mice infected *in vivo* (Cho et al., 1982; Rager-Zisman and Allison, 1973a,b; Takada et al., 1995; Weller et al., 1992). Moreover, in vaccination studies using CVB isolates, mutants, or inactivated virus, protection against challenge with cardiovirulent CVB3

correlates with induction of anti-CVB3 antibodies (Gauntt 1997). The murine strains DBA/2 and BALB/c generally begin to produce virus-neutralizing antibodies approximately 3 days after virus inoculation, which reaches peak levels several days later, and as antibody titers rise in virus infected mice the levels of infectious virus decline the heart (Lodge et al., 1987; Wolfgram et al., 1986). In murine A/ strains, where the onset and peak of antibody responses are delayed, higher levels of viremia and greater disease severity are observed (Lodge et al., 1987). A protective capacity for virus neutralizing antibodies has also been reported in CVB3 infected athymic mice (Sato et al., 1994; Schnurr et. al., 1984). Protection afforded by anti-virus antibodies is affected by the age and gender of the host (Gauntt 1997; Huber and Pfaeffle, 1994a; Rager-Zisman and Allison, 1973a,b; Takada et al., 1995)

Macrophages. Macrophages are present in the mononuclear infiltrates in the heart 5 to 10 days following CVB infection of mice. It has been hypothesized that macrophages may play a role in suppressing virus infection. In support of this hypothesis, injection of silica particles (which are selectively taken up by macrophages and impair their function) increases the mortality of mice infected with CVB3 (Rager-Zisman and Allison, 1973a). Also, cultured peritoneal macrophages stimulated with protease-peptone can inactivate CVB3 infectivity *in vitro*, and when transferred to suckling mice (which presumably possess only immature macrophages) they confer protection from lethal CVB3 infection *in vivo* (Rager-Zisman and Allison, 1973a). Finally, when CVB3 infected mice are pretreated with cortisone (which interferes with the release of monocytes from the bone marrow), myocardial necrosis and mortality is enhanced (Woodruff, 1979). Macrophages may protect against CVB infection through the elaboration of cytokines with antiviral and immunotactic potential (Henke et al., 1991, 1992a,b). However, more recent studies have indicated that macrophages may also play a detrimental role in the development of myocarditis. CVB infected mice with the gene for macrophage inflammatory protein-1α (MIP-1α) knocked out are resistant to the development of myocarditis (Cook et al., 1995). Also, mice depleted of Mac1-

positive macrophages and infected with EMCV fail to develop myocarditis (Hirasawa et al., 1996). Therefore, macrophages may assume a dual role, of protecting the host and also of causing tissue damage as a consequence of their actions.

Natural killer cells. A protective role for natural killer (NK) cells in CVB murine myocarditis is indirectly supported by several observations (Godeny and Gauntt, 1986, 1987b,c). Some strains of mice with decreased NK cell responses exhibit prolonged virus infection, whereas strains with high endogenous levels of NK cell activity are less susceptible to infection. Also, a temporal relationship has been reported between virus-induced NK cell activity in the spleen, and CVB3 replication in the heart. Moreover, in depletion studies, mice treated with asialo-GM_1 antibody, which is cytotoxic and eliminates NK activity in the spleen, and then infected with CVB3 exhibit increased virus titers in the heart and more severe myocarditis, as compared to untreated CVB3 infected littermates. Transfer of asialo-GM_1+ splenic cells from donor mice confers protection against lethal CVB3 infection in NK depleted recipient mice. Myocarditic CVB3 elicits NK activity but inactivated CVB3 does not, indicating that NK cell activation depends on virus replication. Protection by NK cells may be influenced by the gender of the host (Huber et al., 1980, 1981a,b; Wong et al., 1977c). Female mice infected with CVB3 mount a stronger NK cell response early in infection and exhibit less disease severity, as compared to males. The mechanism by which NK cells provide protection is not known, but it may be mediated through interferon or nitric oxide.

Interferon (IFN). A role for IFN in virus clearance during the early phase of CVB heart disease has long been the subject of speculation. *In vitro*, heart cell cultures or MOLT-4 lymphoid cells persistently infected with CVB can be cured of the virus by the exogenous addition of IFNs (Bendinelli et al., 1987; Heim et al., 1992, 1995, 1997b; Kandolf et al., 1985). *In vivo*, administration of IFN to CVB3 or EMCV infected mice around the time of virus inoculation can reduce replication of virus in the myocardium, decrease the inflammation in the heart, and improve survival, as compared to untreated virus infected mice (Kanda et al., 1995; Lutton et al., 1985; Matsumori et al., 1987a,

1988a,b; Yamamoto et al., 1998). The IFN inducer poly(I-C) also prolongs survival time of CVB3-infected mice if it is administered before or shortly after infection (Cho et al., 1982). The mechanism by which IFNs are induced in CVB3 murine myocarditis are not known, as CVB are not known to be strong inducers of IFN directly (Woodruff, 1980). IFN-mediated induction of nitric oxide (NO) production may be important in protecting against CVB infection.

Nitric oxide. Inducible nitric oxide synthase (iNOS) is the enzyme that makes nitric oxide (NO) from L-arginine (reviewed in Lowenstein and Snyder 1992; Nathan 1992; Xie et al., 1992). iNOS is transcriptionally induced by inflammatory cytokines such as IFNs, IL-1β and TNF-α (Karupiah et al., 1993; Lowenstein et al., 1993; Roberts et al., 1992; Xie et al., 1992, 1993). These cytokines are produced during CVB murine myocarditis, and not surprisingly, iNOS expression increases in the myocardium following virus infection (Mikami et al., 1996, Lowenstein et al., 1996). iNOS has been shown to have a protective role in CVB3-induced myocarditis. In support of this hypothesis, administration of high doses of the iNOS inhibitor N^{ω}-Nitro-L-Arginine Methyl Ester (L-NAME) adversely affects CVB3-induced murine myocarditis (Mikami et al., 1997). Moreover, mice more rapidly succumb to CVB3 myocarditis if they are made deficient in iNOS by targeted disruption of the iNOS gene (Zaragoza et al., 1998), or the interferon regulatory gene IRF-1 (unpublished results, and Kamijo et al., 1994), as compared to "intact" CVB-infected littermates. Protection may be conferred through NO inactivation of CVB protease 3C, an enzyme essential for CVB replication (Saura et al., 1999). However, overproduction of iNOS may also be detrimental in CVB3-induced murine myocarditis (Freeman et al., 1998), and treatment with drugs that lower (but don't abolish) iNOS expression can have beneficial effects on the disease outcome (Iwasaki et al., 1999; Mikami et al., 1997; Wang et al., 1997).

iii). Pathogenesis of acute viral myocarditis. Two pivotal factors contributing to the destruction of heart tissue in the acute phase of CVB3-induced murine myocarditis are viral mediated damage and the immune response. Virus replication in myocytes directly destroys infected myocardial tissue. Immune mediated mechanisms such as cytolytic T cells and cytokines can also cause myocyte injury.

The genetics of CVB virulence. Many CVB3 variants are used by laboratories to study viral heart disease and it has been known for some time that they differ in cardiopathogenicity. When CVB3 variants are inoculated into mice, some variants induce heart disease which is virally-mediated, whereas other variants trigger strong immune or autoimmune responses, and yet other variants fail to produce disease (Arola et al., 1995; Chow et al., 1991; Gauntt et al., 1984, 1996a; Godeny et al, 1987a; Huber et al., 1983, 1992b, 1996b; Loudon et al., 1991; Martino et al, 1998; Tracy and Gauntt, 1987; Van Houten et al., 1991a, and others).

It has been postulated that the cardiovirulent CVB3 variants may evolve naturally *in vivo* in hosts with lowered nutritional status. For example, CVB3 passage through mice deficient in selenium or vitamin E promotes transformation of a benign CVB3 variant into a virulent CVB3 strain (Beck et al., 1994a,b, 1995). Since selenium and vitamin E are both antioxidants, it is hypothesized that oxidative stresses contribute to the virus changes. The observation that avirulent CVB3 changes to a virulent phenotype in infected mice with the glutathione peroxidase 1 gene knocked out provides further support for this hypothesis (Beck et al., 1998).

The genetic basis for CVB3 cardiovirulence is not fully characterized, however both noncoding and coding regions of the genome have been implicated as important sites for influencing pathogenicity. Several studies have suggested that the 5'NTR plays a role in determining CVB3 cardiovirulence. In one study, the cardiovirulent CVB3 laboratory strain ($CVB3_{ST}$) was rendered avirulent ($CVB3_{\varnothing}$) when a uridine residue at

nucleotide 234 was mutated to a cytidine residue (Chapman et al., 1994; Tu et al., 1995). This point mutation also occurred when the avirulent CVB3Ø strain was rendered cardiovirulent following its passage through selenium deficient mice (Beck et al., 1995). Unfortunately, although this mutation can influence cardiovirulence in the laboratory setting, it has not been found in CVB3 clinical isolates, and thus it is unlikely to control cardiovirulence in nature (Tracy et al., 1996). In another study with laboratory variants, a cardiovirulent CVB3 laboratory strain ($CVB3_N$) was attenuated following serial passage in skin fibroblast cultures. The change in phenotype was attributed to a mutation at nucleotide 590 (A to U transition), however, mutagenesis studies failed to support this hypothesis (Klump et al., 1990; Zhang et al., 1993). Further support for the hypothesis that the 5'-NTR is important for cardiovirulence comes from studies using chimeric viruses constructed between cardiovirulent and avirulent CVB3 strains ($CVB3_{CG}$ and CVB3Ø; Lee et al., 1997a), ($CVB3_{20}$ and $CVB3_{AS}$/ $CVB3_{20}$ and $CVB3_{CO}$; Dunn et al., 2000). Additional studies using site directed mutagenesis are still required to identify which specific nucleotide sites are important pathogenetically. Finally, sequence comparisons between the 5'NTR of several reference strains and clinical isolates of CVB3 have been performed (Lee et al., 1997b; Gauntt and Pallansch, 1996). An adenine residue was found to be present at nucleotide 565 in 3/4 cardiovirulent CVB3s, whereas a uridine or a cytidine was found at this position in 12/12 avirulent strains (Gauntt and Pallansch, 1996). It was postulated that this site, which is found within the core structure of the CVB3 IRES, controls cardiovirulence by affecting the replicative capacity of the virus.

The capsid region of the CVB3 genome is also believed to be important for determining cardiovirulence. For example, an antibody escape mutant ($CVB3_{H310A1}$) of a myocarditic CVB3 variant ($CVB3_{H3}$) has been isolated (Loudon et al., 1991; Van Houten et al., 1991a). Cloning, sequence analysis, and site directed mutagenesis revealed that a single asparagine to aspartate mutation in amino acid 165 of the VP2 capsid protein was responsible for attenuating the cardiovirulence of the $CVB3_{H3}$ strain

(Knowlton et al., 1996). This mutation maps to the surface of the virion in a region known as the "VP2 puff". It has been postulated that this region contains immunodominant epitopes which may be important pathogenetically (Knowlton et al., 1996). Modified virus receptor binding capabilities may also be involved (Weller et al., 1989). Further support for the hypothesis that the capsid region of CVB3 influences cardiovirulence comes from the study in which the avirulent CVB3ø strain was rendered cardiovirulent following passage through selenium-deficient mice (Beck et al., 1995). Sequence analysis of the two CVB3 strains revealed a mutation in the P1A (VP4) region of the genome at nucleotide 788, which resulted in an Arg-Gly change. In addition, two mutations were found in the P1C (VP3) region of the genome, at nucleotides 2271 (Phe-Tyr) and 2438 (Gln-Glu). A fourth mutation was found in the P1D (VP1) region of the genome at nucleotide 2690, although this did not cause an amino acid change. A role for the capsid proteins in determining cardiovirulence is also indicated in studies using chimeric viruses constructed between cardiovirulent and avirulent CVB3 strains (Lee et al., 1997a), and from studies comparing the sequences of reference strains of CVB3 (Lee et al., 1997b).

Other regions of the CVB3 genome may also be important for cardiovirulence. In the studies on CVB3 attenuation following passage through selenium deficient mice, a mutation was found in the P2A ($2A^{pro}$) region of the genome at nucleotide 3324, which resulted in a Val-Ala change (Beck et al., 1995). In addition, a mutation was found in the 3'-nontranslated region at nucleotide 7334 (Beck et al., 1995). Site directed mutagenesis will help to confirm the pathogenic implications of the mutations found at these sites.

CVB3 killing of myofibers. In murine models of CVB infection, virus is distributed through the bloodstream to target organ sites. Virus can be cultured from the blood within a few hours post inoculation, and it can be cultured from organ sites such as the heart, spleen, pancreas, liver and brain by 1-2 days post inoculation (Gauntt 1997). Virus can also be detected in the myocardium at 2-3 days post inoculation by in

situ hybridization, and it reveals a pattern of random viral distribution in the ventricular myocardium indicative of the hematogenous spread of virus during infection (Klingel et al., 1992).

There are several cell types which may be important for virus distribution following infection. For example, M cells in Peyers patches are postulated to transcytose poliovirus from the lumen of the gut to the underlying tissue for transport to the bloodstream (Melnick, 1990), and a similar mechanism may occur for CVB as well. In addition, immune cells may help traffic virus through the bloodstream (Liu and Opavsky, 2000). Additional factors such as C3 sequestration may influence the pattern of virus deposition in the spleen (Anderson et al., 1997). In the heart and other target organ sites, it is also tempting to speculate that CVB susceptible endothelial cells serve as initial foci of virus infection and replication, and that virus is distributed from these sites to the underlying tissue. In support of this hypothesis, it has been demonstrated that CVB can infect and replicate in cultures of human umbilical vascular endothelial cells (HUVEC) *in vitro* (Carson et al., 1999; Conaldi et al., 1997). HUVEC cells express the human coxsackie-adenovirus receptor (CAR), which presumably mediates CVB infection of the cells (Carson et al., 1999). The virus has also been shown to infect and replicate in vascular endothelial cells derived from murine heart, liver, and lungs (Huber et al., 1984, 1990). Interestingly, virus isolated from specific organ sites exhibits a greater ability to replicate in endothelial cells derived from that organ, as compared for endothelial cells derived from other organ sites, and thus vascular endothelial cells may serve as important mediators of virus tropism *in vivo* (Huber et al., 1990).

CVB3 replication in infected heart tissue is considered to be an important contributing factor to the development of acute myocarditis. The presence of necrotic myofibers at 3 days post inoculation, well before the appearance of an inflammatory cell response, is consistent with CVB3 lysis of myocytes (McManus et al., 1993; Wilson et al., 1969). Histopathological examination of the hearts of CVB infected mice also reveals cytolytic and necrotic changes associated with viral replication well beyond day

3 of infection, as was elegantly described by McManus and colleagues (McManus et al., 1993). Extensive cardiac damage associated with virus replication has also been noted in SCID mice (Chow et al., 1992), athymic mice (Kandolf et al., 1987a; Robinson et al., 1981; Sato et al., 1994; Schnurr et al., 1984), and marasmic mice (Woodruff et al., 1970a,b, 1971); murine models in which the expected inflammatory cell infiltrate is genetically or experimentally suppressed. Finally, immunosuppressive therapies such as adriamycin, cyclophosphamide, and cyclosporin A show no beneficial effect in ameliorating the severity of myocarditis in several strains of inbred mice (see Table 5).

CVB-induced myocytolysis has been examined *in vitro* by use of cultured fetal human (Kandolf et al., 1985) and murine (Yoneda et al., 1979) heart cells. The virus infects myocytes, as evidenced by the presence of viral particles in the cell cytoplasm on ultrastructural examination. Viral replication in myocytes has been confirmed by the production of progeny virus. Damage to the myocytes occurs within 2 days of infection, by which time the majority have lost their contractile ability. Electron microscopy of affected myocytes reveals a pattern of classic enterovirus cytopathic effect, characterized by the destruction of myofibrils and filaments, and the formation of vesicles and vacuoles throughout the cytoplasm. The presence of virus genome in the cytoplasm of infected myocytes, along with cytopathic effects in the infected cells, has also been noted in CVB3 infected murine hearts through the combination of *in situ* hybridization and electron microscopic techniques (Hofschneider et al., 1990; Klingel et al., 1993, 1998; Mall et al., 1991; Ukimura et. al., 1997). Interestingly, it has been recently shown that CVB3 infection of cells leads to caspase activation, and it has been postulated that this too may be important for altering the structural integrity of infected cells (Carthy et al., 1998).

CVB3 infection of myocytes is also postulated to contribute to the pathogenesis of DCM through the disruption of cytoskeletal proteins such as dystrophin (Badorff et al., 1999). Enteroviral protease 2A cleaves dystrophin extracted from cultured rat neonatal ventricular myocytes. Furthermore, dystrophin cleavage products are found in protein

extracts made from CVB3 infected cultured rat neonatal myocytes and CVB3 infected immunocompetant and SCID mice hearts. Virus mediated dystrophin cleavage presumably leads to morphological disruptions in the infected cells, as indicated by the disorganization of dystrophin arrays on immunohistochemical staining, and by an increase in plasma membrane permeability. Abnormalities to cytoskeletal proteins such as dystrophin have also been demonstrated in hereditary forms of DCM, and thus the pathogenic mechanisms involved may be common to both forms of the disease process.

T lymphocyte killing of virus infected myofibers. T cells generally respond to virus infection in the context of molecules encoded by the major histocompatibility complex (MHC) (reviewed in Clark and Ledbetter, 1994; Doherty et al., 1992). Cytotoxic T cells (CTLs/CD8+/Lyt 2+) recognize virus-derived peptides presented by MHC class I, and respond to antigen challenge by lysis of target cells. Helper T cells (CD4+/L3T4+) recognize peptides presented by MHC class II, leading to proliferation of B cells, CTLs, and macrophages. Not surprisingly, if T cells are incapacitated then the hosts' ability to respond to virus infection is hampered (Ahmed et al., 1988; Byrne and Oldstone, 1984; Leist et al., 1987; Moskophidis et. al., 1993; Zinkernagel and Welsch, 1976; and others).

In CVB myocarditis, however, T cells have been shown to be a major cause of damage to myocardial tissue. In a pioneering study, CD-1 mice depleted of T cells by pretreatment with anti-thymocyte serum and then infected with virus exhibit a marked reduction in myocardial disease as compared to immunologically intact littermates (Woodruff and Woodruff, 1974). There was a significant decrease in cardiac inflammation and tissue injury after T cell deprivation, even though there was no change to the pattern of virus replication and clearance from the infected heart tissue. Balb/c mice rendered deficient in T cells by thymectomy, irradiation, and reconstitution with bone marrow cells also exhibited suppressed cardiac inflammation and necrosis, and adoptive transfer of T lymphocytes into these animals restored susceptibility to CVB3 myocarditis (Blay et al., 1989; Huber and Lodge, 1984). The Src-family protein tyrosine

kinase $p56^{lck}$ expressed in all T lymphocytes (Marth et al., 1985; Veillette et al., 1988) has been shown to have a pivotal role in the genesis of T cell responses in CVB3 myocarditis. Mice with the $p56^{lck}$ gene "knocked out" (Molina et al., 1992; Penninger et al., 1993) are resistant to CVB3 myocarditis. These mice fail to generate T lymphocyte responses during CVB3 infection, and there is no apparent pathological damage to the heart tissue, and morbidity and mortality remain at uninfected control levels, despite the presence of virus replication in the heart (Liu et al., 2000).

Characterization of specific T cell populations responsible for CVB-induced myocarditis has been difficult due to the differing cellular responses which occur in inbred strains of mice. In CVB3 infected Balb/c mice, the presence of cytotoxic T lymphocytes in the heart correlates with disease severity (Guthrie et al., 1984; Huber et al., 1986, 1997; Lodge et al., 1987). In CVB infected DBA/2 or MRL+/+ mice the disease is predominantly mediated by helper T cells (Blay et al., 1989; Huber et al., 1986, 1997; Lodge et al., 1987). In CVB infected A/J mice, the disease is mediated by both helper T cells and cytotoxic T cells (Lodge et al., 1987; Opavsky et al., 1999). Diverse host responses also occur in CVB3 infected inbred murine strains treated with prednisone or cyclosporin - treatments aimed at depressing the immunocyte response (see Table 5). Differing cellular responses to CVB infection also occur in sublines of inbred mouse strains (Huber et al., 1999d; Van Houten and Huber, 1991b) prompting the suggestion that it is the expression of specific genetic loci which is important for generating a critical T cell phenotype. In general, activation of Th1-type responses are postulated to promote susceptibility to viral myocarditis, while Th2-type responses confer resistance to the disease, and this may be mediated in part by activated $\gamma\delta$+ T cell populations (Huber et al. 1992b, 1996b, 1999c,d; Huber and Pfaeffle, 1994a; Opavsky et al., 1999).

Female mice are less susceptible to CVB3-induced myocarditis than males (Huber et al., 1981a,b, 1994a, 1999c; Wong et al., 1977c). Gender susceptibility is considered to be due in part to the influence of sex hormones such as estrogens and

androgens on T- cell mediated cytotoxicity (Huber et al., 1994a, 1999c; Lyden et al., 1984). Physiological factors which affect host T lymphocyte responses (and susceptibility to CVB murine myocarditis) are exercise (Gatmaitan et al., 1970; Ilbäck et al., 1989a; Reyes et al., 1981), nutritional status (Beck et al., 1994a,b, 1995, 1997, 1998; Ilbäck et al., 1998; Woodruff, 1970a,b, 1971) and environmental effects (Boring et al., 1956; Cheever, 1953; Woodruff, 1980; Ilbäck et al., 1996). Virulence factors directly attributable to the CVB3 variant used for infection also influence host T cell responses.

It is not known if the T cells activated during acute myocarditis are responding to CVB antigens on the infected myocardial cells, or to upregulated (Huber, 1988, 1992a) or newly synthesized (Paque et al., 1978, 1979) cardiac cell antigens in CVB infected heart cells. Also, although CTLs may kill targeted cells by Fas-dependent apoptosis or through perforin-based pathways (Kägi et al., 1994), the role that these mechanisms play in clearing virus infected cardiomyocytes is unclear. For example, T cell mediated apoptosis of virally infected myocytes is not indicated since apoptotic signals are rarely detected in myocytes inside inflammatory lesions in CVB3 infected CD1 mice, C3H.HeJ mice (Colston et al., 1998), C57 mice (Gebhard et al., 1998), DBA/2 mice, MRL +/+ mice, or BALB/c mice (Huber 1997) during the acute stage of the disease, nor in the myocytes of patients with acute viral myocarditis (Kawano et. al., 1994; Seki et al., 1998). Moreover, it is unlikely that perforin elaboration by T cells represents a critical mechanism for viral clearance from the heart, since levels of infectious virus are the same in the hearts of CVB3 infected perforin knockout mice as in the hearts of "intact" littermates (Gebhard et al., 1998).

Cytokines. Cytokines (reviewed in Curfs et al., 1997) have antiviral properties (see 'interferons' above, Table 5, and Czarniecki, 1993; Dianzani et al., 1988; Matsumori, 1997b), but they can also be important mediators of cardiovascular damage (Barry, 1995; Czarniecki, 1993; Matsumori, 1997b; Meldrum, 1998). The adverse affects of cytokines may be due to their exaggerated or prolonged production within the microenvironment of a heart cell. For example, exposure of cardiomyocytes in culture

to cytokines such as TNF-α, IL-1, IL-2, or IL-6 may alter myocyte contractility (Gulick et al., 1989; Yokoyama et al., 1993), induce hypertrophic growth responses (Palmer et al., 1995; Yokoyama et al., 1997), alter β-adrenergic responsiveness (Gulick et al., 1989), or cause apoptosis (Krown et al., 1996). Negative inotropic effects have also been noted in isolated perfused hearts (Hosenpud et al., 1989; Sobotka et al., 1990; Yokoyama et al., 1993). *In vivo*, elevated levels of TNF-α or TGF-β1 expressed in transgenic mice reproduces the phenotypes of myocarditis and cardiomyopathy (Bryant et al., 1998; Kubota et al., 1997a,b; Sanderson et al., 1995).

Cytokines are also implicated as important agents in the pathogenesis of viral myocarditis. In support of this hypothesis, mice infected with an amyocarditic strain of CVB3 and then administered IL-1 or IL-2 are significantly more susceptible to myocarditis as compared to mice given amyocarditic CVB3 alone (Huber et al., 1994b). Moreover, murine strains naturally resistant to chronic CVB myocarditis become susceptible when cytokine production is stimulated, by administration of CVB3 and lipopolysaccharide (LPS), or CVB3 and interleukin-1 (IL-1), or CVB3 and tumor necrosis factor (TNF) (Lane et al., 1992, 1993). Cytokines are also endogenously elaborated in CVB3 infected mice, and their presence in the heart or spleen tissue is postulated to contribute to disease pathogenesis (Gauntt et al., 2000; Huber et al., 1994a, 1996b, 1999c; Ilbäck et al., 1996; Opavsky et al., 1999; Seko et al., 1997; Shioi et al., 1996).

iv). Chronic heart disease. Chronic myocarditis begins about 14 days after virus infection. In murine strains susceptible to chronic myocarditis, histological examination of the heart reveals continuing myocardial cell necrosis and the presence of inflammatory cell infiltrates, albeit at a lower level of intensity. This leads to replacement fibrosis, calcification, and hypertrophy in remaining myocytes (Leslie et al., 1990; Lodge et al., 1987; Nakamura et. al., 1996a; Schnitt et al., 1993; Wee et al., 1992; Wilson et al., 1969). Apolipoprotein J mRNA synthesis, which is normally restricted to atrial myocytes, is induced in ventricular myocytes, possibly in an attempt to

limit tissue injury (Swertfeger et. al., 1996). Later still, dilated cardiomyopathy similar to that seen in humans becomes evident. The observed pathological changes consist of myocardial scarring in the absence of inflammation, endocardial and subendocardial scarring, and dilation of the cardiac chambers (Matsumori and Kawai, 1982; Reyes et al., 1981). Processes contributing to the ongoing immune response and cardiovascular deterioration during the chronic phase of viral myocarditis include persisting virus and persisting viral RNA, autoimmune T cells, autoantibodies, and apoptotic cell death, and are described in more detail below.

Persisting virus and persisting viral RNA. Low levels of infectious virus persisting in extracardiac sites could continue to fuel the immunocyte response in the chronic phase of the disease. Reservoirs of persisting virus have been noted in the spleen and lymph nodes in CVB3 infected mice (Anderson et al., 1996; Klingel et al., 1996), providing indirect support for this hypothesis. CVB has also been shown to establish low level persisting infections in primary cultures of myocardial cells (Heim et al., 1992, 1995, 1997b; Kandolf et al., 1985, 1987b), fetal aortic organ cultures (Blacklow et al., 1975), vascular endothelial cells (Conaldi et al., 1997), and several established cell lines (Argo et al., 1992; Bendinelli et al., 1987; Crowell, 1963; Matteucci et al., 1985; McLaren et al., 1993; Yiyun et al., 1989), and these systems may be useful for studying the processes involved. However, it is important to note that although infectious virus particles serve as targets for immune responses in the acute phase of the disease process, they may be less than likely to be triggers for the ongoing immune responses observed in later disease stages. Indeed, culturable virus has not been recovered to date from the hearts of patients with chronic myocarditis or DCM, nor from the hearts of immunocompetent CVB3 infected mice beyond day 10 post infection.

Although it is difficult (if not impossible) to culture virus from infected hearts during the chronic stage of the disease, it is possible to detect viral RNA signals in the myocardium. CVB3 RNA can be detected in the hearts of patients with myocarditis and DCM by PCR or hybridization analysis (Table 4). Also, in murine models of CVB3

infection, viral RNA can be detected in the hearts of infected murine strains susceptible to chronic myocarditis, but not in resistant strains of mice, prompting speculation that it can fuel the ongoing attraction of cytolytic T cells to the myocardium. CVB3 RNA has been detected by *in situ* hybridization for up to 56 days in the myocardium of CVB infected athymic mice (Kandolf et al., 1987a), and up to 1 month in A.CA/SnJ [H-2f] (Klingel et al., 1992; Mall et al., 1991), A.BY/SnJ [H-2b], SWR/J [H-2q] (Klingel et al., 1992), and C3H/He mice (Koide et al., 1992). In contrast, DBA/1J mice infected with CVB3 do not progress to chronic myocarditis, and viral RNA is not found to persist in the myocardium (Klingel et al., 1992).

A mechanism by which the presence of viral RNA in the heart is involved in the pathogenesis of disease remains unknown. One possibility is that viral RNA continues to replicate. In support of this hypothesis, biopsy samples from patients were found to contain persisting positive and negative sense viral RNA strands, consistent with the presence of replicative forms of viral RNA (Pauschinger et al., 1998). Moreover, plus-strand and minus-strand CVB3 RNA are detected in the hearts of infected mice beyond the acute phase of the disease (Andréoletti et. al., 1997; Hohenadl et al., 1991; Klingel et al., 1992, 1996). Low levels of virus or viral antigens may be produced (too low to be detected in conventional assays), to fuel an ongoing immunocyte response. Indeed, during the chronic stage of murine CVB3 infection up to 30 cardiac cells are infected in 1 mm^3 of heart tissue, and it has been proposed that this would be sufficient to sustain myocardial inflammation (Klingel et al., 1992).

Alternatively, even if virus replication is defective in all target organs, there are also other mechanisms which can be implicated in fueling the immunocyte response. For example, the presence of non-replicating virus genome in the hearts of genetically engineered mice has been shown to cause myocytopathic effects consistent with the development of DCM (Wessely et. al., 1998a,b). Also, secondary enterovirus infections with the same virus strain (Nakamura et. al., 1999) or a different enterovirus (Beck et al., 1990; Yu et al., 1999) could exacerbate the immune responses in the myocardium.

Autoimmune T cells. A second hypothesis to explain ongoing immune reactivity in chronic myocarditis is that autoreactive T cells are generated during viral myocarditis which can target uninfected heart cells. In support of this hypothesis, T cells isolated from CVB3 infected mice have been shown to lyse uninfected myocardial cells in culture (Huber et al., 1980, 1981a,b, 1984b, 1988; Wong et al., 1977a,b,c). *In vivo*, autoreactive T cells adoptively transferred into T cell deficient mice confer susceptibility to CVB3 myocarditis (Blay et al., 1989; Huber and Lodge, 1984). Moreover, heterotropic cotransplant of a normal mouse heart into a murine host with chronic myocarditis leads to inflammation and myocarditis in the normal transplanted heart (Nakamura et al., 1996b).

Myosin has long been hypothesized to be an antigen target for T cells in autoimmune myocarditis. Murine strains susceptible to chronic CVB myocarditis also develop myocarditis upon injection of cardiac myosin, whereas resistant murine strains do not (Neu et al., 1987). Moreover, adoptive transfer of purified T cells from mice with active cardiac myosin-induced myocarditis to SCID mice recipients successfully transfers the disease into the SCID hosts (Smith and Allen, 1991). Mice lacking the ability to activate T cells or to present myosin to autoreactive T cells are resistant to cardiac myosin induced myocarditis (Bachmaier et al., 1996, 1997).

The mechanism by which autoreactive T cells are activated in CVB infected mice is not known. One explanation is molecular mimicry between an infectious agent and self antigens (reviewed in Huber and Gauntt, 1999a). For example, cross-reactive epitopes have been identified between cardiac specific α myosin heavy chain and the 60-kDa outer membrane protein of chlamydia which are important in the development of myocarditis (Bachmaier et al., 1999a,b; Pummerer et al., 1996), although surprisingly they are not shared with CVB. Also, the peptide "NT4" shares sequence homology with group A streptococcal M5 protein and cardiac myosin β-chain, and immunization with NT4 peptide induces myocarditis in MRL/++ mice, while tolerization with NT4 protects against CVB3-induced myocarditis (Huber and Cunningham, 1996a). Other

mechanisms include a response to excessive amounts of antigen released from myocytes during the inflammatory stage of CVB3 myocarditis, a decrease in immunological tolerance, clonal expansion of T cells which escaped thymic education, T cell mediated stimulation of other immunocytes, or γ/δ TCR+ lymphocyte reversal of clonal anergy of α/βTCR+ cells in immunocompetent hosts *in vivo* (Gauntt et al., 2000, Huber et al., 1992a, 1999b; Moraska and Huber, 1993).

Autoantibodies. Autoantibodies with pathogenic potential may also play a role in the cardiovascular deterioration of chronic myocarditis. This has been reviewed in detail (Gauntt 1997, 2000; Huber and Gauntt, 1999a; Rose and Hill 1996). Briefly, autoantibodies have been identified against the ADP/ATP carrier protein (Schultheiss et al., 1985a,b, 1986; Schwimmbeck et al., 1993), myosin (Beisel et al., 1991; Cunningham et al., 1992; Gauntt et al., 1991, 1995; Latif et al., 1999), and other β-helical coiled-coil molecules (Cunningham et al., 1993; Latif et al., 1999), and miscellaneous cardiac antigens (Latif et al., 1999; Srinvasappa et al., 1986; Wolfgram et al., 1985). Some autoantibodies show evidence of immunopathogenic potential. Autoantibodies against the adenine nucleotide translocator (ANT) penetrate viable myocytes and affect cardiac energy metabolism by inhibiting nucleotide transport across the ADP-ATP carrier *in vitro* (Schulze et al., 1989). Other autoantibodies cause cell lysis in cytotoxicity assays (Cunningham et al., 1992), elicit macrophage chemotactic activity *in vitro* (Gauntt et al., 1991; Gauntt 1995), or induce myocardial pathology when injected into mice (Gauntt et al., 1991, 1993, 1995). One mechanism by which autoantibodies might arise is by antigenic mimicry - first through reactivity to CVB, and later by cross-reacting with proteins released from necrotic cells (Gauntt et al., 1993, 1995, 1996b, 1997; Huber and Gauntt, 1999a).

Apoptosis. Apoptosis of myocytes may contribute to the gradual decline in cardiac function in chronic or autoimmune myocarditis leading to DCM. Apoptosis occurs in myocytes outside areas of evident inflammation or infection in CVB infected immunocompetant BALB/c mice (Huber 1997). Apoptosis has also been noted in

myocytes in perforin "knock-out" mice, where the inflammatory infiltrate is genetically reduced or absent (Gebhard et al., 1998). Heart tissue samples from patients with chronic myocarditis (Kawano et al., 1994), DCM (Seki et al., 1998), and end-stage heart failure (Narula et al., 1996) also show evidence of myocyte apoptosis. The mechanism by which cardiomyocyte apoptosis is triggered in this phase of the disease is not known, but it could occur through cytokine-mediated pathways (Krown 1996), by iNOS activated pathways (Kawaguchi et al., 1997; Shimojo et al., 1999; Stein et al., 1998), or through the actions of other soluble mediators.

v). Treatment. At the present time, there is little effective treatment for patients for myocarditis and DCM. However, animal models of CVB infection (Table 5) have indicated new avenues for the treatment of viral heart disease. The clinical significance of many of these treatments has been extensively reviewed (Caforio and McKenna 1996; Kawai 1999; Liu et al., 1992; Maisch et al., 1993; Martino et al., 1995b; Mason et al., 1995; Matsumori 1997a, Sole and Liu, 1993). Briefly, very exciting experimental results have been noted for the angiotensin-converting enzyme (ACE) inhibitors, antiviral agents, β-blockers with vasodilatory properties, calcium channel blockers, T-cell gene knockout strategies, and protease inhibitors. On the basis of experimental studies, the following are not recommended for use in a clinical setting: corticosteroids, compounds which increase oxidative stresses, cytokines such as IL-1, IL-2, and TNF, nonspecific immunosuppressive agents, iNOS inhibitors, and nonsteroidal anti-inflammatory drugs.

Table 5. Experimental studies of virus-induced heart disease and treatment

Treatment category	[a,b] Susceptiblity to experimental viral myocarditis	References
Alpha1-Adrenergic Antagonist		
Doxazosin	Decreases	Sole 1993
Angiotensin-Converting Enzyme Inhibitor		
Captopril	Decreases	Araki 1995; Rezkalla 1990a,b; Takada 1997
Enalapril	Decreases	Araki 1995
[c]TCV-116	Decreases	Tanaka 1994
Angiotensin II receptor antagonist		
Losartan	No effect	Araki 1995
Antiviral Agents (natural and synthetic)		
IFN-α, β ,γ	Decreases	Cho 1982; Kanda 1995; Lutton 1985; Matsumori 1987a,b,c, 1988a,b; Yamamoto 1998
ribavirin	Decreases	Matsumori 1985, 1987a,b,c, 1988a
WIN 54954	Decreases, No effect	Ilbäck 1993; McKinlay 1993; Pauksen 1993
β-blockers		
Carteolol	Decreases	Tominaga 1991
Metoprolol	No effect, Increases	Rezkalla 1988; Tominaga 1991
β1-adrenergic agonist		
Denopamine	Decreases	Nishio 1998
Calcium channel blocker		
Amlodipine	Decreases	Wang 1997
Verapamil	Decreases	Dong 1992
Corticosteroids		
Cortisone	Increases	Dalldorf 1954; Kilbourne 1951, 1956; Woodruff 1979
Prednisolone	Increases	Kawai 1978; Tomioka 1986
Prednisone	Increases, Decreases	Herzum 1991; Maisch 1993
Gene "knock out"		
CD4 or CD8	Variable	Henke 1995; Opavsky 1999
CD4 and CD8	Decreases	Opavsky 1999
CD45	Decreases	Liu 1995
Glutathione peroxidase 1 (GPX-1)	Increases	Beck 1998
iNOS	Increases	Zaragoza 1998

IRF	Increases	Kimura 1994; Liu 1995
MHC class II IE-	Decreases	Huber 1999b
MIP-1 α	Resistant	Cook 1995
Perforin	Decreases	Gebhard 1998
p56lck	Resistant	Martino 1995b
Severe combined immunodeficiency (SCID; lack T and B cell functions)	Increases	Chow 1992
TCRβ	Decreases	Opavsky 1999
Immunization		
Antiviral antibody	Decreases	Cho 1982
Immunoglobulin serum	Decreases	Matsumori 1987b; Weller 1992
Inactivated virus, attenuated virus, subunit vaccine, or other virus variants	Variable	Beck 1990; Fohlman 1990, 1993; Godeny 1987a; Khatib 1994; Landau 1990; Matsumori 1987b,c; Nakamura 1999; Zhang 1997
Immunomodulation		
Antibody mediated NK cell depletion	Increases	Godney 1986, 1987b
Antibody mediated T-lymphocyte depletion	Variable	Blay 1989; Lodge 1987; Van Houten 1991b; Woodruff 1974
Anti-CD40L/B7-1 mAb	Decreases	Seko 1998a,b
Anti-Mac1 MAb	Decreases	Hirasawa 1996
Anti-vascular cell adhesion molecule (VCAM)-1 MAb	No effect	Seko 1996
BIOPLF-70	Decreases	Gauntt 1985
Granulocyte colony stimulating factor (G-CSF)	No effect	Hiraoka 1995
Interleukin-1 (IL-1)	Increases	Huber 1994b; Lane 1992
Interleukin-2 (IL-2)	Increases, No effect	Huber 1994b; Matsumori 1991
Interleukin-6 (IL-6)	Decreases	Kanda 1996a,b
Interleukin-8 (IL-8)	No effect	Kanda 1996a
Interleukin-12 (IL-12)	Decreases	Shioi 1997
Levamisole	Increases	Gudvangen 1983
Macrophage colony stimulating factor (M-CSF)	Decreases	Hiraoka 1995
Quinoline-3-carboxamide (LS 2616)	Decreases	Ilbäck 1989b; Ilbäck 1993
Tumor necrosis factor (TNF)	Increases	Lane 1992, 1993; Matsumori 1991
Vesnarinone	Decreases	Matsui 1994

Immunosuppressive agents		
Adriamycin	Increases	Huber 1992a
Cyclophosphamide	Increases	Kishimoto 1990; Rager-Zisman 1973b; Rozee 1992
Cyclosporin A	Decreases, Increases	Estrin 1987; Herzum 1991; Maisch 1993; Monrad 1986
Immunosuppressive clinical trials		
prednisone, cyclosporin A	No effect	De Ward 1992; Mason 1995
Nitric oxide inhibition		
[d]L-NAME (high dose)	Increases	Hiraoka 1996; Lowenstein 1996; Mikami 1997
[e]NMMA	Increases	Lowenstein 1996
Nonsteroidal Anti-Inflammatory Drugs		
Ibuprofen	Increases	Costanzo-Nordin 1985
Indomethacin	Increases	Rezkalla 1986
Salicylates	Increases	Rezkalla 1986
Nutritional status		
Selenium deficiency	Increases	Beck 1994a, 1995, 1998; Ilbäck 1998
Vitamin E deficiency	Increases	Beck 1994b
Opiate receptor antagonist		
Naloxone	Decreases	Kishimoto 1995
Phosphodiesterase (PDE) III inhibitor		
Amrinone	Increases, no effect	Matsui 1994; Seta 1997
Pimobendan	Decreases	Iwasaki 1999
Protease Inhibitor		
ZD0892 (serine elastase inhibitor)	Decreases	Lee 1998
Thrombolytic Agents		
Heparin	Decreases	Frizelle 1992

[a]Studies used CVB or EMCV infection

[b]Murine models of virus infection

c(±)-1-(cyclohexyl-oxycarbonyloxy)ethyl 2-ethoxy-1-{[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylate.

[d]L-NAME, N^G-arginine methyl ester

[e]NMMA, N^Gmono-methyl-arginine

C. ENTEROVIRUS RECEPTORS

The concept of an enterovirus receptor was first introduced by Dr. John Holland. He demonstrated that enteroviruses bind to homogenates made from tissues in which they normally propagate *in vivo*, and postulated that the viruses did so by interacting with specific 'receptor' molecules on the cells (Holland, 1961). Over the next 3 decades, many studies were performed to characterize the receptor molecules. In early studies, biochemical assays using detergents or enzymes were performed, and they indicated that most enterovirus receptors were integral membrane protein molecules. Competitive binding assays were also used, to determine the quantity and specificity of the viral receptors. However, specific identification of enterovirus receptors did not take place until recently, and it relied on the use of antibodies for receptor purification, and molecular technology for cloning and expressing the receptor molecule. The first enterovirus receptor to be identified was the poliovirus receptor (PVR). At about the same time, a receptor for the major group of human rhinoviruses and for some group A coxsackieviruses was identified as the intracellular adhesion molecule ICAM-1. Receptor molecules used by echoviruses and group B coxsackieviruses were also subsequently identified, and include the decay accelerating factor receptor (DAF) and coxsackie-adenovirus receptor (CAR).

Today, the study of virus-receptor interactions is a rapidly growing focus in Virology. Several types of receptors have been identified. Some receptors are fully functional, and their expression on the cell surface renders cells susceptible to virus infection. Others are termed co-receptors because they work in conjunction with a primary receptor molecule to permit virus infection of cells. Finally, receptor molecules have also been identified which serve only as attachment sites for virus, and internalization occurs by other mechanisms. The studies performed to date on viral receptors increase our understanding of viral host range, viral tropism, disease

pathogenesis, and provide new avenues for the treatment of viral diseases in a clinical setting.

The initial encounter between enteroviruses and their receptor molecules is presumably a random event, but it is influenced in part by the affinity of a virus for it's receptor molecule, and by the concentration of infecting viral particles and the availability of receptor molecules. Virus-receptor binding is a primary step in the infection process, and it triggers events which lead to the uncoating of the virion capsid, and release of the genome into the cell cytoplasm where viral transcription and translation begin. The specific strategies used by enteroviruses for binding to their receptors, and for uncoating and release of the viral genome are discussed below. The critical role that receptors play in CVB infection and the genesis of viral heart disease is then presented in the studies that constitute this thesis.

C.1. The Poliovirus Receptor

The poliovirus receptor was the first enterovirus receptor to be identified, and is perhaps the best characterized. In early studies on the poliovirus receptor, competitive binding assays were used to demonstrate that all poliovirus serotypes 1-3 bound to the same cellular receptor while other members of *Picornaviridae* did not bind to this receptor molecule (Lonberg-Holm et al., 1976). Monoclonal antibodies were generated against the receptor, and pretreatment of susceptible cultured cells with anti-receptor antibody protected cells from the cytopathic effects of poliovirus (Minor et al., 1984; Nobis et al., 1985; Shepley et al., 1988). Poliovirus was shown to bind to isolated cell membrane extracts, however, further attempts to purify the receptor from these membrane preparations were unsuccessful (Krah and Crowell, 1982).

The cellular receptor for poliovirus was eventually cloned and characterized using molecular techniques (Mendelsohn et al., 1986, 1989). Poliovirus resistant mouse L cells were transformed with DNA from human HeLa cells that were susceptible to

poliovirus. L cell transformants which expressed the poliovirus receptor DNA were located using anti-receptor MAb. DNA from the transformed cells was used to construct a genomic library, which was then screened for the presence of human DNA with a probe for human Alu repeat sequences. Three clones were isolated, and restriction fragments of the genomic DNA clones were used as probes in Northern blot assays on total cell RNA prepared from poliovirus susceptible and poliovirus resistant cell lines. A 1 kb fragment was found which potentially contained the sequences corresponding to the poliovirus receptor DNA, and was used to screen cDNA libraries. Full length cDNA clones were isolated, and upon transfection into non-susceptible cell lines they conferred susceptibility to polioviruses.

Based on cDNA analyses, the gene for the human poliovirus receptor (PVR or CD155) can be expressed as four splice variants. The two variants termed PVRα and PVRδ are membrane bound, and the two variants termed PVRβ and PVRγ are excreted from the cell (Koike et al., 1990; Mendelsohn et al., 1989). The primary structure and amino acid sequence of the PVR has been deduced from their cDNAs (Koike et al., 1990; Mendelsohn et al., 1989), and is shown in Figure 3A. PVR is homologous to immunoglobulin superfamily proteins. It consists of three extracellular domains stabilized by intrachain disulfide bonds, and possesses eight potential N-glycosylation sites. The two membrane bound PVRs each have a transmembrane region of 24 residues, but differ in their carboxy-terminus domains which contain 50 (PVRα) or 25 (PVRδ) residues. The molecular weights of the deglycosylated forms of membrane bound PVR are 45 kDa (PVRα) and 43 kDa (PVRδ), while the fully glycosylated molecule has an apparent molecular weight of 80 kDa (Bernhardt et al., 1994a). The native biological function of the PVR is not known.

Figure 3A

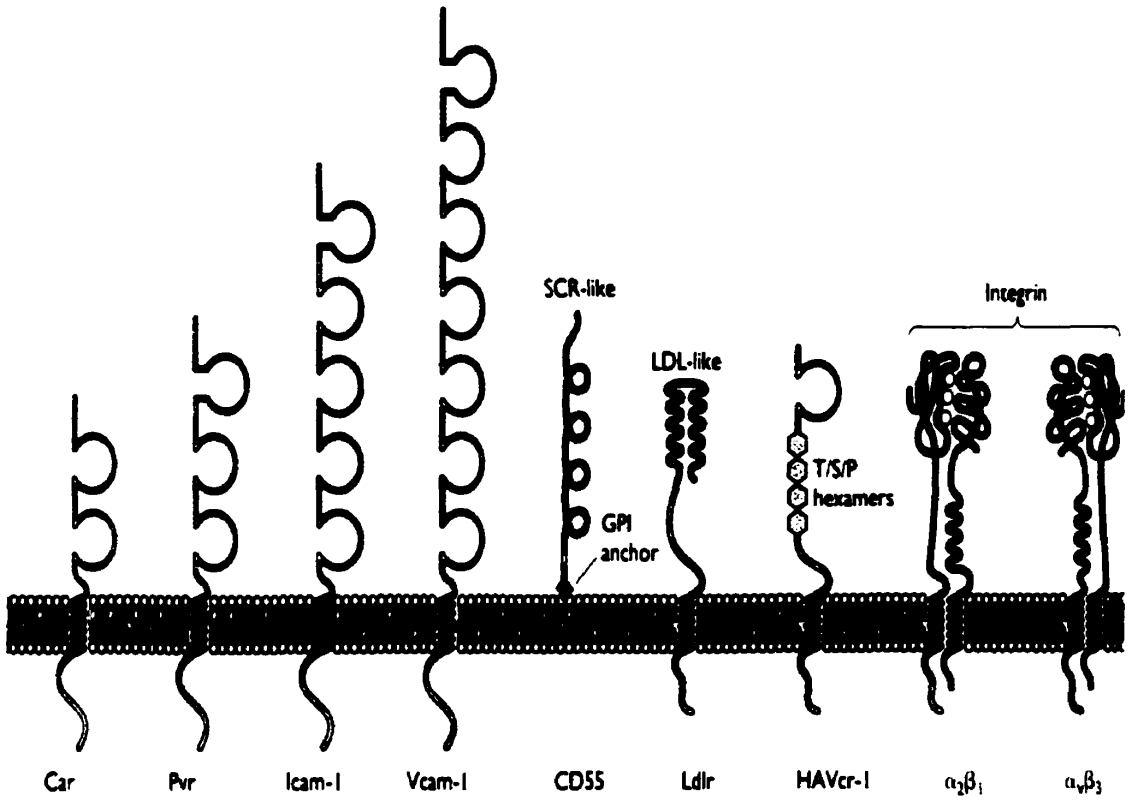


Figure 3. Enterovirus receptors and mode of virus entry into susceptible cells. (A) Schematic diagram of the cellular receptors. Car, coxsackievirus and adenovirus receptor; Pvr, poliovirus receptor; Icam-1, intracellular adhesion molecule 1; Vcam-1, vascular adhesion molecule 1 (receptor for encephalomyocarditis virus); CD55, decay accelerating factor (DAF); Ldlr, low-density lipoprotein receptor; HAVcr-1, hepatitis A virus receptor; α2β1 and αvβ3, integrin receptors. The different domain types are SCR-like, short consensus repeat-like; LDL-like, low density lipoprotein-like; T/S/P hexamers, threonine/serine/proline hexamers. CAR, Pvr, Icam-1, and Vcam-1 have immunoglobulin-like domains. CD55 is anchored to the membrane by a GPI (glycophosphoinositol) linkage. Adapted from D.J. Evans and J.W. Almond, *Trends Microbiol.* 6:198-202, 1998, with permission.

Figure 3C

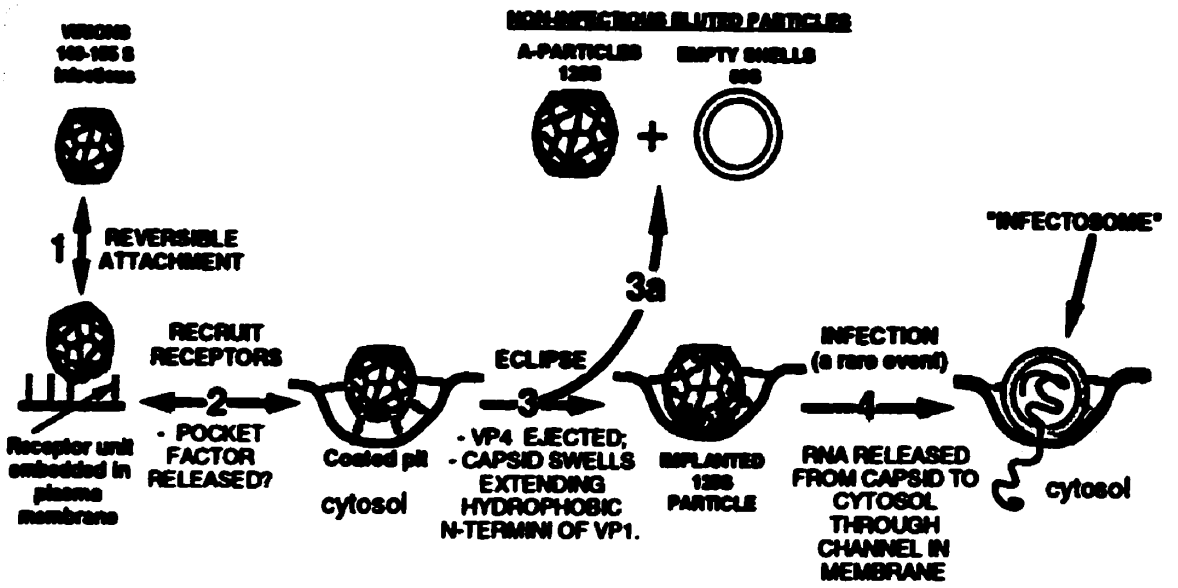


Figure 3C. Model of virus-receptor binding and viral RNA entry into the cell cytoplasm. Step 1, virus attaches to its receptor on the cell membrane. Step 2, virus attaches to multiple receptors, and is located to coated pits for endocytosis. Step 3, conformational alteration of virion leading to formation of A particles. Virus may proceed from this stage to RNA infection of cells, or may be released from the cell surface in the case of abortive entry. Step 4, viral RNA enters the cytoplasm. It is not known whether RNA enters across the cell surface membrane, or across cytosolic endosomes. See Figure 3D for an illustration of RNA release from the virion. Adapted with permission from B.N. Fields et al., (ed)., *Fields Virology* (Lippincott-Raven Publishers, Philadelphia, Pa., 1996), with permission.

Figure 3D

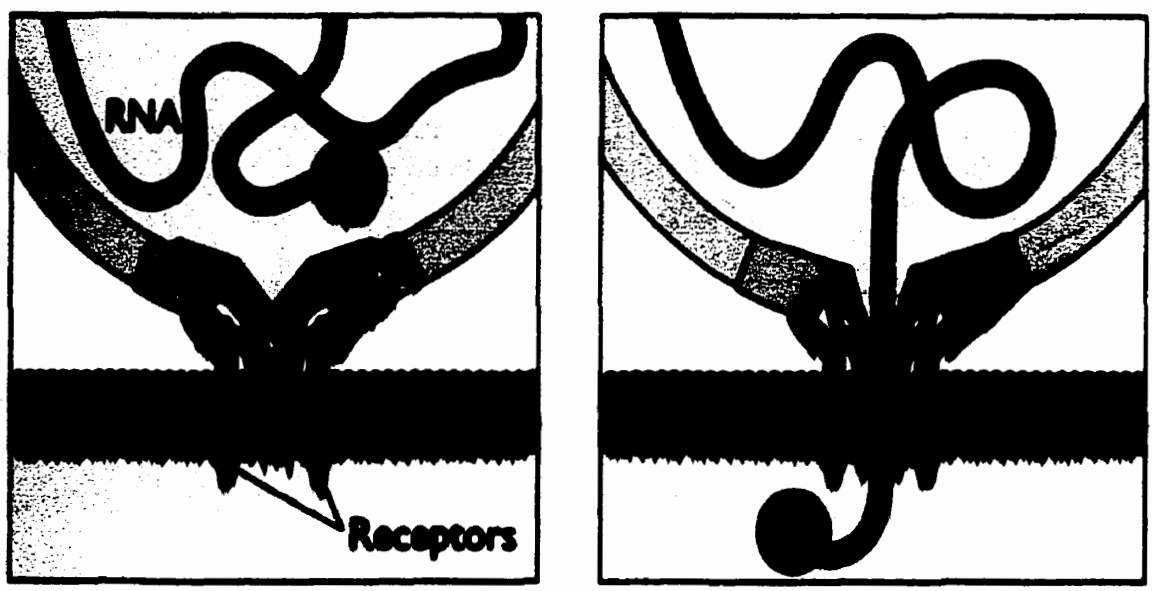


Figure 3D. Model of poliovirus RNA entry into cells. Poliovirus binds to its receptor the PVR, leading to "A" particle formation. The A particles have lost the VP4 protein and irreversibly externalized the N-termini of VP1. The VP1 end termini form a pore in the cell membrane, and viral RNA is released into the cell cytoplasm. Adapted with permission from B.N. Fields et al., (ed)., *Fields Virology* (Lippincott-Raven Publishers, Philadelphia, Pa., 1996), with permission.

On the PVR molecule, it is the N-terminal V domain (domain 1) that binds to the virus, although all 3 external domains of PVR are required for fully functional binding and infectivity (Bernhardt et al., 1994b; Harber et al., 1995; Koike et al., 1991, 1992; Racaniello, 1996; Selinka et al., 1991, 1992; Ziebert and Wimmer, 1992). On the virion, the specific site of binding is the "canyon". This is a surface depression that surrounds each of the twelve 5-fold axes of symmetry on the poliovirion (Figure 2C). The canyon was initially proposed to be the site of PVR binding because mutagenesis of amino acids within the canyon altered the binding phenotype of polioviruses (Colston and Racaniello, 1994, 1995; Harber et al., 1995). Most recently, PVR binding in the canyon region has been demonstrated visually, using cryoelectron microscopy and three-dimensional image reconstruction techniques (Belnap et al., 2000; He et al., 2000). However, although PVR binds in the canyon, adjacent surface residues and internal capsid residues are also important. Through mutagenesis studies, sites in VP1 (Colston and Racaniello, 1995), the hydrocarbon binding pocket (Colston and Racaniello, 1995), the VP1 E-F loop (Liao and Racaniello, 1997), and residues adjacent to neutralization antigenic sites (Harber et al., 1995) have been shown to modulate virus-receptor binding and virus infectivity. Amino acids in the interior of the capsid have also been reported to be important for receptor binding, and these are believed to exert an influence by controlling the flexibility of the viral capsid (Liao and Racaniello, 1997).

Structural changes to the poliovirion occur following binding to the receptor molecule. When poliovirus binds at 37^0C to receptors on susceptible cells (Fenwick and Cooper, 1962; Fricks and Hogle, 1990), membrane extracts (De Sena and Mandel, 1976, 1977; Guttman and Baltimore, 1977), or to soluble receptor (Kaplan et al., 1990a), the virion is conformationally altered to an "A" particle (Figure 3C, 3D). This altered particle has lost the internal protein VP4 and has irreversibly externalized the N terminus of VP1. Moreover, it lacks the ability to bind further to susceptible cells, differs in the sedimentation coefficient from wild-type virus (135S instead of 160S), and is more susceptible to proteases and detergents as compared to native virus. The "A" particle,

or possibly an earlier intermediate form, is hypothesized to play an important role in the infection process because the particles can be found inside susceptible cells soon after virus infection. Further support for this hypothesis comes from studies using antiviral WIN compounds, which insert in the hydrophobic pocket below the receptor binding canyon. In the presence of WIN compounds, polioviruses can still bind to their receptor, but the virion is prevented from converting to the "A" particle and is no longer infectious. Nevertheless, cold-adapted poliovirus mutants bind to receptors and infect cells without changing into "A" particles, indicating that polioviruses may infect cells by other mechanisms as well (Dove and Racaniello, 1997).

Under normal physiological conditions, the exposed lipophilic N-terminus of VP-1 of the "A" particle is believed to be inserted into the cell membrane, forming a pore through which the viral RNA is transported into the cell cytoplasm for transcription and translation (reviewed in Flint et al., 2000; Rueckert, 1996) (Figure 3D). It remains unclear, however, as to whether the RNA is released across endosomal membranes following receptor mediated endocytosis, or whether the RNA is released into the cell cytoplasm from the plasma membrane. In support of the former hypothesis, poliovirus uncoating can be prevented by treating infected cells with reagents which prevent receptor mediated endocytosis from coated pits, such as monensin, chloroquine, or N,N'-dicyclohexyl carbodimide, indicating that endosomal pathways are necessary for infection. However, other studies have indicated that endosomes may not be required for productive infection. For example, bafilomycin A1 blocks endocytosis, but has no affect on poliovirus infection. Moreover, poliovirus antibody complexes bind to cells expressing Fc receptors and are endocytosed, without productively infecting cells. Further studies on enterovirus-receptor binding, virion uncoating, and viral RNA entry into the cell will help to resolve these issues.

C.2. Receptors for group A coxsackieviruses.

Functional cellular receptors for most serotypes of group A coxsackieviruses (CAV) have not yet been identified. However, three receptor molecules that can be used by select CAVs have been reported. Intercellular adhesion molecule-1 (ICAM-1) is used by CAV serotype 21 and possibly CAV13 and CAV18. Decay accelerating factor is also used by CAV21. Finally, the vitronectin receptor termed αvβ3 integrin is a receptor for CAV9.

i). Coxsackievirus A21 and the human rhinovirus ICAM-1 receptor. In 1976, Lonberg-Holm and colleagues used competitive binding assays to show that coxsackievirus type A21 (CAV21) shares a cell surface receptor with human rhinoviruses (HRV) (Lonberg-Holm et al., 1976). Subsequent studies demonstrated that 91 out of 101 HRV serotypes competed for this cellular receptor, and they were designated the major group of human rhinoviruses (Abraham and Colonno, 1984; Colonno et al., 1990). A minor group of HRV consisting of the remaining 10 HRV serotypes compete for a different receptor, which is probably a member of the human low-density lipoprotein receptor (LDLR) family (reviewed in Blaas et al., 1994) (Figure 3A). The CAV/HRV shared receptor hypothesis was further supported by studies using monoclonal antibodies. Pretreatment of cells with antibodies generated against the receptor inhibited infection by CAV21 and the major HRV group, as well as CAV13 and CAV18 (Colonno et al., 1986; Shafren et al., 1997a). Immunoaffinity chromatography with anti-receptor antibody led to the isolation of a 90 kDa glycoprotein receptor molecule (Tomassini and Colonno, 1986). However, attempts to sequence the whole receptor molecule and further characterize it were unsuccessful.

Two approaches were used to ultimately identify this receptor, which is the intercellular adhesion molecule ICAM-1 (Figure 3A). In one approach, the whole receptor protein molecule was enzymatically digested, yielding several fragments for

use in peptide sequencing (Greve et al., 1989; Tomassini et al., 1989). Oligonucleotide probes generated on the basis of the peptide sequences were then used to screen cDNA libraries for clones encoding the receptor molecule. In the second approach, Stauton and colleagues observed that the 90 kDa receptor protein was similar in size and tissue distribution to ICAM-1 (Stauton et al., 1989). Cell transfection studies using ICAM-1 cDNA subsequently demonstrated that it was the viral receptor molecule.

ICAM-1 is a functional receptor for CAV21. Transfecting ICAM-1 cDNA into receptor negative mouse L cells renders the cells susceptible to infection by CAV21 (Shafren et al., 1997a). In contrast, ICAM-1 is only an attachment molecule for HRVs, and additional receptor molecules are required for virus infectivity (Stauton et al., 1989). However, since HRV infection of susceptible HeLa cells can be blocked by anti-ICAM-1 antibodies (Stauton et al., 1989) or soluble ICAM-1 receptor molecule (Marlin et al., 1990), it seems likely that it plays an important role in the infection process.

The deduced primary structure of the ICAM-1 molecule is homologous to immunoglobulin superfamily members, and thus it is structurally related to the receptor used by polioviruses (Figure 3A). ICAM-1 has 5 extracellular immunoglobulin-like domains containing seven or eight potential N-glycosylation sites, a transmembrane anchor, and a short cytoplasmic tail (Simmons et al., 1988; Stauton et al., 1988a,b). The deglycosylated form of ICAM-1 has a molecular mass of 55 kDa, which increases to 76-114 kDa in the glycosylated molecule. The extent of glycosylation depends on the cell type in which it is found (Dustin et al., 1986). Biologically, ICAM-1 is a cell surface ligand for the lymphocyte function-associated antigen-1 (LFA-1) adhesion receptor (Makgoba et al., 1988). Interactions between ICAM-1 and LFA-1 promote leukocyte adhesion and subsequent immunological reactions. ICAM-1 expression on vascular endothelial cells is cytokine inducible, consistent with its role in sequestering leukocytes at sites of inflammation (Dustin et al., 1986). In contrast, the receptors for poliovirus, group B coxsackieviruses, and the minor group HRVs, are not cytokine inducible in similar assay systems (Colonno et al., 1990).

Virus binds to the ICAM-1 molecule at the N-terminal domain (domain 1) of the receptor molecule, and domain 2 helps facilitate this process. This was determined by virus binding studies using ICAM-1 mutants (Register et al., 1991; Staunton et al., 1988a) or soluble forms of ICAM-1 (Greve et al., 1991), and by x-ray crystallography of HRV16 complexed with the ICAM-1 receptor (Olson et al., 1993). On the virion, the site for receptor binding has long been postulated to be the 25-Å deep canyon found at the 12 pentamer vertices. This hypothesis is based on mutagenesis studies which demonstrate that changes affecting the virus canyon structure alter receptor binding capacity (Colonno et al., 1988), and on cryoelectron microscopic visualization of the surface of the HRV14 virion (Rossmann et al., 1985) and HRV16 complexed with ICAM-1 receptor (Olson et al., 1993).

Structural changes occur once virus binds to the ICAM-1 receptor, and they are believed to facilitate cell entry. Following CAV21 binding to ICAM-1 on murine L-cell transfectants (Shafren et al., 1997a), or HRV binding to soluble ICAM-1 (Greve et al., 1991), the infectious 160S viral particles are altered to form 135S A particles. As described above for poliovirus, these altered virions lack the internal coat protein VP4, and the N-terminus of VP1 irreversibly extrudes from the virion surface (Figure 3D).

ii). CAV21 and the DAF receptor. A second receptor molecule for CAV21 is decay accelerating factor (DAF or CD55; Figures 3A, 3B). This was discovered by showing that cells preincubated with anti-DAF MAbs do not bind CAV21, as compared to normal untreated cells to which the virus readily adsorbs (Shafren et al., 1997b). Only MAbs directed against the N-terminal domain (SCR1) of DAF are capable of inhibiting CAV21 binding, indicating that the virus likely attaches to DAF in this region (Shafren et al., 1997b).

Under normal conditions, CAV21 binding to DAF alone is not sufficient to permit virus entry into the cell. The co-presence of ICAM-1 receptor is required for the

formation of the 135S A particle formation and virus infectivity (Shafren et al., 1997a,b). However, when DAF is first cross-linked on the cell surface by MAbs to SCR3 and then CAV21 is added, virus binding to DAF can lead to virus entry and to lytic cell infection. Cross-linked DAF facilitates CAV21 entry into cells in the absence of A particle formation (Shafren, 1998a,b).

iii). CAV9 and RGD-dependent and -independent receptors. The sequence of coxsackievirus A9 (CAV9) has an insertion of approximately 17 amino acids at the C terminus of the VP1 capsid protein in comparison to other enteroviruses, and it contains an arginine-glycine-aspartic acid (RGD) tripeptide motif (Chang et al., 1989, 1992). The RGD motif is postulated to be important for CAV9 binding to cellular integrin receptors. In support of this hypothesis, CAV9 binding to host cells can be inhibited by RGD-containing oligopeptides (Roivainen et al, 1991). Moreover, antibodies specific for the RGD binding $\alpha_v\beta_3$ integrin (the vitronectin receptor) protect susceptible cells from CAV9 infection (Roivainen et al., 1994). Finally, $\alpha v\beta 3$ integrin purifies from plasma membrane preparations by affinity chromatography with CAV9 (Roivainen et al., 1994). A diagrammatic illustration of integrin receptors can be seen in Figure 3A.

RGD-dependent binding to integrins has been noted in a number of other systems as well. The RGD motif is found in the VP-1 protein of foot-and mouth disease virus (FMDV) (Berinstein et al., 1995; Fox et al., 1989; Weddell et al., 1985) and echovirus 22 (Hyypia et al., 1992; Pulli et al., 1997), and promotes virus binding to integrins. Also, adenoviruses infect cells using $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors by an RGD-dependant mechanism (Belin and Boulanger, 1993; Wickham et al., 1993). Moreover, *Borrelia burgdorferi* (the causative agent of Lyme disease) binds to the RGD-recognizing integrin receptor $\alpha_{11b}\beta_3$ (Coburn et al., 1993). And finally, the RGD motif is present in fibronectin, vitronectin, and many other adhesive proteins in the extracellular matrix and blood, and promotes binding to cell surface integrin receptors (Ruoslahti and Pierschbacher, 1987).

However, CAV9 is also capable of infecting cells in a non-RGD dependent manner, since CAV9 can infect human rhabdomyosarcoma (RD) cells and neither RGD oligopeptides nor polyclonal antiserum to $\alpha_v\beta_3$ protects these cells from the virus (Roivainen et al., 1996). Moreover, laboratory mutants of CAV9 which lack the RGD motif are still capable of infecting cells *in vitro* (Hughes et al., 1995). Moreover, CAV9 virions exposed to proteolytic enzymes which cleave the RGD containing sequence (human intestinal fluid, trypsin, plasmin) retain virus infectivity and are not inhibited by RGD peptide blockade (Roivainen et al., 1991). Since CAV9 replicates in the gut and is exposed to host enzymes which may alter the virus in this manner, it is postulated that an RGD-independent pathway is important in the pathogenesis of CAV9 infections *in vivo*. The identity of the receptor molecule used in this pathway is not known.

C.3. Receptors for echoviruses.

Functional cellular receptors for most echoviruses (EV) have not been reported, to date. However, it is likely that many EV serotypes share a cellular receptor(s). Evidence for this was obtained in virus competition assays, which demonstrated that EV serotypes compete with each other for binding to the cell surface (Mbida et al., 1991). In addition, a MAb directed against a 44 kDa cell surface glycoprotein was shown to block most EV serotypes from infecting susceptible cells, as well as CAV9, but not polioviruses or group B coxsackieviruses (Mbida et al., 1992a,b). Further attempts to characterize the EV receptor molecule have not been successful to date, prompting speculation that it is not a single molecule, but rather multicomponent complex. Recently, the β2-microglobulin chain of the class I HLA antigen has also been postulated to form part of this multicomponent receptor complex (Ward et al., 1998).

Although the nature of the shared EV receptor remains unknown, several receptor molecules used by select EVs have been reported. The complement regulatory molecule DAF is used by several EV serotypes. The integrin VLA-2 is used

by echoviruses types 1,8. Finally, the integrin αvβ1 and possibly the matrix protein MMP-9 are used by echovirus type 22.

i). The DAF receptor. Several investigators have identified decay accelerating factor (DAF or CD55; Figures 3A, 3B) as a receptor for echoviruses. To date, echoviruses that bind DAF include strains of EV serotypes 6, 11, 12, 21 (Bergelson et al., 1994), and serotypes 7, 13, 21, 29, 33 (Ward et al., 1994), and serotypes 3, 6, 11, 12, 13, 19, 21, 24, 25, 29, 30, 33 (Powell et al., 1998).

Two approaches were used to demonstrate that DAF is a receptor for enteroviruses. In one approach, preincubation of HeLa cells with anti-DAF MAbs was shown to render the cells resistant to virus infection (Bergelson et al., 1994). Incubation of HeLa cells with phosphatidylinositol phopholipase C (PIPLC) enzyme (which cleaves glycophosphoinositol (GPI) anchored molecules like DAF from the cell surface) also protected against virus infection Finally, CHO cells transfected with DAF cDNA bind to virus, as compared to control cells. In the second approach, mouse cells were transfected with a human cDNA library, and the DAF receptor positive clones were identified by screening with anti-receptor MAb (Ward et al., 1994).

The site of echovirus binding on the DAF molecule is presumed to be domain 3 (SCR3), since MAbs directed against this region are most effective at blocking virus binding (Clarkson et al., 1995; Powell et al., 1998). Neither glycosylation status nor the glycophosphatidylinositol anchor are critical for virus attachment (Clarkson et al., 1995; Powell et al., 1997)

The structural changes in the virus that occur following binding have been examined in studies using echovirus serotype 7 (EV7) (Powell et al., 1997). EV7 forms 135S A particles upon interaction with susceptible cells. However, EV7 does not form A particles when incubated solely with soluble DAF receptor. This is in contrast to the studies described above in which poliovirus incubation with soluble PVR, or rhinovirus

incubation with soluble ICAM-1, lead irreversibly to A particle formation. One possible explanation for these differences is that echoviruses may require an additional receptor molecule, such as the "common echovirus receptor" for virion A particle formation. Alternatively, DAF may need to present on the cell surface in order to interact with virus in a manner that produces A particle formation. Three dimensional structure studies of echovirus-DAF receptor binding may provide further insight into the interactions that occur.

ii). Echoviruses type 1,8 and the VLA-2 receptor. A receptor for echovirus type 1 (EV1) and the related echovirus type 8 (EV8) has been identified as the integrin very late antigen-2 (VLA-2, α2β1 integrin, CD49b/CD29) (Bergelson et al., 1992, 1993) (Figure 3A). To identify the receptor, MAbs were generated which protected cells from infection by EV1 and EV8, but not by other echovirus serotypes. Immunoprecipitation using the antibodies yielded two cell surface proteins, with molecular weights of 125 kDa and 145 kDa. It was observed that the same protein patterns were obtained using antibodies to the α2 or β1 integrin subunits of VLA-2. To confirm receptor identity, RD cells which lack α2 but express β1 were transfected with α2 subunit cDNA, leading to the cell surface expression of VLA-2. Transfected cells bound significant quantities of radiolabeled EV1 and were susceptible to virus infection, as compared to untransfected controls.

Biologically, VLA-2 mediates cell interactions with extracellular matrix proteins such as collagen and laminin (reviewed in Hemmler, 1990). Binding triggers the activation of immediate-early genes (reviewed in Schwartz et al., 1995). Since the binding sites for both collagen and for EV1 are found within the first domain of the α2 subunit (King et al., 1997), it has been postulated that EV1 binding to VLA-2 also triggers signaling pathways, and that these are important pathogenetically. Indeed, EV1 infection of cells through VLA-2 leads to phosphorylation of the stress protein p38,

which in turn activates the expression of immediate-early genes such as c-jun, thus providing support for this hypothesis (Huttunen et al., 1997, 1998).

iii). Echovirus type 22 interacts with αvβ1 and MMP-9. The integrin αvβ1 is used as a receptor by echovirus type 22 (EV22) (Pulli et al., 1997). EV22 binding to cells can be inhibited by anti-αv and anti-β1 antibodies. Moreover, virus binding to host cells can be inhibited by RGD-containing oligopeptides, consistent with the RGD binding nature of αvβ1, and the presence of the RGD tripeptide motif in the VP1 capsid protein of EV22 (Hyypia et al., 1992). EV22 infection of cells can also be blocked by antibodies to matrix metalloproteinase 9 (MMP-9), indicating that it too may serve as a receptor for this virus (Pulli et al., 1997).

C.4. Receptors for group B coxsackieviruses.

Two receptor molecules have been identified for the group B coxsackieviruses (CVB). They are coxsackie-adenovirus receptor (CAR) and decay accelerating factor (DAF). Identification and characterization of these receptor molecules is described in detail below. The important roles that they play in CVB infection, virus virulence, and disease pathogenesis is presented in the thesis sections that follow.

i). The coxsackie-adenovirus receptor (CAR). It has long been postulated that all CVBs share a common receptor molecule which is not used by other enterovirus groups. To this effect, all CVB serotypes were found to compete with each other for binding to receptors on the cell surface, but they did not compete with polioviruses, human rhinoviruses, or group A coxsackieviruses (Crowell, 1966, 1976; Lonberg-Holm et al., 1976). Surprisingly, adenovirus type C fiber protein was also found to compete with CVB for binding, indicating that it uses a common receptor as well (Lonberg-Holm et al., 1976). Further support for a common CVB group receptor comes from

biochemical studies. Treatment of HeLa cells with the proteolytic enzyme chymotrypsin inactivates the CVB receptor, whereas treatment with trypsin enzyme is required for poliovirus receptor inactivation (Zajac and Crowell, 1965a,b). Moreover, CVB receptor activity is less labile at elevated temperatures and low pH than poliovirus receptors (Zajac and Crowell, 1969).

Biochemical studies have shown that the common CVB receptor can be inactivated by proteases and glycosidases, but is unaffected by lipases, indicating that it is a glycoprotein molecule (Krah and Crowell, 1985; Zajac and Crowell, 1965a). Moreover, the receptor can be dissociated from the membrane only by detergents or solvents, indicating that it is an integral membrane component (Crowell and Siak, 1978; Krah and Crowell, 1982). Virus attachment to the receptor occurs optimally at 37^0C, and attachment is more rapid for CVB1, 3, and 5 serotypes than for CVB2, 4, and 6 (Crowell, 1976). Virus binding to susceptible cells leads to virus eclipse, formation of conformationally altered A particles, and progeny virus production (Crowell, 1966; Crowell and Philipson, 1971). This occurs optimally when virus infected cell cultures are incubated in medium at pH 4.5 (Crowell, 1976). It is estimated that there are approximately 10^5 receptor molecules on the surface of a HeLa cell (Crowell, 1966; Hsu et al., 1988).

A CVB receptor molecule has been purified from HeLa cells (Mapoles et al., 1985) and YAC-1 cells (Hsu and Crowell, 1989). To do this, CVB3 was bound to the cells to form a virus-receptor complex (VRC). The VRC was then extracted with detergents, purified on sucrose gradients, radiolabeled with ^{125}Iodine, and analyzed by electrophoresis on SDS-polyacrylamide gels. The VRC consists of CVB proteins, and a cellular protein approximately 50 kDa in size termed Rp-a. A monoclonal antibody generated against Rp-a protects HeLa cells from infection by all six CVB serotypes, consistent with it being directed against the common CVB receptor (Hsu et al., 1988)

The CVB group receptor was recently cloned and characterized by several investigators, and was named the coxsackie-adenovirus receptor (CAR) (Figures 3A,

3B). In one approach, the Rp-a/CAR receptor was isolated from HeLa cells, then enzymatically digested to yield several protein fragments for sequencing (Bergelson et al., 1997a; Carson et al., 1997). Oligonucleotide probes generated on the basis of the protein sequences were then used to screen for full-length cDNA's from eukaryotic cell libraries. In the second approach, a cDNA library was constructed in the prokaryotic expression vector λ ZAP Express, and then receptor expression was screened for using anti-receptor antibody p46 (Tomko et al., 1997; Xu and Crowell, 1996).

CAR is a fully functional receptor for CVBs. Transfecting CAR cDNA into receptor negative hamster cells (Bergelson et al., 1997a) or mouse cells (Tomko et al., 1997) renders the cells susceptible to infection by selected strains of CVB3, CVB4, CVB5, and presumably by all six CVB serotypes.

CAR is also a receptor for adenoviruses (Ad), thus confirming the original observations that CVB and Ad share a receptor molecule (Lonberg-Holm et al., 1976). CAR is used as a cellular fiber receptor by Ad subgroups A, C, D, E, and F (Bergelson et al., 1997a; Roelvink et al., 1998; Tomko et al., 1997). However, CAR functions only as an attachment molecule for these viruses, and additional receptor molecules are required for productive infection of cells. For example, for group C Ad, the viral penton base protein binds to αv-integrins through an RGD motif, leading to rapid internalization of the virus particle (Wickham et al., 1993). The fiber knobs can also bind with low affinity to the α2 domain of human MHC class I molecule (Davison et al., 1999; Hong et al., 1997).

The gene for CAR is encoded on human chromosome 21 at 21q11.2 and consists of seven exons (Bowles et al., 1999). The CAR RNA transcripts are found in many human tissues by Northern blot analyses (Bergelson et al., 1997a; Tomko et al., 1997; and many others). Levels of expression are strongest in the human heart, brain, and pancreas, weaker in the liver and lung, and undetectable in kidney, placenta, or skeletal muscle (Bergelson et al., 1997a). This tissue distribution is consistent with the tissue tropism and disease manifestations associated with CVB infections in humans.

CAR homologues have also been found in the tissues of many animals including mice, rats, pigs, and dogs (Bergelson et al., 1998; Fechner et al., 1999; Tomko et al., 1997), consistent with the ability of CVBs to experimentally infect many animal species (reviewed in Woodruff, 1980).

Analysis of the sequence encoding CAR predicts a protein with homology to the immunoglobulin superfamily (Bergelson et al., 1997a; Tomko et al., 1997) (Figure 3A, 3B). The deduced primary structure of CAR consists of a short amino terminal signal peptide, followed by 2 extracellular immunoglobulin-like domains containing two putative N-linked glycosylation sites and one potential tyrosine phosphorylation site, a single transmembrane anchor, and a cytoplasmic tail. CAR homologues expressed in mice and other animals share the same general structural motifs, although they differ slightly in nucleotide sequence or the length of the cytoplasmic tail (Bergelson et al., 1998; Fechner et al., 1999; Tomko et al., 1997).

The region of the CAR molecule required for CVB binding is not known, but mutagenesis studies have indicated that the extracellular domains alone are sufficient to permit virus attachment and cell entry (Wang and Bergelson, 1999). The site on the CVB virion which is used for binding to CAR is postulated to be the canyon found at the fivefold vertices of the 12 pentamer units. This is similar to the canyon site described above, which poliovirus and rhinoviruses use for binding to their cellular receptors. However, confirmation of this binding site will likely not be resolved until cryoelectron microscopic studies are performed. In contrast, the primary binding sites for CAR and Ad fiber protein have been extensively examined, through sequence analysis, mutagenesis, and x-ray crystallography of Ad fiber knob complexed with the CAR receptor (Bewley et al., 1999; Roelvink et al., 1999).

ii). The DAF receptor. It was hypothesized that some coxsackie B viruses interact with at least one additional receptor molecule, which is found on HeLa cells. Evidence for this was that a MAb termed RmcA blocked selected strains of CVB1, 3, and 5 from infecting HeLa cells, by interacting with a 70 kDa protein on the cell surface (Bergelson et al., 1997b; Crowell et al., 1986; Hsu et al., 1988; Mohanty and Crowell, 1993; Reagan et al., 1984). This receptor molecule was also expressed on human red blood cells, consistent with the hemagglutinating capacity of many of these virus strains (Reagen et al., 1984).

This second CVB receptor was identified as the decay accelerating factor (DAF or CD55; Figures 3A, 3B). This was determined by noting that the 70 kDa protein recognized by MAb RmcA was the same size as the DAF molecule (Bergelson et al., 1995). Moreover, MAb RmcA, which is directed against the receptor molecule, protected cells from infection by a DAF binding strain of echovirus type 6, in addition to CVB 1, 3, and 5 variants (Bergelson et al., 1995). Furthermore, a second antibody termed MAb854 blocked echovirus type 7 from binding to DAF on human red blood cells, and also prevented CVB1, 3, and 5 serotypes from binding to and infecting cells in culture (Shafren et al., 1995).

Although DAF is considered a second receptor for CVBs, it functions only as an attachment site, and additional receptors such as CAR are required for productive infection of cells (Bergelson et al., 1995; Pasch et al., 1999; Shafren et al., 1995). The site of attachment on the DAF receptor has been examined using MAbs and DAF deletion mutants, and is located within the second (SCR2) and third (SCR3) of the molecule (Bergelson et al., 1995; Shafren et al., 1995). The site of attachment on the CVB virion is not known, but has been postulated to contain residues from a surface depression found uniquely at the twofold axes of the CVB structure (Lindberg et al., 1992).

DAF serves as a receptor for many enteroviruses, in addition to CVBs. As noted above, DAF is also a receptor for CAV21 and several echovirus serotypes. DAF is also a receptor for enterovirus type 70 (Karnauchow et al., 1996), and for DR adhesin-positive *Escherichia coli* (Nowicki et al., 1993). Moreover, DAF is often found on the membrane of enveloped viruses, presumably to facilitate virus evasion of host immune responses (Spear et al., 1995; Vanderplasschen et al., 1998). Since DAF interacts with such a broad array of microbial pathogens, and since DAF is important to the studies presented in this thesis, a detailed description of the molecule is presented below.

Biologically, DAF regulates complement at the level of the C3 and C5 convertases (reviewed in Lublin and Atkinson, 1989) (Figure 4). In the alternative complement pathway, DAF binds to C3b and prevents formation of the C3 convertase (C3bBb) or the C5 convertase (C3bBb3b). It can also bind to a preformed C3 or C5 convertase and facilitate its dissociation by releasing the Bb subunit from its binding site. In the classical pathway, DAF binds to C4b and prevents assembly of the C3 convertase (C4b2a) and C5 convertase (C4b2a3b), or causes preformed C3/C5 convertases to dissociate their 2a subunits. Thus, DAF effectively blocks the amplification steps in the complement cascade, and protects cells from damage by complement mediated lysis.

DAF is present on the surface of most cells that are exposed to serum complement, including many blood cell types and those of the vascular endothelium, although many lymphocyte and NK subpopulations are DAF deficient (reviewed in Nicholson-Weller and Wang, 1994). Individuals with paroxysmal nocturnal hemoglobinuria, an acquired condition, lack DAF on their erythrocytes, and as expected their cells are significantly more sensitive to complement mediated lysis *in vitro* than those from normal individuals (Nicholson-Weller et al., 1983). DAFs role in complement regulation leads to the expectation that DAF-like molecules would exist in other species with complement systems too. To date, DAF molecules which are genetically and/or

Figure 4

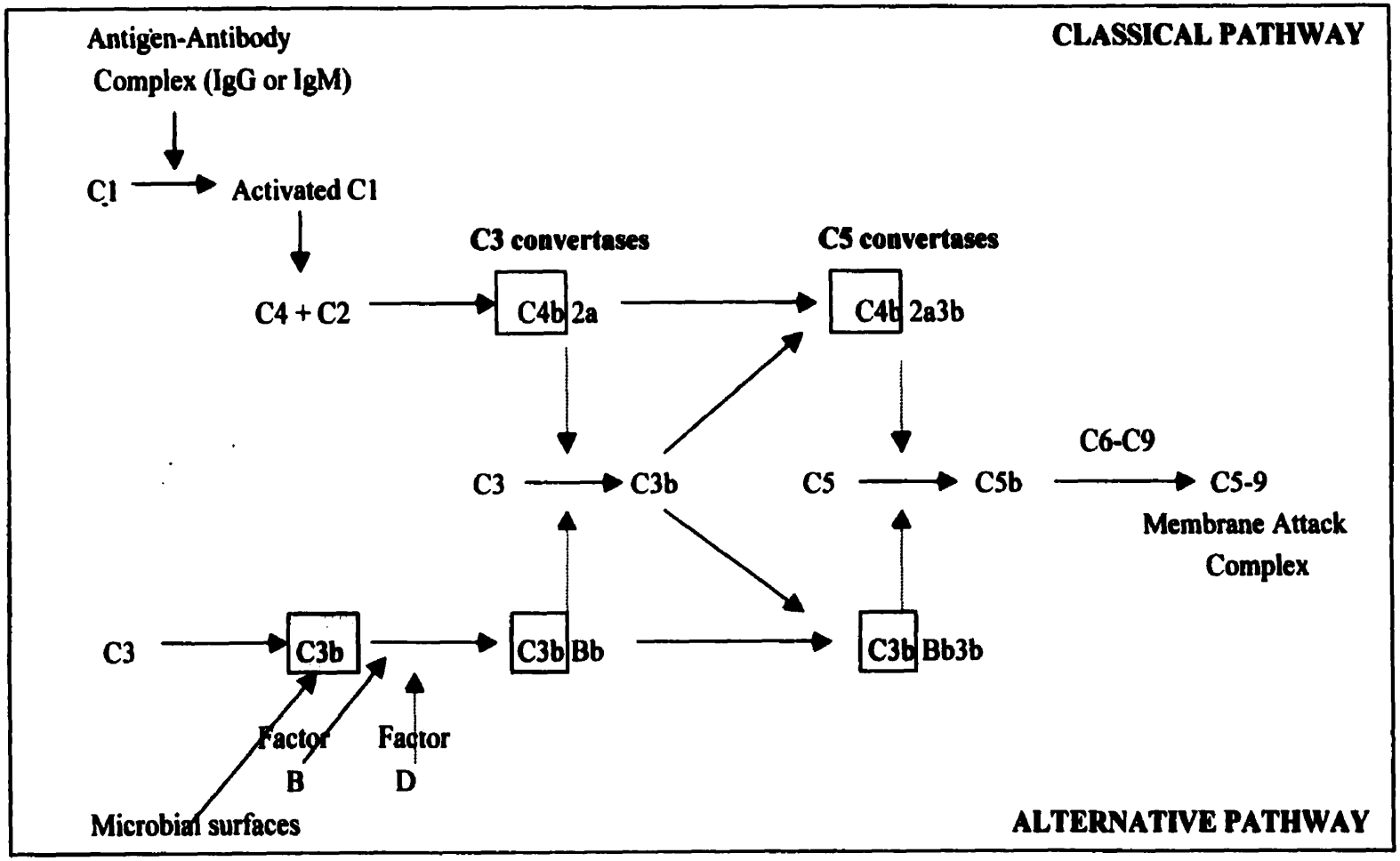


Figure 4. The classical and alternative complement pathways. The classical pathway (top) and the alternative pathway (bottom) converge leading to the formation of membrane attack complexes and cell lysis. DAF regulates complement by preventing formation or causing dissociation of C3 convertases and C5 convertases, through interactions with C4b (yellow boxes) or C3b (purple boxes). Adapted with permission from A.K. Abbas et al., (ed)., *Cellular and Molecular Immunology*, Second Edition. (W.B. Saunders Company, Philadelphia, P.A., 1994).

functionally similar to human DAF have been found in mice (Fukuoka et al., 1996; Kim et al., 1995; Song et al., 1996; Spicer et al., 1995), guinea pigs (Nicholson-Weller et al., 1981), and rabbits (Sugita et al., 1987).

The biosynthesis and glycosylation of DAF has been studied in detail (reviewed in Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994). Briefly, DAF is synthesized as an approximately 40 kDa protein containing a single N-linked mannose moiety. As it moves through the Golgi, the N-linked oligosaccharide is changed to a complex sugar type, and multiple O-linked oligosaccharide chains are added near the carboxy terminus. The mature DAF form has a molecular weight of approximately 70 kDa, varying only slightly in size on different cell membranes due to the extent of glycosylation.

The structure of DAF (Figures 3A, 3B) has been deduced based on its genomic sequence, which is encoded for by a gene on the long arm of chromosome 1 in a region containing a cluster of genes for homologous complement regulatory proteins (the RCA gene family) (reviewed in Hourcade et al., 1989). DAF consists of four homologous short consensus repeat (SCR) domains, each containing approximately 60 amino acids and four cysteine residues (Caras et al., 1987). The cysteine residues are alternatively paired in disulfide bonding to give rise to the characteristic structure of the SCR domains (Nakano et al., 1992). SCR3 is most important for complement regulation, although SCR2, 3, and 4 are required for proper conformation of the active SCR3 site (Coyne et al., 1992). The carboxy terminus of DAF is covalently attached to a glycophosphoinositol (GPI) anchor, which is inserted into the outer lipid bilayer of the cell membrane (reviewed in Lublin and Atkinson, 1989). The GPI anchor may allow for faster lateral mobility of the molecule on the cell membrane, although this does not appear to enhance its ability to regulate complement at the cell surface (Lublin and Coyne, 1991). The GPI anchor may also be important for intercellular signaling pathways. Indeed DAF (and other GPI anchored molecules) associates with protein

tyrosine kinases such as $p56^{lck}$ and $p59^{fyn}$, which regulate cell activation and signal transduction (Davis et al., 1988; Shenoy-Scaria, 1992; Stefanova et al., 1991).

In summary, group B coxsackieviruses are important pathogens in human diseases such as myocarditis and dilated cardiomyopathy. The pathogenesis of these diseases involves the interaction of the virus with target cells, mediated by the host cell surface receptors. Investigating virus-receptor interactions has enriched our understanding of the pathogenesis of other viruses (reviewed in Flint et al., 2000), and has great potential to help understand the pathogenesis of CVB mediated diseases as well, and is therefore the subject of this thesis.

D. RESEARCH FOCUS

Group B coxsackievirus (CVB) are members of the enterovirus genus, family *Picornaviridae*. These viruses are etiologically associated with many human diseases. Of primary interest to our laboratory is the role of CVB in the pathogenesis of two cardiac conditions, namely viral myocarditis and it's chronic sequelae dilated cardiomyopathy.

The pathogenesis of CVB-induced heart disease is characterized by virus infection of susceptible cells in tissues such as the heart. This is likely mediated by virus binding to receptor molecules on the surface of myocyte cells, and leads to virus internalization, progeny production, and destruction of the host myocardial cells. Virus infection also leads to the triggering host immune responses, some of which have pathogenic consequences.

In the following studies in this thesis, findings will be presented which characterize interactions between CVB and two cell surface receptors, namely decay accelerating factor (DAF), and the coxsackie-adenovirus receptor (CAR). These findings are analyzed in light of the interactions that occur, and in the context of a broader role for CVB receptors in the pathogenesis of viral heart disease.

E. HYPOTHESIS AND SPECIFIC AIMS

The central hypothesis of this work is that receptors play an important role in CVB infections and in the genesis of CVB-induced heart disease.

The following aims were addressed experimentally:

1. To characterize interactions between cardiovirulent coxsackieviruses and the decay accelerating factor (DAF) receptor, and identify mechanisms by which CVB-receptor interactions may play a role in the pathogenesis of viral heart disease.

2. To characterize interactions between virus and the coxsackie-adenovirus receptor (CAR).

3A. To identify sites on the CVB3 virion which interact with virus receptors.

3B. To establish a model system which can be used to confirm these interactions.

The experiments in the studies that follow were performed in the laboratory of Dr. Peter Liu, Toronto General Hospital, Canada. The collaborative efforts of other investigators also involved in this work are described in detail below.

Study #1, the DAF receptor. Cardiovirulent CVB3 viruses were contributed by Dr. Charles Gauntt at the University of Texas Health Science Center, Texas, USA and Dr. Larry Chow at the University of Western Health Science Center, London, Canada. The adenovirus was from Dr. Martha Brown at the University of Toronto, Canada. The $p56^{lck}$ knockout mice were from the laboratories of Dr. Tak Mak and Dr. Josef Penninger, Princess Margaret Hospitals/OCI/Amgen Institute, Toronto, Canada. Animal infections and sacrifices were performed in our laboratory by Dr. Fayez Dawood and Dr. Wen-Hu Wen. Data on animal mortalities and virus titers in the heart were provided by Karen Aitken in our laboratory.

Study #2, the CAR receptor. The clinical CVB viruses were provided by Dr. Martin Petric at the Hospital for Sick Children, Toronto, Canada, and by Dr. John Modlin at the Dartmouth Medical School, NH, USA. The Canadian samples were typed by Dr. Spencer Lee at the Canadian Enterovirus Reference Center in Halifax, Canada. The CHOP cells were from Dr. Jim Dennis at Mount Sinai Hospital, Toronto, Canada. The stable CHO-CAR transfectants, anti-CAR MAb RmcB, and soluble CAR were obtained from the laboratories of Dr. Jeff Bergelson and Dr. Robert Finberg, Children's Hospital of Philadelphia, PA, USA, and Dana Farber Cancer Institute, MA, USA. Anti-CAR rabbit polyclonal antibody antiserum was prepared with the help of Dr. Martin Petric and Dr. Anne Opavsky at the Hospital for Sick Children, Toronto, Canada. Experiments using SVDV were performed under isolation by Dr. Hana Weingartl at the National Center for Foreign Animal Diseases, Winnipeg, Canada.

Study #3, the CVB3 virion receptor binding sites. The CVB3 primers were provided by Dr. Michael Sole and Dr. C. C. Liew, Toronto General Hospital, Canada. The map of the CVB3 protomer unit was obtained from Dr. Jodi Muckelbauer through Dr. Charles Gauntt at the University of Texas Health Science Center, Texas, USA. Phylogenetic alignments were performed by Dr. David Irwin, Toronto General Hospital, Canada. CVB6 amplicons were generated by RT-long-PCR and cloned and transcribed to viral RNA by Dr. Raymond Tellier, Hospital for Sick Children, Toronto, Canada. Experiments using SVDV were performed under isolation by Dr. Ahmad Afshar at the Animal Diseases Research Institute, Nepean, Canada.

Thanks also to the summer students in Dr. Peter Liu's laboratory and to Barbara Kellam for their help with sequencing, and to David Hou for the graphic illustrations in Figures 3B, 28, and 29.

F. STUDY #1
THE DAF RECEPTOR

F.1. Purpose.

To characterize interactions between cardiovirulent coxsackieviruses and the decay accelerating factor (DAF) receptor, and identify mechanisms by which CVB-receptor interactions may play a role in the pathogenesis of viral heart disease.

Based on the publications -

Martino, T. A., M. Petric, M. Brown, K. Aitken, C. J. Gauntt, C. D. Richardson, L. H. Chow, and P. P. Liu. (1998). Cardiovirulent coxsackieviruses and the decay-accelerating factor (CD55) receptor. *Virology* **244**, 302-314.

Martino, T. A., Aitken, K., Penninger, J., Mak, T., Sole, M. J., Dawood, F., Wen, W. H., and Liu, P. (1995a). Mice with p56lck T-lymphocyte tyrosine kinase knocked out are resistant to coxsackieviral myocarditis. *Can. J. Cardiol.* **11** (Suppl. E):78E.

Liu, P., K. Aitken, Y. Y. Kong, M. A. Opavsky, T. Martino, F. Dawood, W. H. Wen, I. Kozieradzki, K. Bachmaier, D. Straus, T. W. Mak, and J. M. Penninger. (2000). The tyrosine kinase $p56^{lck}$ is essential in coxsackievirus B3-mediated heart disease. *Nature Medicine*, **6**, 429-434.

Figures 5, 6, 7, 8, 9, 10, 11, and 12, by T. A. Martino. The $p56^{lck}$ knockout mice were from Dr. T. W. Mak and Dr. J. M. Penninger's laboratories. Figures 13c, 13d, 14a, 14b, and 14c were done by T. A. Martino, and represent only part of the study on $p56^{lck}$ by Liu et al., 2000 (Nature Medicine). The $p56^{lck}$ animal experiments and Figures 13a, 13b were done by F. Dawood, W. H. Wen, K. A. Aitken. Thanks to Shelley Rotman for her help with tissue sectioning and staining.

F.2. Abstract

Group B coxsackieviruses are etiologically linked with many human diseases including acute myocarditis and associated chronic dilated cardiomyopathy. Well-established CVB3 cardiovirulent strains ($CVB3c_{(s)}$) with known phenotypic differences have been used to study the pathogenesis of virus-induced heart disease. The receptor binding characteristics of *cardiovirulent* CVB3 are not known, but may represent an important mechanism accounting for differences in disease virulence. In this study, interactions between $CVB3c_{(s)}$ and the decay-accelerating factor (DAF or CD55) cell surface receptor were examined. Anti-DAF monoclonal antibodies (MAbs) blocked virus binding and infection of susceptible HeLa cells. Virus binding was significantly reduced by treatment of these cells with phosphatidylinositol phospholipase C enzyme, which rendered them DAF-deficient. $CVB3c_{(s)}$ exhibited a differential propensity for the DAF receptor, as several cardiovirulent strains interacted more strongly than others. However, virus binding and infection was always most effectively blocked by MAbs directed against the SCR 2, 3 domains of DAF, suggesting that binding occurs at a similar site(s) on the molecule for all strains. Virus binding and internalization were associated with DAF down-regulation at the cell surface, as monitored by flow cytometry analysis. Cardiovirulent CVB3 did not interact with molecules functionally and/or structurally related to DAF including CD35, CD46, Factor H, or C4 binding protein. Adenovirus type 2 (Ad2) does not use the DAF receptor. However, competitive binding assays between Ad2 and CVB1-6, $CVB3c_{(s)}$, anti-DAF MAbs, or DAF-reduced cells, indicated that DAF is associated with Ad2 receptors on the HeLa cell membrane.

Since DAF interacts with the T cell signaling molecule $p56^{lck}$, and T cell activation is critical to the development of myocarditis, a mechanism for these interactions in the pathogenesis of viral heart disease was examined. Transgenic knockout mice lacking $p56^{lck}$ were resistant to CVB3-induced heart disease. There was a lack of inflammatory infiltration in the heart tissue, and no apparent pathologic damage, even though virus replicated in the heart. Virus was cleared from the

myocardium of $p56^{lck}$ knockout mice by intact host defense mechanisms such as NK cells. In summary, these findings indicate that DAF is an attachment receptor for cardiovirulent CVB3, and that DAF interaction may be important in the pathogenesis of CVB-mediated heart disease.

F.3. Introduction

Myocarditis is defined as heart disease in which there is inflammation of the myocardium with myocardial cell necrosis (Aretz 1987; Aretz *et al.*, 1987). It is the most common cause of acquired heart failure in children, and is regarded as an important predisposing condition of dilated cardiomyopathy (DCM) in adults (Liu *et al.*, 1996; Martino *et al.*, 1994a,b, 1995b; Woodruff 1980). Yet despite its importance in both the pediatric and adult populations, there is little effective treatment available, and mortality remains high. A recent prospective clinical trial indicated that 56% of patients die within 4.3 years of diagnosis (Mason *et al.*, 1995). Coxsackievirus group B (CVB) is the most commonly identified pathogen in patients with myocarditis and DCM, having been implicated in more than 50% of cases with infectious etiology (Liu *et al.*, 1996; Martino *et al.*, 1994a,b, 1995b; Woodruff 1980).

The initial event in these disease processes is the binding of virus to its receptor(s) on host cells. The first CVB receptor to be identified was the decay-accelerating factor (DAF or CD55) (Bergelson *et al.* 1995; Shafren *et al.* 1995). DAF is a ~70 kDa glycophosphatidylinositol-anchored membrane protein with broad tissue distribution. It functions in the complement regulatory system, by preventing formation or causing dissociation of C3 convertases in both the classical and alternative system pathways (Hourcade *et al.*, 1989, Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994).

CVB serotypes differ in their ability to bind to the DAF receptor. Monoclonal antibodies (MAb) to DAF blocked binding to and infection of HeLa cells *in vitro* by CVB 1, 3, and 5 protoserotypes (Bergelson *et al.* 1995; Shafren *et al.* 1995). DAF expression on transfected rodent cells promoted virus binding (Bergelson *et al.* 1995; Shafren *et al.* 1995). In contrast, protoserotypes CVB 2, 4, and 6 did not bind to DAF (Bergelson *et al.* 1995; Shafren *et al.* 1995).

Virus strains, which occur within each CVB serotype, differ in their ability to bind to the DAF receptor. One prototype CVB3-VR30 strain was found to bind to transfected cells expressing the DAF molecule (Shafren *et al.*, 1995), whereas a second CVB3-VR30 strain did not bind to DAF on transfected cells (Bergelson *et al.*, 1995). Serial passage of the non-DAF-binding CVB3-VR30 strain through human rhabdomyosarcoma cells selected a strain (CVB3-RD; Reagan *et al.*, 1984) that was capable of binding to DAF on transfected cells (Bergelson *et al.*, 1995). Moreover, CVB strains isolated from infants with aseptic meningitis have been shown to differ in their ability to bind to DAF (Bergelson *et al.* 1997b). Some clinical isolates of CVB 1, 3, and 5 were inhibited from infecting HeLa cell monolayers by an anti-DAF MAb (MAb IF7), whereas several other strains did not appear to interact with DAF (Bergelson *et al.*, 1997b).

CVB3 cardiovirulent strains ($CVB3c_{(s)}$) are used in a number of laboratories to study viral heart disease. These strains differ in cardiopathogenicity in several murine model systems (Chow *et al.*, 1991). The characteristics of the CVB3 strains used in this study are listed in Table 6. The capacity of each of these strains for binding receptors and infecting cells is expected to be an important part of the disease process. However, it is not known if receptor molecules such as DAF have a role in the binding and infection of host cells by *cardiovirulent* CVB3. Accordingly, the interactions of $CVB3c_{(s)}$ with the DAF receptor molecule were explored in the efforts described below. Since it had previously been reported that prototype CVB3 and Ad2 fiber compete for receptor binding (Lonberg-Holm *et al.*, 1976), the ability of $CVB3c_{(s)}$ to compete with Ad2 binding and the ability of Ad2 to bind to DAF was also investigated. Finally, since DAF interacts with the T cell signaling molecule p56lck (Davis et al., 1988; Shenoy-Scaria et al., 1992), this pathway was postulated to be important for the development of myocarditis *in vivo*. To examine this, p56lck+/- and p56lck-/- knock-out mice were infected with cardiovirulent CVB3, and monitored for endpoints of mortality, and cardiac virus titers and pathology.

F.4. Materials and Methods

i). Cell lines. HeLa (ATCC CCL-2) and Vero (ATCC CCL-81) cells were grown in RPMI 1640 media supplemented with $NaHCO_3$ to pH 7.5, 0.5% penicillin and streptomycin, and 10% fetal calf serum (FCS). All media and supplements were purchased from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada.

ii). Viruses. The CVB3 cardiovirulent strains ($CVB3c_{(s)}$) -SH, -NR, -N, -CG, -20, and -Ø were obtained from the laboratories of Charles Gauntt and Larry Chow. CVB3-VR30 strain was obtained from the American Type Culture Collection. The passage strain CGP1V refers to the CVB3-CG strain passaged once through Vero cells. The characteristics of these strains are summarized in Table 6. Adenovirus type 2 was obtained from the American Type Culture Collection. Stocks of these viruses were prepared by passaging them through HeLa cell cultures (with Vero cells used for CGP1V). Stocks were freeze-thawed 3x, clarified by centrifugation, titered by plaque assay on HeLa cells, aliquoted and stored at -70°C.

iii). Virus radiolabeling and purification. HeLa cell monolayers in 10 cm in diameter culture dishes were incubated with the infecting CVB virus (10 PFU/cell) for 30 minutes at 37°C. Monolayers were washed, then incubated at 37°C in media deficient in methionine for 2.75 hours. To each dish, 6 mls of media containing 100 µCi of [^{35}S] methionine (Amersham Life Science, Oakville, Ontario, Canada) was added for an additional 3.75 hours. The infected cell monolayers were washed, collected by scraping into 2 mls of collection buffer (1.5% sucrose, 0.01M NaCl, 0.01M Tris HCl, 0.05M $MgCl_2$), and subjected to three freeze-thaw cycles to release the virus. The lysate was centrifuged at 12,000xg for 15 minutes to remove the cell debris. The supernatant was overlaid on sucrose gradients (15-45% [wt/wt] in collection buffer) and centrifuged at

24,000 rpm at 25°C for 3 hours in an SW28 rotor (Beckman, Mississauga, Ontario, Canada). Fractions with peak radioactivity and viral titer were pooled and dialyzed in 10K Slide-A-Lyzer Cassettes (Pierce, Rockford, Il.) against RPMI media. Virus titer was determined by plaque assay and aliquots were stored at -70°C.

For adenovirus type 2, HeLa cell monolayers were incubated at an MOI of 5 PFU/cell for 1 hour at 37°C, washed, then incubated in regular media. After 16 hours, the media was removed and replaced by 6 mls of methionine deficient RPMI which contained 100 μCi of [^{35}S] methionine, and cell monolayers were further incubated for 6 hours. Finally, the media was replaced by a 10 mls volume of a 1:1 mixture of RPMI and methionine deficient RPMI supplemented with 300 μCi of [^{35}S] methionine, and cells were incubated fora further 48 hours. The cells were collected by scraping into 2 mls of collection buffer, then subjected to five freeze-thaw cycles to release virus, and the lysate was centrifuged at 12,000xg for 15 minutes to remove cell debris. The supernatant was overlaid on sucrose gradients (15-60% [wt/wt] in collection buffer), and centrifuged as described above. Virus containing fractions were pooled, dialyzed, titred and stored as described above.

iv). Plaque assays. HeLa cells plated in 6 well Costar dishes at a density of 1x10^6 cells/well were incubated with serial ten-fold dilutions of CVB3c$_{(s)}$ in 0.2 ml of RPMI media (without FCS), for 1 hour at 37°C. Unbound virus in the inoculum was removed and an overlay of 2x RPMI media supplemented with 20% FCS and 1.4% agarose (1:1 [vol/vol]) was added. The monolayers were incubated for 3 days, fixed in 2% formalin and stained with 0.1% crystal violet dye. Adenovirus type 2 was titred in the same manner, except that the monolayers were incubated for 10 days to allow for plaque formation.

v). $TCID_{50}$ assays. HeLa cells plated in 96 well Costar dishes at a density of $6.25x10^4$ cells/well were incubated with serial two-fold dilutions of $CVB3c_{(s)}$ in 0.02 ml of RPMI media (without FCS), for 1 hour at 37^0C. Unbound virus in the inoculum was removed, and RPMI media supplemented with 10% FCS was added. The monolayers were incubated for 3 days, fixed in 2% formalin and stained with 0.1% crystal violet dye. The endpoint was taken as the highest dilution in which 50% CPE was noted.

vi). Monoclonal Antibodies (MAbs) and complement proteins. Anti-DAF MAb 1A10 was obtained from Cedarlane Laboratories Ltd., Hornby, Ontario, Canada. Anti-DAF MAb 914 (clone BRIC 216) and anti-DAF MAbs 737 and 737F (FITC conjugated) (clone BRIC 110) and MAb 695 to membrane cofactor protein (anti-MCP/CD46; clone J4-48), MAb 554 (anti-CR1/CD35; clone E11), and MAb 653 (anti-CD4; clone B-B14) were obtained from Serotec Canada, Toronto, Ontario, Canada. Complement Factor H and C4-binding protein were purchased from Sigma Immunochemicals.

vii). Anti-DAF MAb inhibition of $[^{35}S]$-$CVB3c_{(s)}$ binding to HeLa cells. HeLa cells were dispersed from confluent cell monolayers by incubation with EDTA, washed with media, and aliquoted at 5×10^6 cells/test in media containing 50 μg/ml of anti-DAF MAb 914. Anti-CD4 MAb 653 served as control. Radiolabeled $[^{35}S]$-$CVB3c_{(s)}$ was then added to the MAb-treated cells. Supernatant fluid and two subsequent washes were collected, as was the cell pellet. Unbound and cell bound virus were determined by monitoring the $[^{35}S]$ cpm in supernatants and cell pellets, respectively, by scintillation spectroscopy.

viii). Anti-DAF MAb inhibition of $CVB3c_{(s)}$ infection of HeLa cells. HeLa cells plated in Costar dish wells were preincubated with anti-DAF MAb 914, or anti-DAF

MAb IA10, or anti-CD4 MAb 653 as control, then infected with 50 PFU/ml of each respective virus. Infection was monitored by plaque assay for all strains except CVB3-20. This strain was monitored by a $TCID_{50}$ assay since it proved difficult to quantitate by plaque assay.

ix). Effects of DAF depletion on binding of $CVB3c_{(s)}$. HeLa cells dispersed from cell monolayers by EDTA were preincubated with phosphatidylinositol phospholipase C enzyme (PI-PLC, from *Bacillus cereus*; Boehringer Mannheim, Laval Quebec, Canada). Cells were then labeled with FITC conjugated anti-DAF MAb 737F as described above. DAF expression/loss was monitored by flow cytometry. DAF-deficient HeLa cells were then used in binding assays with $[^{35}S]$-$CVB3c_{(s)}$ by methods described above.

x). $CVB3c_{(s)}$ binding and DAF expression. HeLa cells dispersed from cell monolayers by EDTA were incubated with each CVB3 strain. The cells were washed and then further incubated in RPMI media. Cells were labeled with FITC conjugated anti-DAF MAb 737F and processed through flow cytometry, as described above.

xi). Complement studies. HeLa cells dispersed from cell monolayers with EDTA were preincubated with anti CD46-MAb 695, by the same methods described for anti-DAF MAbs. After washing, radiolabeled $[^{35}S]$-CVB3-CG was added to the MAb-treated cells. Virus binding was monitored by scintillation spectroscopy, as described above. CD35 binding assays were performed using human blood cells, since the CD35 molecule is not expressed on HeLa cells but is present on erythrocytes (unpublished observations). These were collected from 1 ml of whole human blood by centrifugation at 1000xg for 5 minutes, washed in RPMI, then aliquoted at $5x10^6$ cells/test. Binding assays with $[^{35}S]$-CVB3-CG were performed as described above. 50 PFU/ml of CVB3-

CG was incubated with Factor H or C4 binding protein for 1 hour at 37^{0}C, before being used to infect HeLa cell monolayers under plaque assay conditions as described above.

xii). Ad2-CVB competition binding assays. HeLa cells dispersed with EDTA were pre-incubated with unlabeled CVB virus or unlabeled Ad2, for 1 hour at 37^{0}C. The cells were washed and further incubated with radiolabeled [^{35}S]-Ad2 for 1.5 hours at 37^{0}C. [^{35}S]-Ad2 binding was monitored by scintillation spectroscopy.

xiii). Ad2 binding to DAF. HeLa cells dispersed with EDTA were preincubated with anti-DAF MAbs 914 or anti-DAF MAb IA10 or PI-PLC enzyme, as described above. The cells were washed, and radiolabeled [^{35}S]-Ad2 was then added. [^{35}S]-Ad2 binding was monitored by scintillation spectroscopy.

xiv). Murine models of CVB3 infection in p56lck knockout mice. p56^{lck} transgenic mice have been described previously (Molina et al., 1992). Heterozygous (p56^{lck}+/-) or homozygous (p56^{lck}-/-) mice were inoculated intraperitoneally with 10^5 PFU of CVB3-CG and monitored for endpoints of mortality, cardiac pathology, and cardiac virus titers. Some mice were also depleted of NK cells prior to virus infection, using the methods described previously (Godney and Gauntt, 1986).

F.5. Results

i). MAb inhibition of [^{35}S]-CVB3c$_{(s)}$ binding to HeLa cells. The inhibition of cardiovirulent CVB3 virus binding to susceptible cells by anti-DAF MAbs was first examined (Fig. 5). Pretreatment with MAb 914 to DAF blocked the binding of all cardiovirulent [^{35}S]-CVB3 strains to HeLa cells. However, the degree of binding inhibition differed for each strain. Preincubation with MAb 914 strongly reduced the binding of radiolabeled CVB3-CG, CVB3-CGP1V, CVB3-SH, CVB3-Ø, and CVB3-VR30 to the cell surface, moderately reduced binding of CVB3-N and CVB3-NR, and only weakly reduced binding of CVB3-20 to HeLa cells. In contrast, there was no reduction of [^{35}S]-CVB3-CG binding when cells were pretreated with control MAb 653 (anti-CD4) (inhibition=0%, results not shown).

ii). MAb inhibition of CVB3c$_{(s)}$ infection of HeLa cells. The inhibition of cardiovirulent CVB3 virus from infecting susceptible cells by anti-DAF MAbs was then examined (Fig. 6). Anti-DAF MAb 914 strongly inhibited plaque formation by CVB3-CG, CVB3-CGP1V, CVB3-SH, CVB3-Ø, and CVB3-VR30 strains, and moderately inhibited plaque formation in HeLa cells by CVB3-N, CVB3-NR, and CVB3-20 strains. In contrast, anti-DAF MAb IA10 was generally less effective at inhibiting CVB3c$_{(s)}$ plaque formation in HeLa cells, when used at the same concentration as MAb 914. Control MAb 653 (anti-CD4) did not affect CVB3c$_{(s)}$ plaque formation in HeLa cells (%inhibition of plaques = 0%, results not shown).

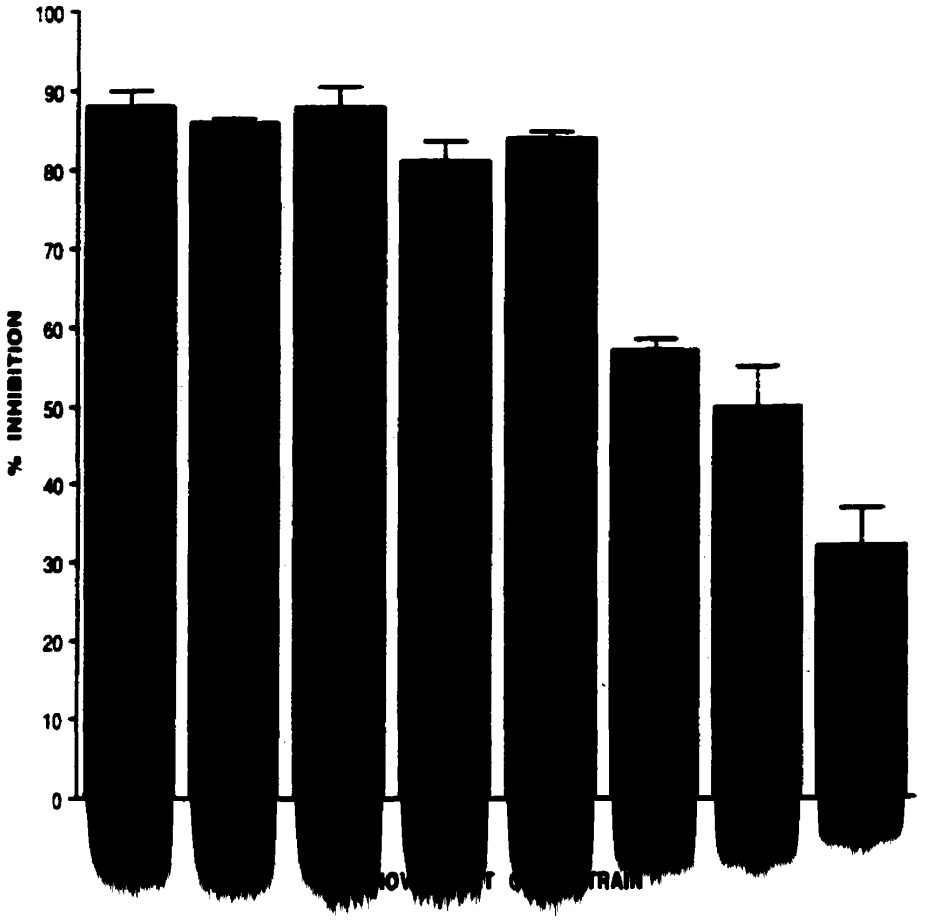


Figure 5. Inhibition of [^{35}S]-CVB3c$_{(s)}$ binding to HeLa cells by anti-DAF MAb 914. Versene dispersed HeLa cells washed and aliquoted at 5x10^6 cells/test were resuspended in 200 µl media containing 50mg/ml of anti-DAF MAb 914, or 200 µl media only, at 37°C for 1 hour. After washing, 10^3-10^4 cpm of [^{35}S]-CVB3c$_{(s)}$ was added to the cells for 1 hour at 37°C. Cells were centrifuged at 1000xg for 3 minutes and the supernatant fluid and two subsequent washes were collected, as was the cell pellet. Unbound virus and cell bound virus were determined by monitoring the [^{35}S] cpm in supernatants and cell pellets, by scintillation spectroscopy. Experiments were performed in triplicate for each virus strain. Inhibition of [^{35}S]-CVB3c$_{(s)}$ binding to cells was calculated as:

$$100\% - \left[\frac{\text{fraction of added (35S)-CVB3c(s) that binds to treated cells}}{\text{fraction of added (35S)-CVB3c(s) binding to untreated (control) cells}} \times 100 \right]$$

Figure 6

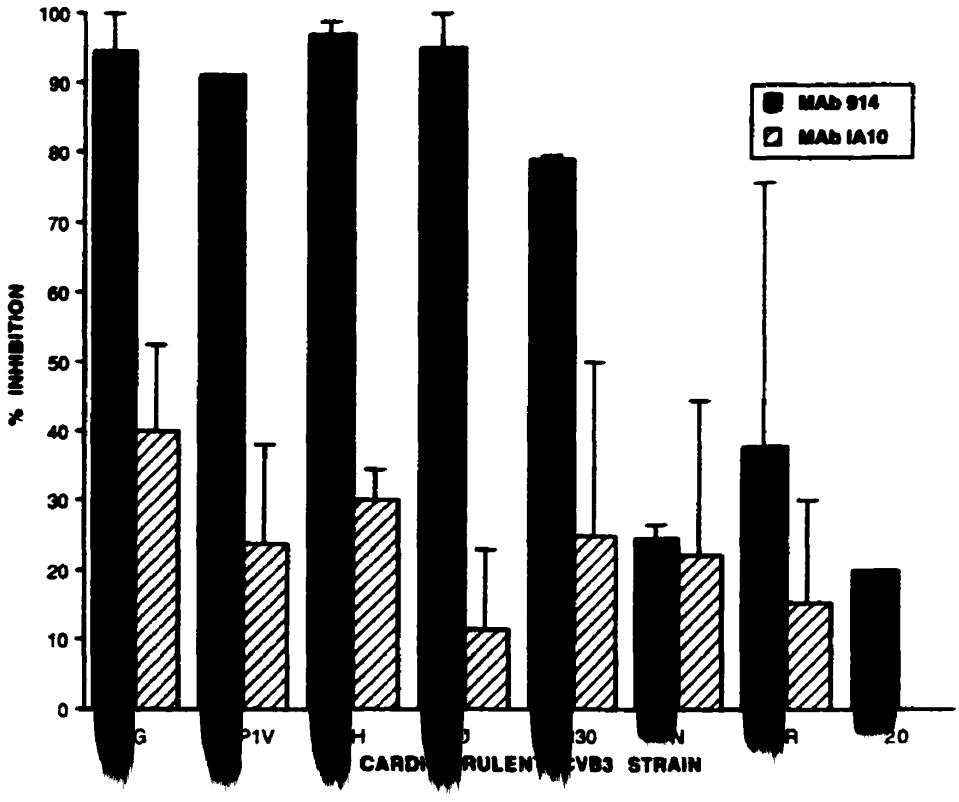


Figure 6. Inhibition of CVB3c$_{(s)}$ plaque formation on HeLa cells pretreated with anti-DAF MAbs 914 or IA10. HeLa cell monolayers plated in 6 well Costar dishes at a density of 1x10^6 cells/well were preincubated with 1 ml media containing 50µg/ml of MAb IA10 or 914, or 1 ml of media only, at 37^{0}C for 1 hour. After washing, 1 ml containing 50 PFU/ml of each virus was added at 37^{0}C for 1 hour. The monolayers were washed, plaque overlays were added for 3 days, and then plaques were enumerated. The CVB3-20 strain plaques inefficiently on HeLa cells, so infectivity for this strain was monitored by a TCID$_{50}$ assay. All experiments were performed in triplicate. Inhibition of CVB3c$_{(s)}$ infection of HeLa cells was determined as follows:

$$100\ \% - \left[\frac{\text{No. CVB3c(s) PFU on MAb treated cells}}{\text{No. CVB3c(s) PFU on untreated cells}} \times 100 \right]$$

iii). [^{35}S]-CVB3c$_{(s)}$ binding to PI-PLC treated HeLa cells. Since MAbs against DAF could theoretically cause steric hindrance of the binding of virus to other cell surface molecules, the ability of CVB3c$_{(s)}$ binding to DAF-depleted HeLa cells was explored. Cells were pretreated with PI-PLC, an enzyme which cleaves GPI-anchored molecules such as DAF from the cell surface (Davitz *et al.*, 1986; Medof *et al.*, 1986). DAF depletion of HeLa cells by PI-PLC enzyme was confirmed by flow cytometry analysis (Fig. 7). Following PI-PLC treatment, the intensity of DAF staining decreased (left shift in the peak) by approximately 75%. A reduction in binding of [^{35}S]-CVB3-CG, CVB3-SH, CVB3-Ø, and CVB3-CGP1V strains to the PI-PLC treated HeLa cells was also observed (Fig. 8). In contrast, PI-PLC treatment of HeLa cells reduced binding of [^{35}S]-CVB3-NR and CVB3-N strains by only 30%, and the binding of [^{35}S]-CVB3-VR30 and CVB3-20 strains by less than 20% (Fig. 8).

iv). Effect of virus infection on DAF expression on HeLa cells. The effect of CVB3c$_{(s)}$ on DAF expression at the cell surface was also monitored. Infection of HeLa cells by all CVB3c$_{(s)}$ for 3 hours led to a reduced expression of DAF on the cell surface, by 10-25%, as compared to uninfected control cells (Fig. 9). Binding of virus most likely internalizes the DAF receptor, since eclipse of bound CVB3 in HeLa cells generally requires only several minutes at 37^{0}C (Zajac and Crowell, 1969). Since host protein synthesis is shut off in virus infected cells, then the cells may be unable to synthesize new DAF for replacement on the cell surface.

v). Complement studies. CVB interactions with the DAF-related complement molecules CD46, CD35, Factor H, and C4bp were examined. The cardiovirulent CVB3-CG strain did not interact directly with any of these molecules (no detectable inhibition, results not shown). There was no reduction in virus infection on HeLa monolayers

Figure 7

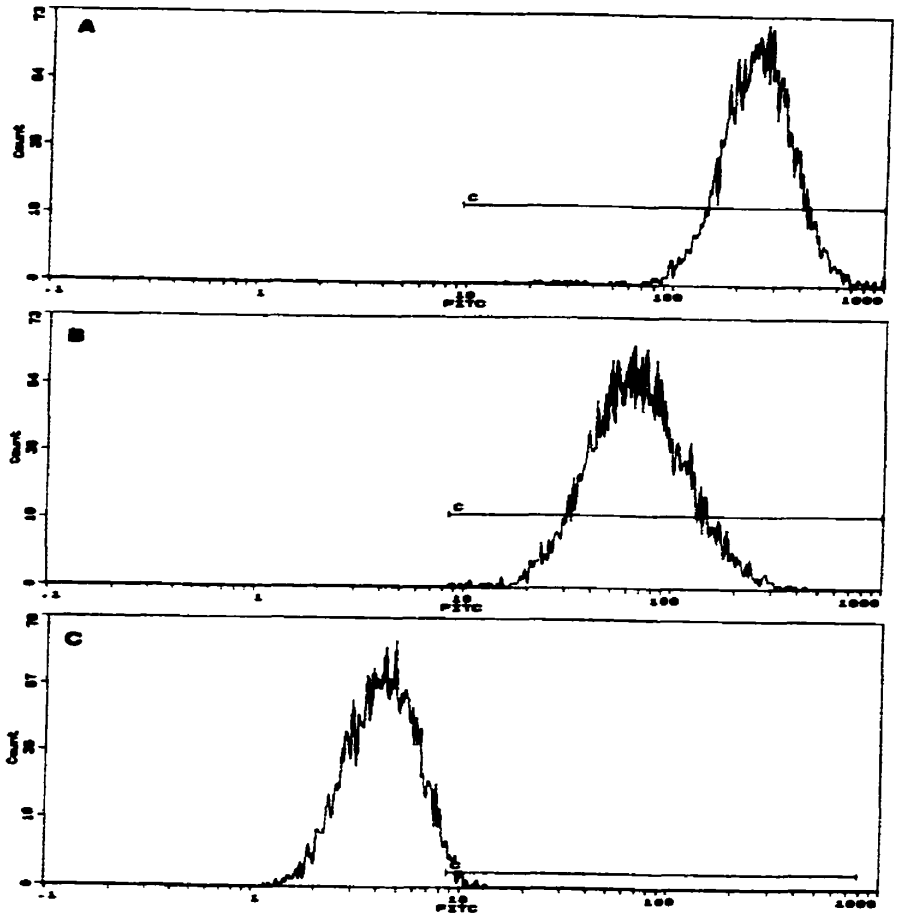


Figure 7. Characterization of DAF-depleted HeLa cells by flow cytometry analysis. Versene dispersed HeLa cells aliquoted at $5x10^6$ cells/test were treated with 0.2 ml RPMI containing 0.5 units of phosphatidylinositol phospholipase C (PI-PLC, from *Bacillus cereus*; Boehringer Mannheim, Laval Quebec, Canada) at 37°C for 1 hour. Cells were washed three times, and labeled with 10 µl of undiluted FITC conjugated anti-DAF MAb 737F on ice for 30 minutes. The cells were washed and resuspended in 1 ml PBS, and DAF expression/loss was monitored by flow cytometry. A) Untreated HeLa cells. B) HeLa cells treated with PI-PLC. C) HeLa cells labeled with control MAb (FITC conjugated rabbit (Fab')2 anti-rat IgG; Serotec).

Figure 8

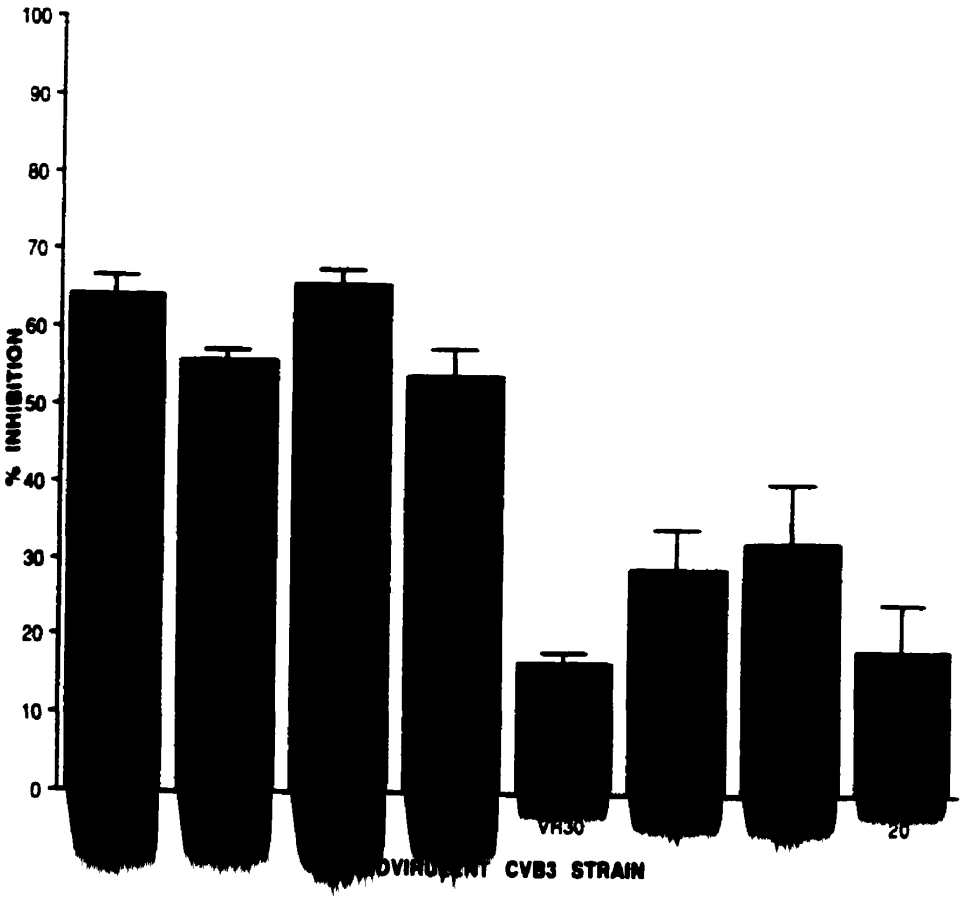


Figure 8. Reduction in [^{35}S]-CVB3c$_{(s)}$ binding to PI-PLC-treated DAF-depleted cells. PI-PLC treated HeLa cells (unlabeled) were prepared as described in Fig. 7. They were used in binding assays as described in Fig. 5. The experiments were performed in quadruplicate for each virus strain.

Figure 9

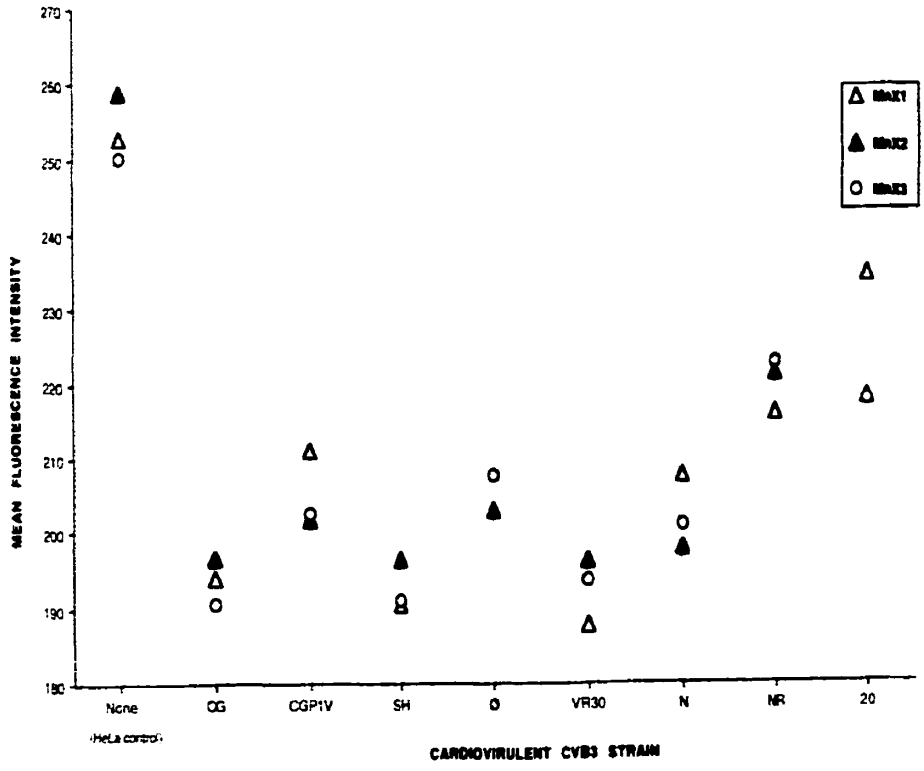


Figure 9. DAF reduction on the surface of CVB3c$_{(s)}$ infected cells. Versene dispersed HeLa cells aliquoted at 1x10^6/test were resuspended in 200µl RPMI containing 1x10^8 PFU of virus, for 1 hour at 25^{0}C. Cells were washed and further incubated in complete media for 2 hours at 37^{0}C. Cells were labeled with FITC conjugated anti-DAF MAb 737F as described in Fig. 7, and DAF expression/loss was monitored by flow cytometry. MnX refers to the mean fluorescence intensity of the sample under analysis. The assay was performed in triplicate, and each symbol represents one value obtained.

pretreated with anti-CD46 MAb. Moreover, there was no detectable binding of [^{35}S]-CVB3-CG to human blood cells. Finally, when virus was pretreated with Factor H or C4bp, there was no reduction in infection in HeLa cell plaque assays, as compared to untreated virus controls.

vi). Ad2-CVB competition binding assays. Binding interactions between Ad2 and CVB were performed by using competition assays. Pretreatment of HeLa cells with unlabeled Ad2 virus strongly inhibited radiolabeled [^{35}S]-Ad2 binding to the cells (Fig. 10). [^{35}S]-Ad2 binding to cells was also inhibited in cells pretreated with CVB serotypes 1-6 (Fig. 10). Virus binding to HeLa cells was most strongly inhibited by pretreatment with odd-serotypes CVB 1, 3 and 5, whereas even-CVB serotypes 2, 4, and 6 were less competitive. [^{35}S]-Ad2 binding to HeLa cells was also reduced in cells pretreated with CVB3c$_{(s)}$ (Fig. 11). Virus binding was strongly inhibited by strains CVB3-CG, CVB3-Ø, CVB3-VR30, and moderately inhibited by pretreatment with CVB3-CGP1V and CVB3-SH, but was not inhibited by strains CVB3-N, CVB3-NR, and CVB3-20.

vii). Ad2 binding to DAF. The binding of Ad2 to DAF was examined with HeLa cells pretreated with anti-DAF MAbs, or with DAF-reduced (PI-PLC pretreated) HeLa cells. [^{35}S]-Ad2 binding to HeLa cells was moderately inhibited by pretreatment of HeLa cells with anti-DAF MAb IA10 (Fig. 12). In contrast, virus binding was not inhibited by anti-DAF MAb 914 (Fig. 12). [^{35}S]-Ad2 binding to HeLa cells rendered DAF-deficient by treatment with the enzyme PI-PLC was significantly increased, as compared to virus binding to untreated HeLa cells (Fig. 12).

Figure 10

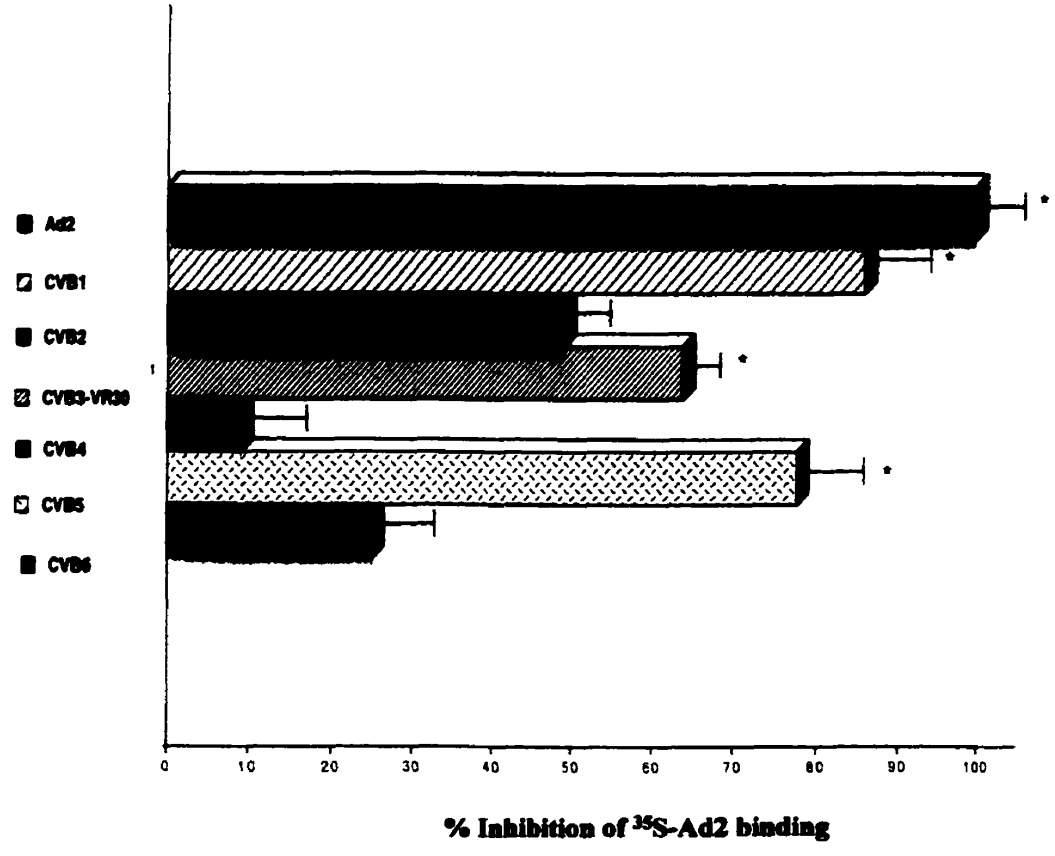


Figure 10. Adenovirus 2 and CVB serotypes 1-6 competitive binding assays. Versene dispersed HeLa cells aliquoted at $5x10^6$ cells/test were preincubated in 0.2 ml of media containing $1x10^8$ PFU of unlabeled virus strain (Ad2 or CVB1-6) or 0.2 ml of media, for 1 hour at 37°C. Cells were washed and further incubated with $5x10^4$ cpm of [^{35}S]Ad-2 virus, for 1.5 hours at 37°C. [^{35}S]-Ad2 binding was monitored by scintillation spectroscopy, as described in Fig. 5. The experiments were performed at least 3 times for each virus serotype. * $p<0.001$

Figure 11

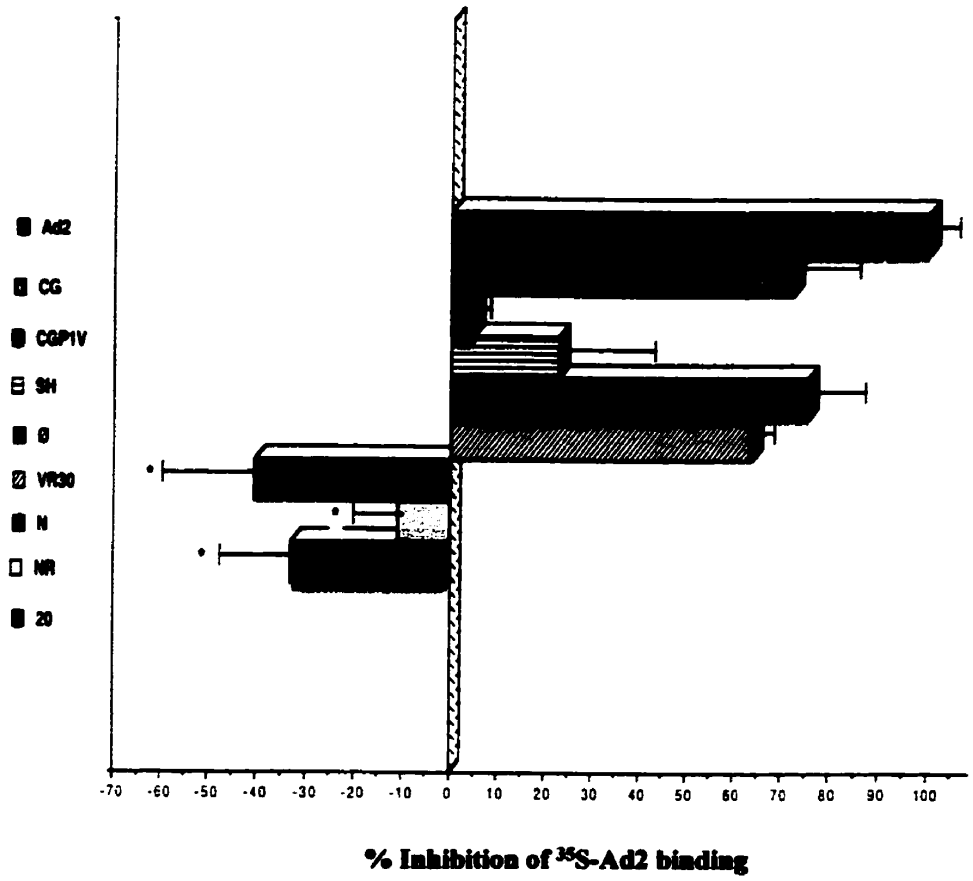


Figure 11. Adenovirus 2 and CCVB3c$_{(s)}$ competitive binding assays. Assays were performed as described in Fig. 10, but used CVB3c$_{(s)}$ instead of CVB1-6. The experiments were performed in triplicate for each virus strain. • $p<0.001$

Figure 12

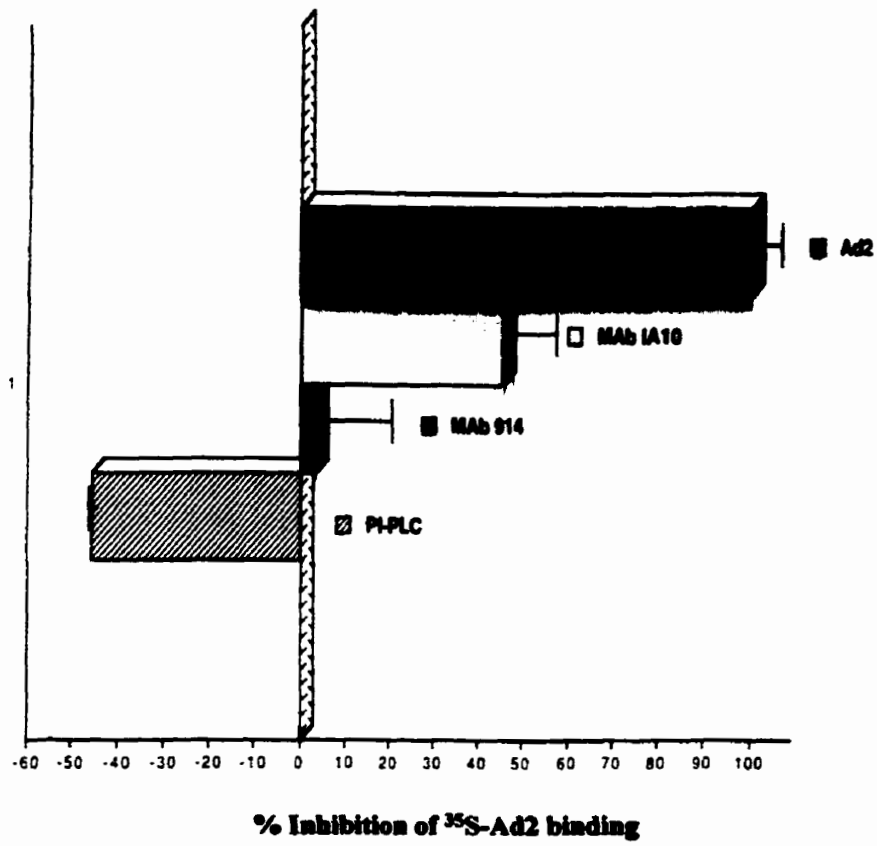
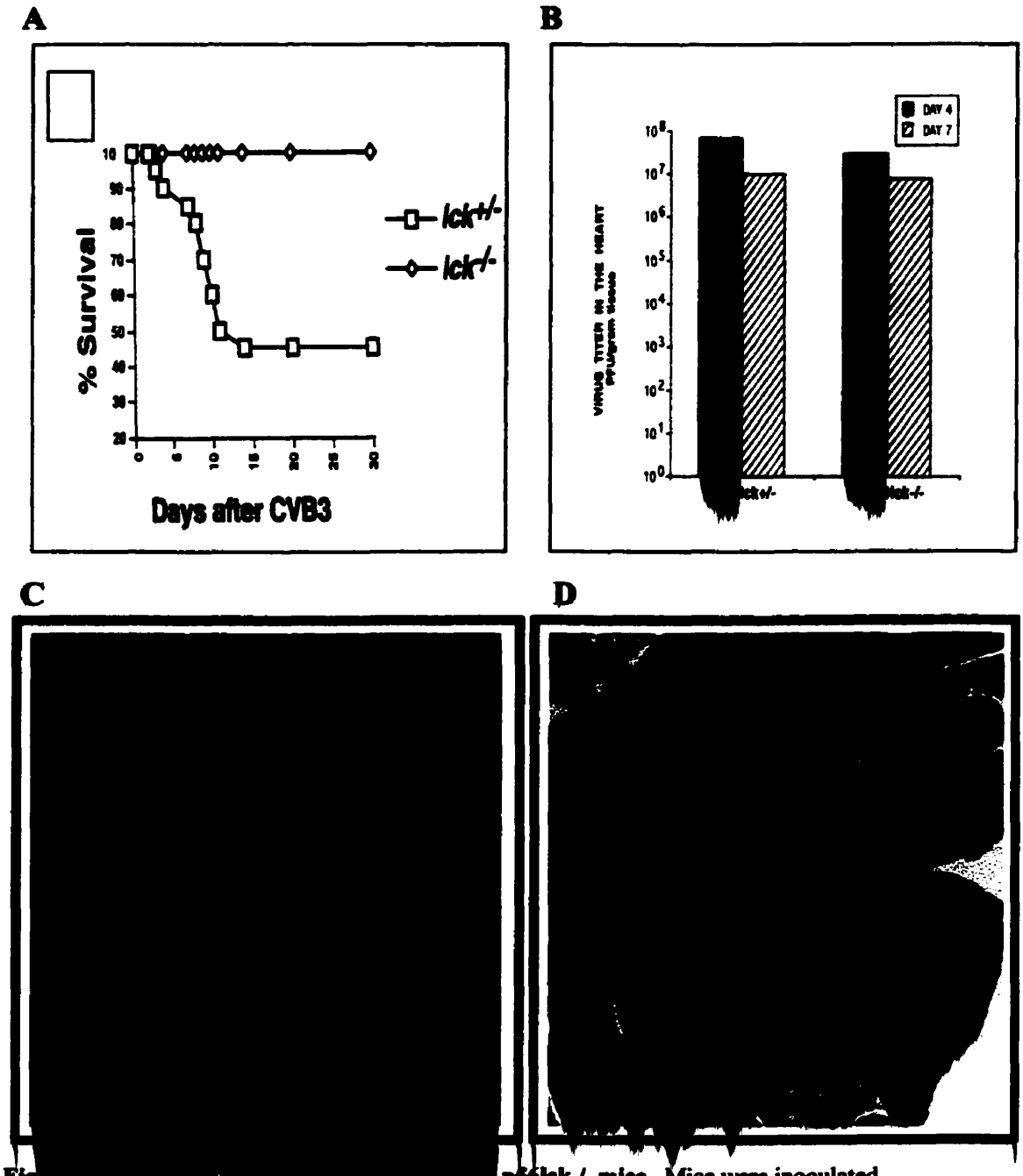


Figure 12. Inhibition of [^{35}S]-Ad2 binding to HeLa cells. HeLa cells pretreated with anti-DAF MAbs 914 and IA10 were prepared as described in Fig. 6. PI-PLC treated cells were prepared as described in Fig. 7 and Fig. 8. Pretreated cells were then incubated in 200 µl RPMI containing $5x10^4$ cpm of [^{35}S]-Ad2, for 1.5 hours at 37^{0}C. Virus binding was determined as described above. The experiments were performed at least 3 times for each assay.

viii). CVB3 infection in p56lck knockout mice. To gain an insight into the mechanism by which DAF might contribute to the pathogenesis of CVB-induced heart disease, the role of T cell activating tyrosine kinase $p56^{lck}$ which associates with DAF was examined. When the $p56^{lck}$ molecule is knocked out, a critical T cell signaling pathway is inactivated. Mice deficient in the p56lck gene were shown to be resistant to CVB3 myocarditis. There was no mortality (Fig. 13a), despite virus replication in the heart (Fig. 13b). Moreover, there were minimal pathologic changes in the myocardium and few inflammatory cells, as compared to mice with a functional copy of the $p56^{lck}$ gene (Fig. 13c,d).

NK cells have been implicated in host defense mechanisms against the virus infection (reviewed in Martino et al., 1995b), and presumably retain this capacity even in the $p56^{lck}$ knockout mice. However, $p56^{lck}$ knockout mice that were also depleted of NK cells were less able to control the virus infection (Fig. 14a,b), and damage to the ventricular myocardium became evident (Fig. 14c). Thus a loss of both T cells and NK cells rendered the knockout animals susceptible, once again, to CVB3-induced heart disease.

FIGURE 13



Fig[illegible] p56lck-/- mice. Mice were inoculated intraperitoneally with 10e5 PF[illegible] of CVB3-CG, and randomized to monitor endpoints of mortality, cardiac virus titers, and cardiac pathology. At least 3 samples were taken for each endpoint. A). Survivorship curves. B). Virus replication in the hearts of CVB3 infected p56lck+/- and p56lck-/- mice. C). Pathologic changes in the ventricular myocardium of a p56lck+/- mouse, day 7 PI. Note myocardial cell necrosis and inflammatory cell infiltrate. Hematoxylin and eosin stain. Magnification @40x. D) Ventricular myocardium of a p56lck-/- mouse, day 7PI. Minimal pathologic changes and few inflammatory cells. Hematoxylin and eosin stain. Magnification @40x.

FIGURE 14

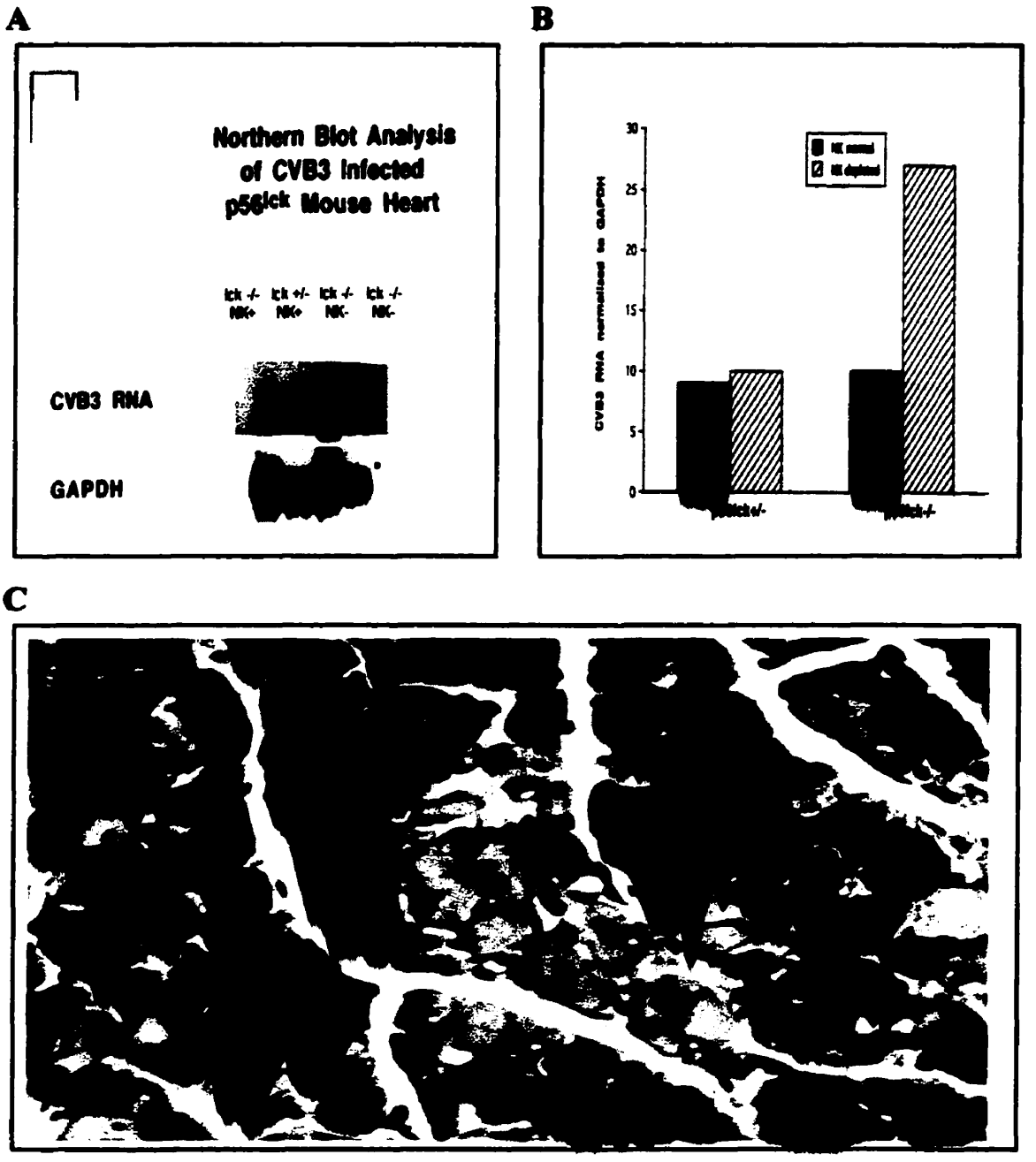


Figure 14. CVB3 myocarditis in p56lck+/- and p56lck-/- mice depleted of Natural Killer (NK) cells. Mice administered anti-asialo GM1 antibody to deplete NK cells were injected intraperitoneally with 10e5 PFU of CVB3-CG as described in Fig. 13. At least 3 samples were taken for each endpoint. A). Northern blot analysis of virus load in the heart. Upper band - CVB3 RNA. Lower band - GAPDH RNA control. B). Densitometry plot. Virus RNA load increases slightly in hearts of p56lck+/- NK- mice, and more significantly in p56lck-/- NK mice. NK cells are likely protective against virus infection, even in p56lck-/- mice. C). Pathologic changes in the ventricular myocardium of a p56lck-/-NK- mouse, day 7. Regions of light staining myocytes presumably containing foci of replicating virus. Masson's stain, @100x magnification.

F.6. Discussion

CVB3 is implicated in the pathogenesis of myocarditis and dilated cardiomyopathy (Liu *et al.*, 1996; Martino *et al.*, 1994a,b, 1995b; Woodruff 1980). Many CVB3 strains can infect and replicate in the heart, as demonstrated in murine model systems. However, only some strains are found to be cardiovirulent, in that they initiate disease processes which lead to inflammation of the myocardium, and severe destruction of myocardial tissue. The genesis of viral heart disease likely represents a complex interplay of both virus-encoded and host-derived factors. In this study, the capacity of cardiovirulent CVB3 strains to interact with the DAF cell surface receptor was examined.

The characteristics of the CVB3 strains used in this study are summarized in Table 6. Three lines of evidence show that the cardiovirulent CVB3 strains used in this study interacted with DAF on the cell surface. 1). Binding of radiolabeled [^{35}S]-CVB3c$_{(S)}$ to HeLa cells decreased when cells were preincubated with anti-DAF MAbs, as compared to control MAbs or media. 2). Plaque formation by CVB3c$_{(s)}$ was reduced when HeLa cell monolayers were preincubated with anti-DAF MAbs, showing that blockage of DAF reduced CVB3c(s) infection. 3). Binding of CVB3c$_{(s)}$ to DAF-depleted PI-PLC treated HeLa cells was reduced, as compared to untreated HeLa cell controls.

The present study showed that although all the strains tested showed interactions with DAF, some CVB3c$_{(s)}$ interacted more strongly with DAF than others. DAF interactions were most pronounced for the viruses which produce severe disease in the murine model (CVB3-CG, CVB3-SH), as compared to those which produce less severe disease (CVB3-20, CVB3-NR) *in vivo*.

Interestingly, the CVB3-Ø strain interacted significantly more strongly with DAF than the parental CVB3-20 strain. There are several mutations in the capsid region of CVB3-Ø relative to CVB3-20 (Chapman *et al.*, 1994; Tracy *et al.*, 1992; Tu *et al.*, 1995),

and it is possible that one or more of these mutations, (in combination with 234-U transition which restores cardiovirulence), could result in a significantly stronger DAF binding phenotype.

The CVB3-N strain exhibited a weaker DAF binding phenotype, similar to the interactions observed for CVB3-NR. Interestingly, nucleotide sequencing and phylogenetic analysis of the (receptor binding) capsid region of the strains indicated that CVB3-NR and CVB3-N are most closely related to each other, as compared to the other CVB3c$_{(s)}$ (Martino *et al.*, 1997), consistent with the similar binding patterns observed for these two viruses.

The CVB3-VR30 strain is presumably non-cardiovirulent, and its interactions with DAF were distinct from that noted for the cardiovirulent strains of virus. Anti-DAF MAbs strongly blocked virus binding (Fig. 5) and infection (Fig. 6) of HeLa cells. However, CVB3-VR30 binding was only weakly affected by PI-PLC digestion of cells (Fig. 8). Although the reason for this discrepancy is unclear, it may indicate that of all the strains tested, the CVB3-VR30 strain most requires additional receptor(s) other than DAF for efficient binding to the cell surface. This is consistent with previous reports that MAbs can block CVB3-VR30 binding to the cell surface, but that this strain binds to DAF only weakly (Shafren *et al.*, 1995) or not at all (Bergelson *et al.*, 1995).

The extent to which anti-DAF MAbs interfered with binding/infection of HeLa cells by a CVB3c$_{(s)}$ depended on the epitope to which the antibody was directed. MAb 914 (clone BRIC 216) which binds to the DAF molecule at SCR3 (Coyne *et al.*, 1992) was most effective at reducing CVB3c$_{(s)}$ infection of HeLa cells. In preliminary studies, MAb 737 (clone BRIC 110) which binds to SCR2 (Coyne *et al.*, 1992), produced similar results when tested with the CVB3-CG strain (unpublished observations). In contrast, MAb IA10, which binds DAF at SCR1 (Coyne *et al.*, 1992), does not block CVB3 efficiently. These findings are consistent with previous reports that anti-DAF MAbs recognizing SCR2 and SCR3 are most effective at blocking virus attachment and

internalization, and that a CVB binding site is likely located within or near these domains (Bergelson *et al.*, 1995; Shafren *et al.*, 1995).

Complement regulatory molecules which are structurally and/or functionally related to DAF, including CR1 (CD35), MCP (CD46), C4-binding protein and Factor H (Hourcade *et al.*, 1989), do not interact with the cardiovirulent CVB3-CG strain, indicating that the site of virus binding is unique to the DAF molecule. The possibility exists that other strains not tested could interact with DAF-related ligands.

The ability of Ad2 to bind to DAF, as well as the competitive binding characteristics between Ad2 and CVB serotypes 1-6 and CVB3$c_{(s)}$ were also examined. These investigations stem from the original reports that CVB and Ad2 fiber can compete for a receptor (Lonberg-Holm *et al.*, 1976). It was found that the odd-numbered CVB 1,3,5 serotypes competed most strongly with Ad2 for binding to the HeLa cell surface, as compared to the even-numbered CVB 2,4,6 serotypes (Fig. 10). One possible explanation for this observation is that the odd-numbered CVB serotypes exhibit greater binding affinities than the even numbered ones (Crowell 1976), and thus more rapidly saturate available cell surface receptors. Additionally, it has been noted that CVB1,3,5 can bind to DAF, whereas CVB2,4,6 do not bind DAF (Bergelson *et al.*, 1995; Shafren *et al.*, 1995), and the ability to saturate the DAF receptor could be important in competing with Ad2 for cell surface binding. In support of this latter hypothesis, it was observed that cardiovirulent CVB3$c_{(s)}$ strains which interacted most strongly with DAF were more effective at inhibiting Ad2 binding to HeLa cells than cardiovirulent strains which showed weaker interactions with DAF (Fig. 11).

It seems unlikely that Ad2 binds directly to DAF. Anti-DAF MAb 914, which binds to the third domain (SCR3) near the site of CVB attachment, does not affect Ad2 binding to the cell surface (Fig. 12). Anti-DAF MAb IA10, which binds to the outermost domain (SCR1) does block approximately 50% of Ad2 binding (Fig. 12), but this may well be due to steric hindrance. The observation that DAF-reduced HeLa cells are significantly more capable of binding Ad2 than normal HeLa cells (Fig. 12) also points to the notion

that pretreatment with DAF-binding viruses or antibodies sterically blocks the ability of Ad2 to bind to its natural receptors. It also indicates that DAF and Ad2 binding receptors (such as coxsackie adenovirus receptor (CAR) (Bergelson *et al.*, 1997a; Carson *et al.*, 1997; Tomko *et al.*, 1997) or the Ad2 binding integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (Wickham *et al.*, 1993) or Ad2 fiber binding human fibronectin type III (Hong and Boulanger, 1995) or MHC class I α2 domain (Hong *et al.*, 1997)) probably co-localize on the HeLa cell membrane. Indeed, it has previously been reported that DAF also co-localizes with ICAM-1 (Shafren *et al.*, 1997b), the receptor shared by coxsackievirus A21 (Shafren *et al.*, 1997a) and the major group rhinoviruses (Greve *et al.*, 1989; Stauton *et al.*, 1989; Tomassini *et al.*, 1989).

This study demonstrated that only cardiovirulent CVB3 strains can bind strongly to DAF. In light of this observation, an important issue arises. DAF is not a receptor in the classical sense, because it alone does not promote lytic infection of cells (Bergelson et al., 1995, Shafren et al., 1995). How then, does CVB3c(s) binding to DAF play a role in the pathogenesis of CVB-induced heart disease?

One proposed mechanism by which DAF binding could play a role role in the disease process is through the triggering of signaling cascades. DAF is a glycolipid-anchored protein which associates with the T-cell activating tyrosine kinase $p56^{lck}$ (Davis et al., 1988; Shenoy-Scaria et al., 1992). T cell activation, which is a hallmark feature of myocarditis, does not occur in transgenic mice lacking the signaling molecule $p56^{lck}$. These mice are resistant to CVB3-induced heart disease (Figs 13, 14). Future studies to examine the signaling pathways triggered by virus-receptor binding may well enhance understanding of the pathogenesis of CVB myocarditis.

There are also a number of other mechanisms which can be elaborated from the observations of this study, by which the DAF receptor may play an important role in the pathogenesis of CVB-induced heart disease. For example, DAF may act as a sequestering site for virus and facilitate virus presentation to a second cell surface receptor, thus enhancing virus virulence. Indeed, co-localization of DAF and a second

receptor such as CAR was indicated by the competitive binding studies with adenovirus (Figs. 10-12). Moreover, it has been recently demonstrated that a DAF binding strain uses both DAF and CAR to productively infect cells (Shafren et al., 1997c), thus providing further support for this hypothesis. Finally, it was observed in this study that DAF expression was reduced on CVB infected cells (Fig. 9). Since DAF is a complement regulatory molecule (reviewed in Hourcade *et al.*, 1989, Lublin and Atkinson, 1989; Nicholson-Weller and Wang, 1994), it is also tempting to speculate that loss of DAF from the cell surface renders the cells more susceptible to complement-mediated lysis. This could facilitate the release of progeny virus from infected cells, thus further enhancing virus virulence.

A second important issue to be addressed is whether all DAF binding viruses are cardiovirulent. Although all the strains tested here bound to DAF and are cardiotropic in well-defined model systems, this does not presume that all DAF binding viruses are cardiovirulent. Indeed, prototype strains of CVB1, CVB5, CAV21 and several echoviruses bind to DAF (Bergelson et al., 1994, 1995, Shafren et al., 1995, 1997b), and it is not known whether these virus strains can cause heart disease in the model systems. Nevertheless, future studies examining the cardiopathogenicity of these viruses may be important, particularly in light of the observation that strains including CVB1, CVB3, CVB5, CAV and echoviruses have all been associated with heart disease in humans (WHO/ISFC Task force, 1980).

In summary, cardiovirulent strains of CVB3 interact with the DAF receptor, whereas Ad2 virus does not, shedding some light on the different tissue tropism's, disease manifestations, and host ranges noted for each virus. It is likely that CVB binding to DAF is only one of several virus-determined factors involved in viral heart disease. Additional mechanisms by which CVB targets heart tissue and initiates aberrant immune responses will undoubtedly become clearer once reagents to recently identified CVB receptor molecules like CAR (Bergelson et al., 1997a; Carson *et al.*, 1997; Tomko *et al.*, 1997) and other less characterized putative receptor molecules

(Raab de Verdugo *et al.*, 1995) become available. Understanding these interactions is important for an understanding of the disease processes that occur, and perhaps will open new avenues for the treatment of CVB heart disease.

G. STUDY #2
THE CAR RECEPTOR

G.1. Purpose

To characterize interactions between virus and the coxsackie-adenovirus receptor (CAR).

Based on the publication -

Martino, T. A., M. Petric, H. Weingartl, J. M. Bergelson, M. A. Opavsky, C. D. Richardson, J. F. Modlin, R. W. Finberg, K. C. Kain, N. Willis, C. J. Gauntt, and P. P. Liu. (2000). The coxsackie-adenovirus receptor (CAR) is used by reference strains and clinical isolates representing all six serotypes of coxsackievirus group B and by swine vesicular disease virus. *Virology*, 271, 99-108.

Figures 15, 16 (CVB data), 17a, 17b, 17c, and Tables 7, 8 (all viruses except those of Dr. John Modlin) by T. A. Martino. SVDV data in Figures 16, 18, 19, and Table 9 used our protocols, but were performed under isolation conditions at the National Center for Foreign Animal Diseases by Dr. Hana Weingartl. All Figures and Tables compiled by T. A. Martino. Thanks to Dr. Jim Dennis for the CHOP cells, JenniElizabeth Petrella and Dr. John Young for the sCAR protein and ALV-env protein, Dr. Michael Heffernan for the eukaryotic expression cloning materials, Dr. Peter Backx for the GFP vector, Barbara Kellam for sequencing, John Nishikawa and Rose Cheung for help with the HSC clinical enteroviruses and GMK cells, and Dr. S. Lee of the Canadian Enterovirus Reference Center in Halifax NS for typing studies.

G.2. Abstract

Group B coxsackieviruses are etiologically linked to many human diseases, and cell surface receptors are postulated to play an important role in mediating their pathogenesis. The coxsackievirus adenovirus receptor (CAR) has been shown to function as a receptor for selected strains of Coxsackievirus group B (CVB) serotypes 3, 4, and 5, and is postulated to serve as a receptor for all six serotypes. In this study, it was demonstrated that CAR can serve as a receptor for laboratory reference strains and clinical isolates of all 6 CVB serotypes. Infection of CHO cells expressing human CAR results in a 1000-fold increase in CVB progeny virus titer compared to mock transfected cells. CAR was further shown to be a functional receptor for swine vesicular disease virus (SVDV), as CHO-CAR cells but not CHO mock transfected controls were susceptible to SVDV infection, with the production of progeny SVDV, and the development of cytopathic effects. Moreover, SVDV infection could be specifically blocked by monoclonal antibody to CAR (RmcB). SVDV infection of HeLa cells was also inhibited by an anti-CD55 MAb, suggesting that this virus, like some CVB, may interact with CD55 (decay accelerating factor) in addition to CAR. Finally, pretreatment of CVB or SVDV with soluble CAR was found to effectively block virus infection of HeLa cell monolayers.

G.3. Introduction

Group B coxsackieviruses are etiologically implicated in human disease, whose clinical manifestations include mild gastrointestinal or upper respiratory tract symptoms, myocarditis, meningitis, encephalitis, and pulmonary disease (Chonmaitree and Mann, 1995; Grist *et al.*, 1978; Liu *et al.*, 1996; Martino *et al.*, 1994a,b, 1995b; Rotbart, 1995; Woodruff, 1980). Infection is mediated by cell surface receptors, which facilitate binding and entry of CVB into susceptible host cells. Receptors likely have a major role in determining organ and cell tropism in patients infected with these viruses, and may account for some of the clinical manifestations and disease sequelae (Crowell and Landau, 1983; Holland, 1961; Rotbart and Kirkegaard, 1992).

It had been proposed that all CVB1-6 serotypes shared a common receptor molecule. This hypothesis was based on the observation that prototype strains of CVB1-6 competed with each other for binding to the HeLa cell surface, but did not interfere with cell binding by other enterovirus types (Lonberg-Holm *et al.*, 1976). A monoclonal antibody termed RmcB, directed against this putative common receptor molecule, was subsequently shown to block binding and infection by CVB reference strains (Hsu *et al.*, 1988). However, it was also demonstrated that RmcB blocked only some clinical isolates of CVB from infecting HeLa cells (Bergelson *et al.*, 1997b), throwing into question the nature of MAb blocking studies and the premise of the common CVB receptor molecule.

A common receptor molecule termed the coxsackievirus adenovirus receptor (CAR) was recently cloned and characterized. It functions as a cell surface receptor for CVB, as well as an attachment molecule for adenovirus fiber proteins (Bergelson *et al.*, 1997a; Carson *et al.*, 1997; Roelvink *et al.*, 1998; Tomko *et al.*, 1997). CAR is a 46-kDa transmembrane glycoprotein with two extracellular immunoglobulin-like domains. Selected strains of CVB3 and CVB4, and CVB5 were shown to bind and to productively

infect nonsusceptible hamster cells transfected with CAR cDNA, but not control cells lacking the CAR molecule (Bergelson *et al.*, 1997a; Tomko *et al.*, 1997).

At the inception of this study, it still remains to be proven that CAR can function as a common host cell receptor for CVB. To address this, CAR was examined for its ability to serve as a receptor for a variety of clinical and prototype isolates representing the six CVB serotypes. This investigation was then extended to include the swine vesicular disease virus (SVDV), which is antigenically and genetically related to CVBs (Graves, 1973; Martino et al., 1999; Zhang *et al.*, 1993, 1999).

G.4. Materials and Methods

i). Cell lines. HeLa cells (CCL-2) and Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC). They were grown in RPMI 1640 medium supplemented with 0.5% penicillin and streptomycin and 10% fetal calf serum (FCS). CHOP cells (chinese hamster ovary cells expressing polyoma T antigen) were from the laboratory of Dr. Jim Dennis (Heffeman and Dennis, 1992), and were non-permissive to infection with CVB. CHOP-CAR cells were grown in DMEMα medium supplemented with 0.5% penicillin and streptomycin and 10% FCS. Cells were transiently transfected with CAR cDNA (CHOP-CAR) or control cDNAs. CHO-CAR cells are CHO cells stably transfected with CAR cDNA (Bergelson *et al.*, 1997a), and CHO-control cells are stably transfected with empty pcDNA3.1 vector (Wang and Bergelson, 1999) or with human integrin α2 subunit (Bergelson *et al.*, 1993). Stable CHO-CAR transfectants and CHO-control cells were grown in nucleoside-free alpha minus MEM supplemented with $NaHCO_3$ to pH 7.5, 0.5% penicillin and streptomycin, and 10% FCS. GMK cells (primary tube cultures of African green monkey kidney) were obtained from Viromed, Minneapolis, USA. They were grown in ELY medium (Earle's Balanced Salt Solution, supplemented with lactalbumin, yeast hydrolysate and 0.5% penicillin and streptomycin). Swine testis (ST) cells were obtained from the ATCC. Porcine kidney (PK-15) cells were from NVLS, Ames, Iowa, USA. ST and PK-15 cells were maintained in alpha MEM supplemented with 0.5% gentamycin and 10% FCS. Media and supplements were purchased from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada.

ii). Viruses. Reference virus strains CVB1 (Conn-5), CVB2 (Ohio), CVB3-VR30 (Nancy), CVB4 (Benschoten), CVB5 (Faulkner) and CVB6 (Schmitt) were obtained from ATCC. Virus stocks were prepared by one passage in HeLa cells (with Vero cells used for CVB6). Stocks were frozen and thawed 3X, clarified by centrifugation, titered by

plaque assay on HeLa cells, and stored as aliquots at -70^{0}C. Selected enterovirus isolates collected over a five year period at the Hospital for Sick Children, Toronto, Canada are shown in Table 7. These viruses were isolated by culture on GMK cells, and typed using reference antisera at the Canadian National Centre for Enteroviruses, Halifax, Canada. Additional CVB clinical specimens were maintained in the laboratory of J.F.M, and some of these samples were described previously by Bergelson *et al.*, 1997b (see Table 8). CVB3 strains known to be cardiovirulent in mice (CVB3cs) -SH, -NR, -N, -CG, -20, -Ø were maintained in our laboratory, as previously described (Martino *et al.*, 1998). SVDV (UK 27/72) was obtained from the Institute for Animal Health, Pirbright, UK, and maintained at the National Center for Animal Diseases, Winnipeg, Manitoba, Canada.

iii). Antibodies. Anti-CAR RmcB monoclonal antibody was prepared as mouse ascites fluid (Hsu *et al.*, 1988), and provided by J.M.B. Anti-CAR rabbit polyclonal antiserum was prepared as described below. Anti-DAF MAb 914 (clone BRIC 216) was obtained from Serotec Canada, Toronto, Ontario, Canada. Rabbit affinity purified anti-bovine albumin antiserum was obtained from Cappel/ICN, Toronto, Ontario, Canada.

iv). Primers. Primer TM39 was used for first strand cDNA synthesis of CAR. TM39 (5'-TTGAGGCTAGTAACACAAT-3') was derived from the CAR 3' non-translated region (Bergelson *et al.*, 1997a). Primers TM31 and TM32 were used for PCR of CAR. Primer TM31 (5'-ACTTATCTAGGATCCATGGCGCTCCTGCTGTGCTTCGTGCTCCTG TGC-3') consists of a *BamH1* restriction site (underlined), followed by the first 33 nucleotides encoding hCAR protein (Bergelson *et al.*, 1997a). Primer TM32 (5'-CGTTATGTACTCGAGCCTATACTATAGACCCATCCTTGCTCTGTGCTGG-3') consists of an *XhoI* restriction site (underlined), a single cytosine nucleotide, and 33 nucleotides

TABLE 7.
Enterovirus isolates from patients at the Hospital for Sick Children, Toronto, Canada

VIRUS TYPE	SPECIMEN	YEAR ISOLATED	ISOLATE NUMBER
CVB1	CSF	1995	CVB1 (5010107)
CVB1	Throat swab	1995	CVB1 (5010607)
CVB2	CSF	1995	CVB2 (5010159)
CVB3	Stool	1994	CVB3 (4012530)
CVB3	Stool	1997	CVB3 (19141255)
CVB3	Stool	1997	CVB3 (19281109)
CVB3	Pleural fluid	1998	CVB3 (20270361)
CVB3	Stool	1997	CVB3 (20310308)
CVB4	CSF	1995	CVB4 (5010936)
CAV9	CSF	1994	CAV9 (4012720)
EV9	CSF	1997	EV9 (22030957)

complementary to the CAR coding sequence upstream of the termination codon (Bergelson *et al.*, 1997a).

v). PCR amplification of CAR cDNA. RNA extracted from HeLa, Vero, transiently transfected CHOP-CAR cells, untransfected CHOP cells, and the porcine cells lines ST and PK-15 was subjected to reverse transcription followed by PCR to amplify the CAR cDNA. Total cell RNA was purified using TRIzol reagent according to the manufacturer's directions (Gibco BRL). For first strand cDNA synthesis, a 15 µl aliquot of RNA was heated for 5 min at 65^{0}C, chilled on ice, then added to a 25 µl volume containing 1 x AMV reverse transcription buffer (Pharmacia), 1 µl RNasin (Pharmacia), 4 µl of 10 mM dNTP mixture (Pharmacia), 2 µl of 10 mM Spermidine HCl, 2 µl of 80 mM sodium pyrophosphate, 4 µl of downstream primer TM39 and 1 µl of AMV reverse transcriptase (Pharmacia), and samples were incubated at 37^{0}C for 1 h. For PCR, a 4 µl aliquot of this reaction mixture was added to a 46 µl volume containing 1 x reaction buffer (Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada), 0.2 mM each of dNTP (Pharmacia) and 2 µM each of primers TM31 and TM32. The preparation was denatured for 10 minutes at 94^{0}C, then 2.5 U of Taq polymerase (Pharmacia) and 50 µl light mineral oil were added. PCR was performed on a DNA Thermal Cycler (Perkin Elmer Cetus) for 39 cycles of annealing 50^{0}C, 2 min; extension 72^{0}C, 2 minutes; denaturation 94^{0}C,1 minute.

vi). Expression of CAR and production of rabbit polyclonal antisera. HeLa cell CAR cDNA was amplified by PCR and purified by agarose gel electrophoresis. A band corresponding to the ~1.1 Kb coding region of CAR was excised from the gel and purified with Sephaglas Band Prep (Pharmacia), digested with *BamH1* and *Xho1* (Pharmacia), ligated into a pET28a(+) cloning vector (Novagen, Madison, WI), and transformed into INVαF' One Shot cells (Invitrogen, Carlsbad, CA). The presence of CAR in the transfected cells was confirmed by extraction and purification of hCAR-

pET28a(+) DNA on Qiagen chromatography columns (Qiagen Inc, Santa Clarita, CA, USA), and by sequencing the CAR portion using Thermosequenase (Amersham Canada Ltd, Oakville, Ontario, Canada). Full-length in-frame clones were transformed into BL21(DE3) expression host cells (Novagen). To induce CAR protein expression, transformed cells were grown in the presence of IPTG, in accordance with the manufacturer's instructions (Novagen). Proteins extracted from these cells were fractionated by SDS-PAGE analysis, and transferred electrophoretically to a polyvinylidene fluoride (PVDF) nylon membrane (Millipore, Bedford, MA). A band corresponding to CAR protein was detected by Western blot immunohistochemical analysis, using a 1:3000 dilution of T7*Tag Antibody-Alkaline Phosphatase Conjugate (Novagen) and BCIP-NBT (5-bromo-4chloro-3indolyl phosphate-nitro blue tetrazolium; SigmaFAST, Sigma chemicals). Rabbit polyclonal antisera were raised against the CAR protein. SDS-PAGE gel bands corresponding to expressed CAR protein were excised, soaked in PBS to remove staining solutions, mixed with Freund's incomplete adjuvant, and injected subcutaneously into two young adult male rabbits once every two weeks for six successive weeks. Sera collected from these animals was stored at -20^{0}C. Reactivity to CAR was confirmed by Western blot and flow cytometry analysis using HeLa cells, or CHO cells transfected with CAR cDNA as described below.

vii). CAR expression in eukaryotic cells, and CVB replication in transfected cells. HeLa cell CAR cDNA (1.1 Kb) amplified by PCR was digested with *BamH1* and *Xho1* restriction endonucleases (Pharmacia), ligated into pcDNA-1.1/Amp vector (Invitrogen, Carlsbad, CA, USA), and transformed into INVαF' One Shot cells (Invitrogen). hCAR-pCDNA-1 was purified by Qiagen chromatography (Qiagen Inc) and sequenced by Thermosequenase (Amersham Canada Ltd.). Two clones were selected, of which one, hCAR7, contained full length coding sequence, and the other, hCAR2, encoded the extracellular region only (amino acids 1-189). CHOP cells were transfected with hCAR7-pcDNA-1, or hCAR2-pcDNA-1, or mock transfected, using

Lipofectamine reagent (Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada) according to the manufacturers instructions. CAR expression was monitored by immunohistochemical staining and Western blot analysis of transfected cells, using rabbit anti-CAR polyclonal antibody. Transfection efficiency was also monitored using a reporter vector containing GFP (jellyfish green fluorescent protein) (kindly supplied by Dr. Peter Backx, University of Toronto, Canada). CVB replication in transiently transfected CHOP-CAR cells (or controls) was monitored by inoculating cell monolayers with each test virus at room temperature for 1 h, washing 3x, and further incubating in growth medium for 0 h (to monitor for residual virus in the inoculum), or 24 h at 37^0C. Dishes containing cells and virus were frozen and thawed, and progeny virus titer was determined by $TCID_{50}$ assay on HeLa cells.

viii). CVB-induced cytopathic effect on CAR positive cells. Confluent monolayers of HeLa cells, stably transfected CHO-CAR cells, or CHO-control cells transfected with empty pcDNA3.1 vector were plated in 96-well Costar dishes. Cell monolayers were exposed to a 50 µl aliquot of serial 2-fold dilution's of virus, and incubated at room temperature for 1h. Cell monolayers were washed and further incubated at 37^0C. Cytopathic effect was monitored after 3 days by removing the supernatant and staining the cells with a 2% formaldehyde / 0.1% crystal violet dye solution. For SVDV, the experiments were performed as described above except that serial 10-fold dilution's of virus were tested.

ix). Soluble CAR blocking of CVB-induced cytopathic effect. For production of soluble CAR immunoadhesin (sCAR), DNA encoding the CAR extracellular domain (ending with PPSNK) was fused to DNA encoding the Fc region of rabbit immunoglobulin, derived from plasmid pKZ374 (provided by Dr. John Young, Harvard Medical School) and inserted in the mammalian expression vector pcDNA 3.1 (Invitrogen). Fusion protein was purified from the supernatant of transiently transfected

293 cells by chromatography on protein A Sepharose (Zingler and Young, 1996). The control immunoadhesin, avian leukosis virus envelope glycoprotein fused to rabbit Fc (ALV-env) was produced using plasmid pKB201 provided by Dr. Young. For sCAR blocking of CVB3-induced CPE, sCAR (or control) was added to a fixed concentration of virus and warmed to 37^{0}C for 1 hr. The mixture was then added to confluent monolayers of HeLa cells plated in 96-well plates. Monolayers were examined after 24 hours for cytopathic effect, by staining cells using the methods described above.

x). Studies with SVDV. SVDV induced cytopathic effect was monitored on CAR-positive monolayers of HeLa cells, porcine ST and PK-15 cells, stably transfected CHO-CAR cells, and CAR-negative CHO-control cells transfected with empty pcDNA3.1, using the methods described above for CVB. For antibody blocking experiments, stable CHO-CAR transfectants or HeLa cells were preincubated with anti-CAR MAb RmcB, or anti-DAF MAb 914, or antibody to bovine albumin (control), and then infected with ~50 PFU/ml of SVDV. Virus yield was determined by plaque assay. The ability of soluble CAR to block SVDV-induced CPE of HeLa cells was performed as described above for CVB.

G.5. Results

i). Confirmation of CAR mRNA in cells. It was first necessary to confirm that the cell lines used in this study which were susceptible to virus infection expressed CAR RNA, while cell lines not susceptible to virus did not express CAR. CAR transcription was monitored by RT-PCR analysis using CVB susceptible HeLa cells, Vero cells, and transiently transfected CHOP-CAR cells, and the nonsusceptible untransfected CHOP cells. SVDV susceptible porcine cell lines ST and PK-15 were also examined in this manner. Figure 15 is a composite demonstrating that CAR cDNA was amplified from all the cell lines that were susceptible to CVB (HeLa, Vero, CHOP-CAR) and SVDV (ST, PK-15), but not from nonsusceptible untransfected CHOP cells. Although not shown, positive controls using cloned CAR cDNA were also amplified by PCR and analyzed by electrophoresis, and were consistently positive as expected. Also, negative water controls containing all reagents except for CAR cDNA were consistently negative. PCR amplification of the entire region encoding CAR produced an amplicon of just over 1 Kb (Fig. 15, see arrow), consistent with the approximately 1.1 Kb size of the mRNA coding region of CAR (Bergelson et al., 1997; Tomko et al., 1997).

ii). CVB induces cytopathic effect on CAR positive cells. All CVB strains tested induced cytopathic effect (CPE) on monolayers of HeLa cells and stably transfected CHO-CAR cells (Fig. 16 and Table 8). Some CVB strains also produced CPE on Vero cell monolayers, although only CVB5 (Faulkner), CVB3cs-N, CVB3cs-NR, and CVB3cs-20 were observed to produce a comparable CPE in Vero cells as they did in HeLa cells (not shown). Genetically manipulated chimeric viruses generated from the cardiovirulent CVB3cs-CG strain and the avirulent CVB3cs-Ø strain (Lee et al., 1997a) also produced CPE on monolayers of HeLa cells and stable CHO-CAR cells (not

Figure 15

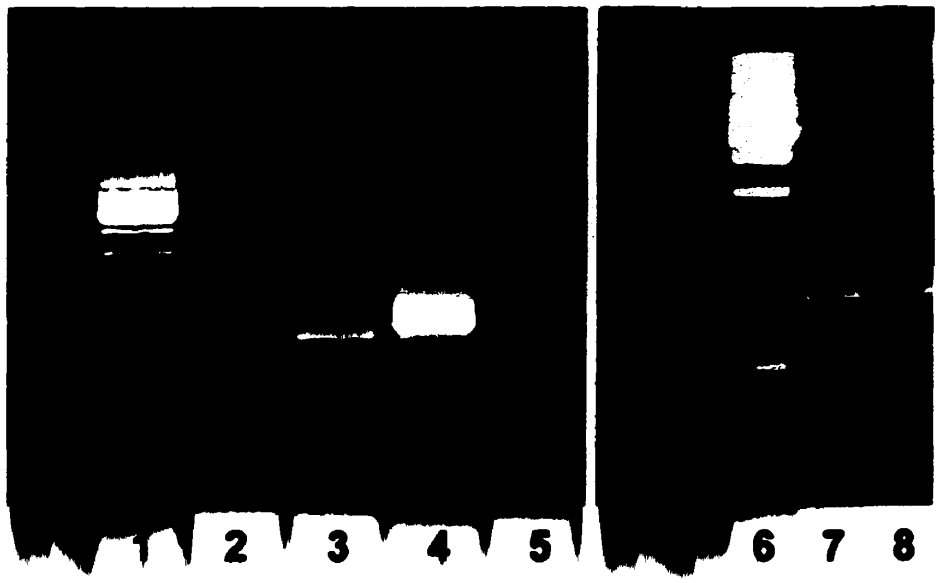


Figure 15. Amplification of CAR mRNA by RT-PCR. PCR products were electrophoresed on 1.5% EtBr agarose gels. Lanes 1 and 6 contain the DNA marker GeneRuler 1Kb (MBI Fermentas). The lowest 5 bands shown denote DNA sizes of 500, 750, 1000, 1500, and 2000 base pairs, as per the manufacturers specifications. Lanes 2-5, 7, 8 contain a 10 µl aliquot of each PCR reaction mix. Lane 2, HeLa cells; Lane 3, Vero cells; Lane 4, CHOP cells transfected with CAR cDNA; Lane 5, control CHO cells; Lane 7, porcine ST cells; Lane 8, porcine PK-15 cells. The major band at approximately 1.1 Kb (arrow) is the anticipated size of the amplicon containing the coding region of CAR.

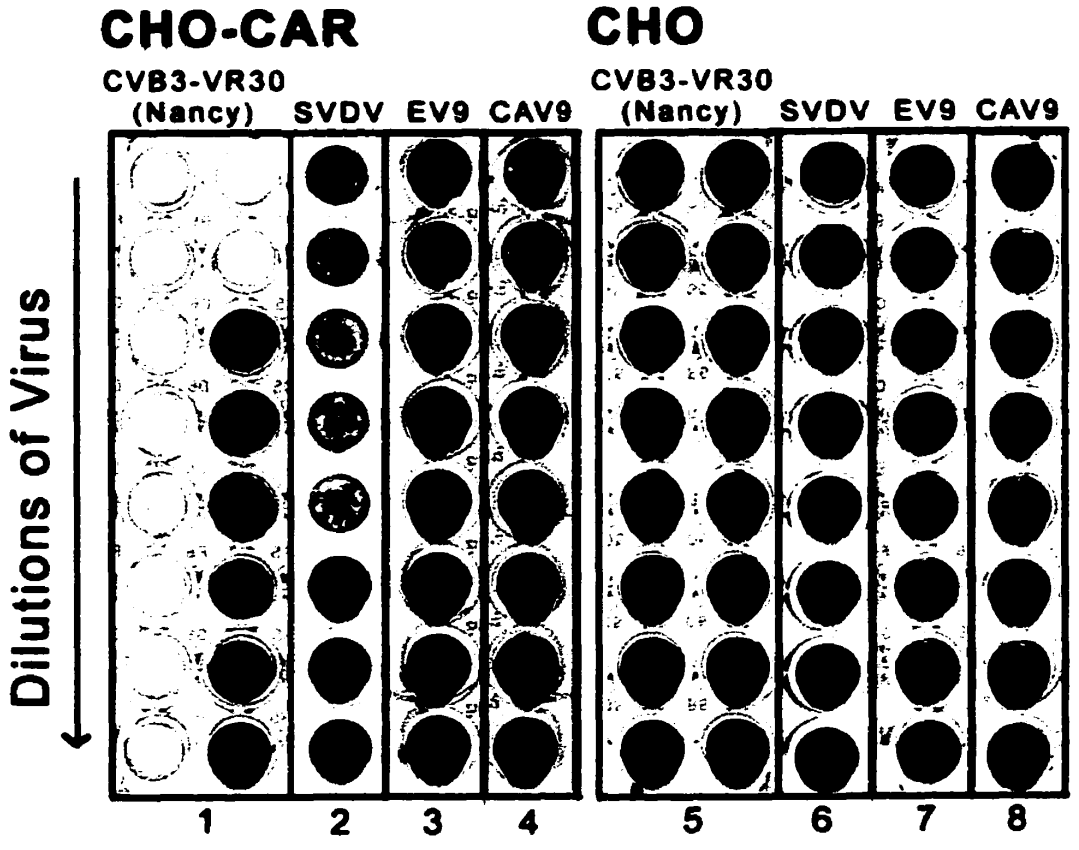


Figure 16. Virus-induced cytopathic effect on CAR positive cells. Stable CHO-CAR transfectants or CHO-control cells plated in 96-well Costar dishes at a density of 6 x 10^4 cells/well were incubated with 50 µl of serial 2-fold dilution's of each test virus (10-fold dilution's for SVDV), at room temperature for 1 h. Monolayers were washed, and growth medium was added for 3 days. To monitor for CPE, cell monolayers were fixed in 2% formalin and stained with a 2% formaldehyde / 0.1% crystal violet dye solution. Panels 1,5 - CVB3-VR30 (Nancy); panels 2,6 - SVDV; panels 3,7 - EV9; panels 4,8 - CAV9.

TABLE 8
Cytopathic effect in designated cell lines[a]

Virus	Cell line		
	HeLa	CHO-CAR	[b]CHO
[c]Reference strains			
CVB1 (Conn-5)	+	+	-
CVB2 (Ohio)	+	+	-
CVB3 (VR30-Nancy)	+	+	-
*CVB3-N (RLC)	+	+	-
CVB4 (Benschoten)	+	+	-
CVB5 (Faulkner)	+	+	-
CVB6 (Schmitt)	+	+	-
[d]Cardiovirulent CVB3			
CG	+	+	-
SH	+	+	-
Ø	+	+	-
N	+	+	-
NR	+	+	-
20	+	+	-
Clinical isolates			
‡CVB1 (5010107)	+	+	-
‡CVB1 (5010607)	+	+	-
*CVB1 (86-2299)	+	+	-
*CVB1 (94-0503)	+	+	-
+CVB1 (95-0004)	+	+	-
‡CVB2 (5010159)	+	+	-
*CVB2 (90-1376)	+	+	-
*CVB2 (90-1445)	+	+	-
‡CVB3 (4012530)	+	+	-
‡CVB3 (19141255)	+	+	-
‡CVB3 (19281109)	+	+	-
‡CVB3-R (20270361)	+	+	-

‡CVB3 (20310308)	+	+	-
*CVB3 (86-1799)	+	+	-
*CVB3 (86-1990)	+	+	-
*CVB3 (86-2327)	+	+	-
*CVB3 (86-2424)	+	+	-
*CVB3 (86-2751)	+	+	-
+CVB3 (95-003)	+	+	-
‡CVB4 (5010936)	+	+	-
*CVB4 (87-1026)	+	+	-
*CVB4 (88-0658)	+	+	-
*CVB4 (88-0842)	+	+	-
*CVB5 (88-0578)	+	+	-
*CVB5 (88-0870)	+	+	-
*CVB5 (88-0973)	+	+	-
*CVB5 (89-0895)	+	+	-
+CVB5 (89-0516)	+	+	-
+CVB6 (95-0005)	+	+	-
+CVB6 (95-0006)	+	+	-
‡CAV9 (4012720)	-	-	-
‡EV9 (22030957)	-	-	-
Swine vesicular disease virus			
SVDV UK 27/72	+	+	-

[a]Experiments were performed as described in Fig. 2.

[b]Enterovirus specimens maintained in the laboratory of P.P.L. and SVDV were tested on CHO-control cells transfected with empty pcDNA3.1 vector. CVBs maintained in the laboratory of J.F.M. were tested on CHO-control cells transfected with human integrin α2 subunit.

[c]Reference strains obtained directly from ATCC by T.A.M. unless otherwise noted.

[d]CVB3 that are cardiovirulent in mice, as described in Martino et al., 1998.

‡Specimens isolated at the Hospital for Sick Children, Toronto, Canada.

*Specimens described in Bergelson *et al.*, 1997b, maintained in the laboratory of J.F.M.

+Additional specimens from the laboratory of J.F.M.

shown). In contrast, none of the CVBs caused CPE in CHO-control cells lacking the CAR receptor molecule. The control enteroviruses, coxsackievirus A9 and echovirus 9, did not cause CPE on any cell line tested (Table 8 and Fig. 16).

iii). CVB1-6 reference strains, wild-type clinical isolates and laboratory variants produce progeny virus in transiently transfected CHOP-CAR cells. The yield of reference CVB1-6 strains was 3-6 $\log_{10}$ greater in transiently transfected CHOP-CAR cells, as compared to control CHOP cells transfected with partial CAR sequence cDNA or to mock transfected cells (Fig. 17a). Similarly, the yield of all 9 clinical CVB isolates was 2-3 $\log_{10}$ greater in cells expressing the entire CAR protein, as compared to controls (Fig. 17b). Finally, the yield of the six CVB3 strains known to be cardiovirulent in mice was 2-6 $\log_{10}$ greater in cells expressing full-length CAR, as compared to control cells (Fig. 17c). These results indicate that all CVB1-6 strains tested use CAR as a functional cellular receptor.

Small amounts of virus could be detected at 24 hours in control cell cultures infected with CVB clinical isolates and cardiovirulent variants CVB3-N, CVB3-NR, and CVB3-20 (Fig. 17b,c). To confirm if this was due to virus replication in control cells, virus titers at 24 hours and 1 hour post infection were compared. Virus titers were generally unchanged or lower at 24 hours as compared to those detected immediately after virus adsorption, with the exception of a few clinical isolates (Fig. 17b,c). However, even for these isolates, virus titer changes were much lower than when the cells were expressing the CAR receptor. Moreover, CPE was never detected in control CHO cell monolayers for any of the virus strains tested (Table 8). Based on these observations, it is hypothesized that the low virus titers in control cultures at 24 hours post infection most likely reflects residual input virus from the inoculum. Since the cell monolayers were washed 3 times, it is unclear as to whether further washing would help to decrease the residual virus levels. It is tempting to speculate that they may even remain constant,

Figure 17A

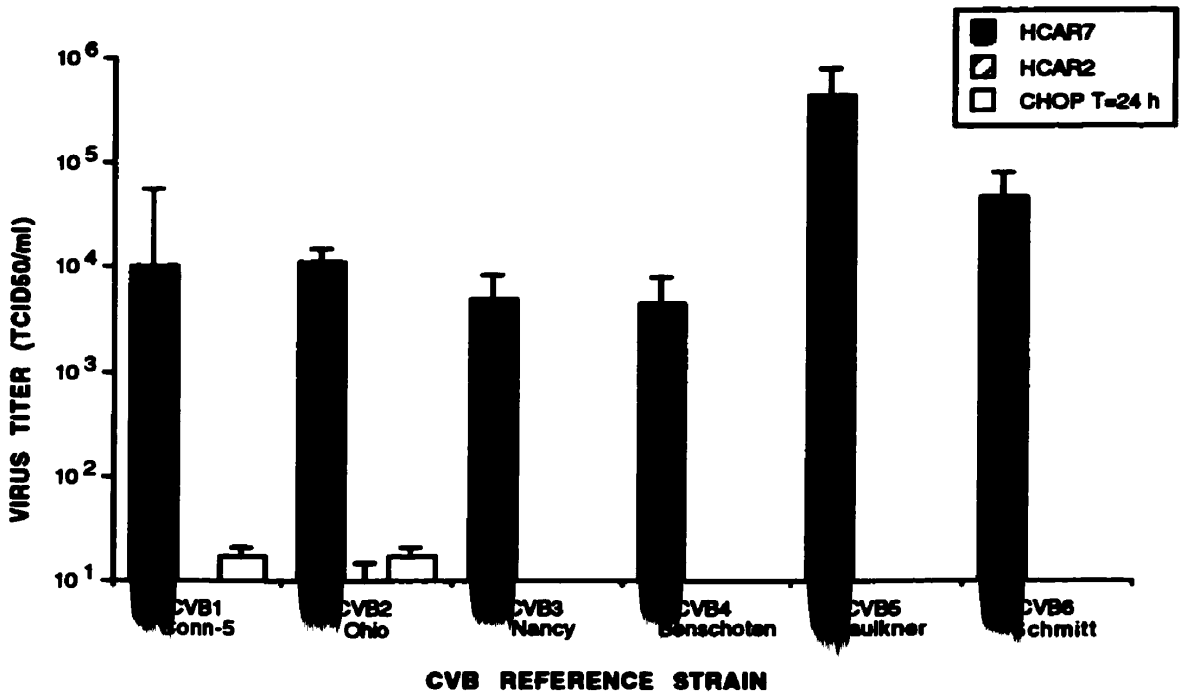


Figure 17. CVB1-6 reference strains, clinical isolates, and cardiovirulent variants replicate in CAR transfected cells. CHOP cells plated on 24-well Costar dishes at a density of 2.5 x 10^5 cells/well were transfected with 1.6 µg/well of CAR cDNA (or controls) using 2.5 µl/well of Lipofectamine reagent for 5 h., washed and incubated overnight in growth medium. CAR transfected cells were inoculated with each test virus at an MOI of 5 PFU/cell, at room temperature for 1 h. Monolayers were washed, and cells were further incubated for 0 h (to monitor for residual virus in the inoculum) or 24 h at 37^0C (to allow for progeny virus production). Dishes containing cells and progeny virus were frozen and thawed, and progeny virus titer was monitored by $TCID_{50}$ assay on HeLa cells. HCAR7, full length CAR cDNA clone. HCAR2, cDNA control coding for AA 1-189 of CAR. CHOP T=0 h or T=24 h, mock transfected cells inoculated with virus and incubated for 0 or 24 hours, respectively. Experiments were performed in triplicate. (A) Progeny production by CVb1-6 reference strains. (B) Progeny production by CVB clinical isolates. (C) Progeny production by cardiovirulent CVB3.

Figure 17B

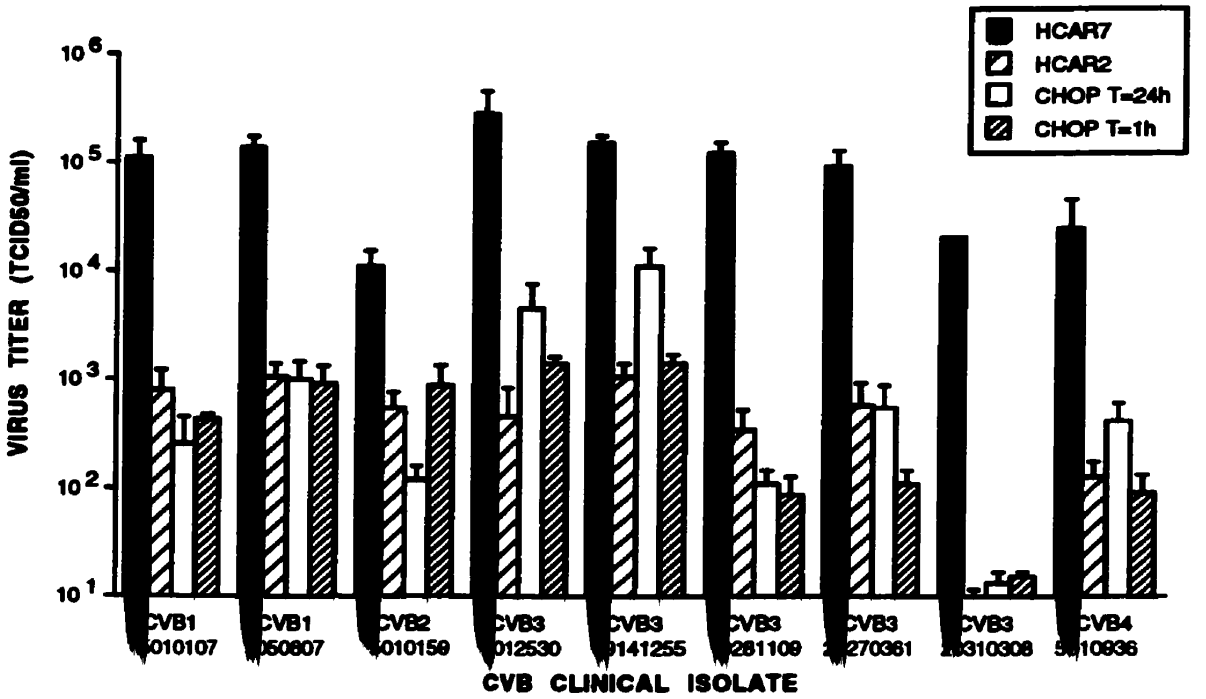
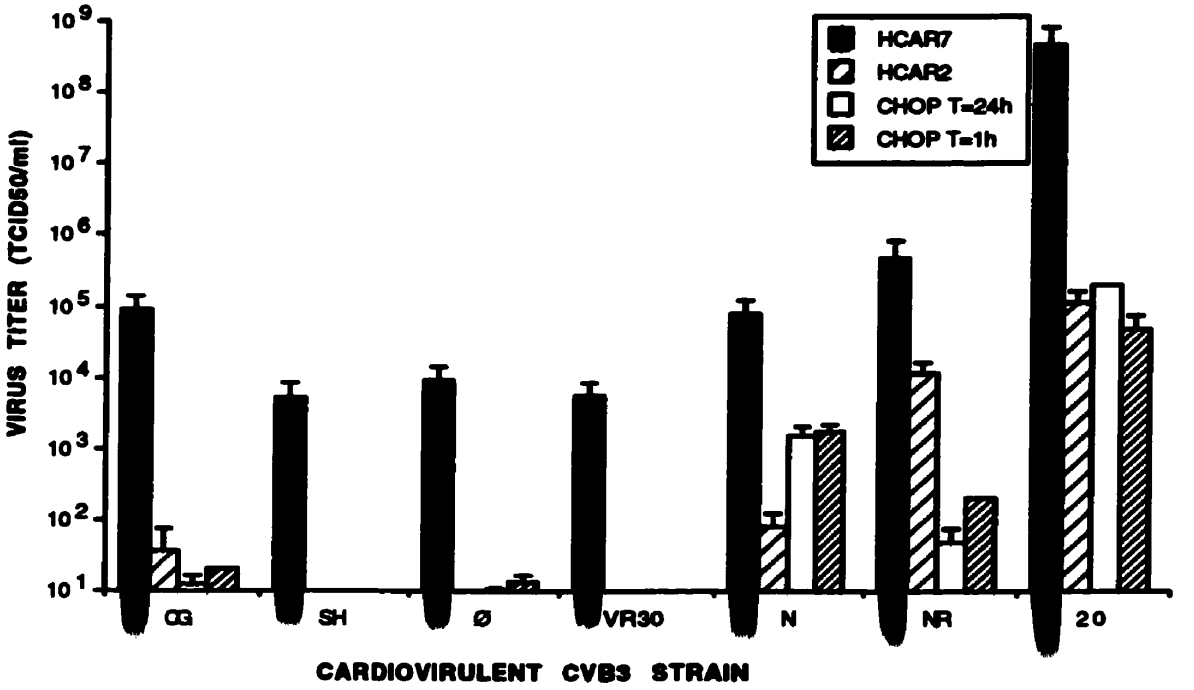

HCAR7
HCAR2
CHOP T=24h
CHOP T=1h
VIRUS TITER (TCID50/ml)
10^6
10^5
10^4
10^3
10^2
10^1
CVB1
CVB1
CVB2
CVB3
CVB3
CVB3
CVB3
CVB3
CVB4
CVB CLINICAL ISOLATE

Figure 17C


HCAR7
HCAR2
CHOP T=24h
CHOP T=1h
VIRUS TITER (TCID50/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
CG
SH
Ø
VR30
N
NR
20
CARDIOVIRULENT CVB3 STRAIN

because of stable interactions between some virus strains and the CHO cell surface. A report of a putative molecule on CHO cells which interacts with the cardiovirulent CVB3-N strain but does not allow for productive lytic infection provides some support for this hypothesis (Kramer et al., 1997).

iv). SVDV utilization of CAR and DAF receptors. Strong antigenic and phylogenetic relationships between CVB and swine vesicular disease virus (SVDV) raised the question of whether CAR can also serve as a functional receptor for SVDV. As shown in Table 8 and Fig. 16, SVDV infection produced cytopathic effects on monolayers of stably transfected CHO-CAR cells, and on monolayers of HeLa cells. In addition, SVDV produced CPE on monolayers of porcine ST and PK-15 cells. A CAR homologue was amplified from these cells by PCR (Fig. 15). In contrast, SVDV did not cause CPE in the receptor-negative CHO-control cells (Table 8 and Fig. 16). Anti-CAR MAb RmcB decreased SVDV plaque formation by approximately 75% in stably transfected CHO-CAR cells, and in HeLa cells (Table 9 and Fig. 18). In contrast, control serum did not decrease SVDV plaque formation (Table 9).

DAF is a co-receptor molecule used by some CVB strains (Bergelson *et al.*, 1995; Martino *et al.*, 1998; Shafren *et al.*, 1995). Previous studies have shown that HeLa cells express DAF at the cell surface and that anti-DAF antibodies specifically block CVB interactions with DAF on HeLa cells (Bergelson *et al.*, 1995, 1997b; Martino *et al.*, 1998; Crowell *et al.*, 1986; Mohanty and Crowell, 1993; Shafren *et al.*, 1995). Because SVDV, like the CVBs, was able to use the CAR receptor, its ability to also use DAF as a receptor was explored. As shown in Table 9, pretreatment of HeLa cell monolayers with anti-DAF MAb 914 resulted in a reduction of SVDV yield by 75%. The antibody to DAF was therefore as efficient at suppressing the replication of SVDV as antibody to CAR. These findings are consistent with the hypothesis that, as is the case for CVB1,3,5, both DAF and CAR have receptor functions for SVDV.

TABLE 9.

Anti-CAR and anti-DAF MAbs block SVDV infectivity[a]

	% SVDV plaque reduction		
	Anti-CAR	Anti-DAF	Anti-albumin
Cell line	RmcB	MAb 914	(control)
CHO-CAR	72 ± 7	n/a	6 ± 1
HeLa	72 ± 15	75 ± 5	9 ± 7

[a] CHO-CAR or HeLa cells plated in 6-well Costar dishes at a density of 1 x 10^6 cells/well were preincubated with 1 ml of media containing 1:1000 dilution RmcB antibody as described previously (Bergelson *et al.*, 1997), or 50 μg/ml anti-DAF MAb 914 (Martino *et al.*, 1998), or 50 μg/ml of control rabbit anti-bovine albumin antiserum, at room temperature for 1 h. After washing, 1 ml containing ~50 PFU/ml of SVDV was added and cells were incubated at room temperature for 1 h. Monolayers were washed, plaque overlays were added for 2-3 days, and the plaques were counted. All experiments were performed in triplicate.

Figure 18

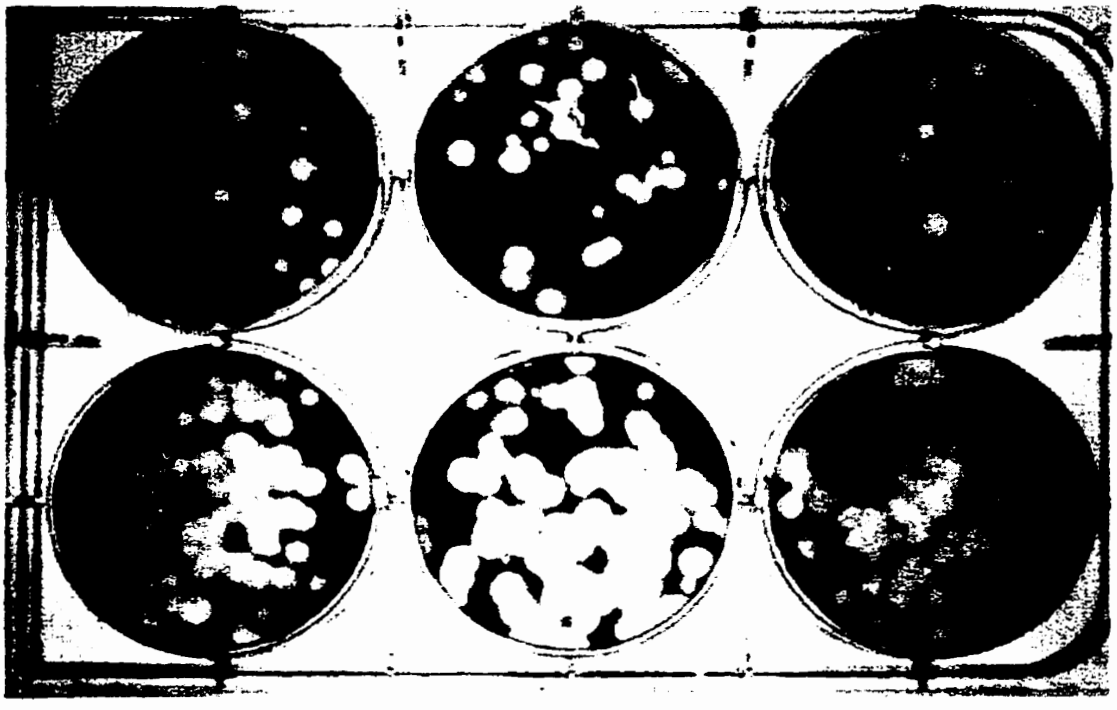


Figure 18. Inhibition of SVDV plaque formation on CHO-CAR cells pretreated with anti-CAR MAb RmcB. CHO-CAR cells plated in 6-well Costar dishes at a density of 1 x 10^6 cells/well were preincubated with 1 ml media containing a 1:1000 dilution of RmcB ascites (or controls) at room temperature for 1 h. After washing, 1 ml containing ~ 50 PFU/ml of SVDV was added at room temperature for 1 h. The monolayers were washed, plaque overlays were added for 36 h., then cells were stained with crystal violet dye solution. Upper wells, plaques on CHO-CAR cell monolayers pretreated with anti-CAR MAb RmcB, then infected with 50 PFU SVDV. Lower wells, plaques on untreated CHO-CAR monolayers infected with 50 PFU SVDV only.

v). Soluble CAR blocks CVB3 and SVDV infection of HeLa cells. To examine whether CAR alone can block virus infection of cells, a soluble form of the CAR molecule (sCAR) was produced, which was shown to be a specific inhibitor of CVB and SVDV infection. sCAR lacking the transmembrane and cytoplasmic domains was produced by fusing DNA encoding the extracellular portion of CAR to DNA encoding the Fc region of rabbit immunoglobulin. The DNA was inserted into a eukaryotic expression vector and transiently transfected into 293 cells. sCAR secreted into the supernatant was purified by chromatography. The ability of sCAR to inhibit CVB and SVDV infection of cells was examined using a quantitative *in vitro* assay for virus cytopathic effect (CPE). sCAR was preincubated with virus, and then the mixture was added to HeLa cell monolayers. As shown in Figure 19, sCAR blocked CVB3 and SVDV induced CPE on the HeLa cell monolayers, in a dose dependent manner. In contrast, a control immunoadhesin consisting of avian leukosis virus envelope glycoprotein fused to rabbit Fc (ALV-env) did not block virus infection of the cells.

Figure 19

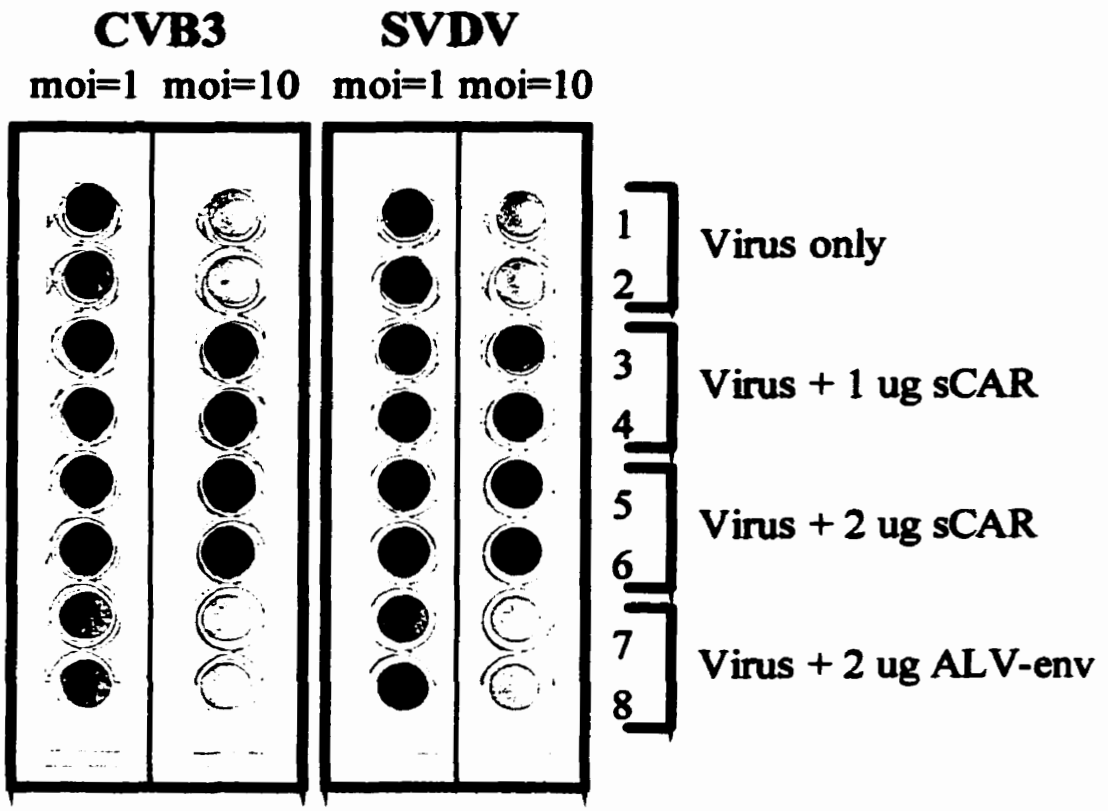


Figure 19. Decrease of CVB3 and SVDV induced cytopathic effect on CAR-positive HeLa cells by soluble CAR (sCAR). HeLa cells were plated in 96 well dishes at a density of 3 x 10^4 cells/well and incubated overnight under standard culture conditions. CVB3 (strain Nancy from Dr. Richard Crowell, as described in Bergelson *et al.*, 1995), or SVDV was diluted in α-MEM medium to 1 moi or 10 moi, and added to 1μg or 2 μgs of sCAR (or ALV-env as control), and samples were incubated for at 37^{0}C for 1 h. Medium from the cell wells was removed, and replaced with the mixtures of virus and sCAR protein (or controls), in duplicate wells. CPE was monitored after 24 hours by fixing cell monolayers with crystal violet dye solution.

G.6. Discussion

In these studies, evidence is provided that reference strains and clinical isolates representing all six serotypes of group B coxsackieviruses (CVB) interact with the coxsackievirus adenovirus receptor (CAR) during infection. All CVB strains were capable of replicating and causing CPE in hamster cells expressing CAR, but not CAR negative control cells. In contrast, other enteroviruses such as coxsackievirus A9 and echovirus 9 did not infect CAR expressing cells. These findings are consistent with the hypothesis that all CVB use a common receptor molecule (Bergelson *et al.*, 1997a; Hsu *et al.*, 1988; Lonberg-Holm *et al.*, 1976). Moreover, use of CAR by CVB clinical isolates strongly suggests that CAR may be a CVB receptor *in vivo*. Virus strains isolated by only one passage through primary GMK cells were capable of utilizing CAR, as well as virus strains which had been maintained for many years in cell culture.

In these experiments, CAR was shown to be a functional receptor for all CVB strains tested. In a previous study using a panel of CVB clinical and prototype isolates (Bergelson *et al.*, 1997b), it was found that infection by many - but not by all - isolates was inhibited by the anti-CAR MAb RmcB. Eighteen of these isolates were subsequently shown to infect stably transfected CHO-CAR cells and CHO-control cells, and all were found to use CAR as a receptor. Why some of these viruses are not inhibited by RmcB is not certain. It is possible that they interact with CAR in a way that cannot be inhibited sterically by RmcB; it is also possible that they make use of additional receptor molecules. Co-receptors or accessory molecules for CVBs which have been identified to date include decay accelerating factor (Bergelson *et al.*, 1995; Shafren *et al.*, 1995) and the integrin αvβ6 (Agrez *et al.*, 1997). Two additional putative receptors are a 100 kDa nucleolin-like membrane protein (Raab de Verdugo et al., 1995), and a hamster cell binding molecule (Kramer et al., 1997), although a role for these molecules in CVB infections in humans is not known.

Previous studies have indicated that SVDV is antigenically and phylogenetically related to CVB, and may have arisen within the past century from a single transfer of CVB5 into pigs (Graves, 1973; Zhang *et al.*, 1993, 1999). These observations prompted an examination as to whether SVDV could use the CVB receptor CAR. It was shown that SVDV could replicate and cause CPE in hamster cells expressing CAR, but not mock transfected cells lacking CAR. Moreover, pretreatment of CAR positive cells with anti-CAR monoclonal antibody RmcB blocked virus infection in plaque assays. These observations indicate that CAR can serve as a functional receptor for SVDV, a characteristic shared only with CVBs, to date. It is tempting to speculate that the ability of SVDV to use CAR is an inherent characteristic from the evolutionary past which may have facilitated virus entry into porcine hosts. Indeed, when two porcine cell lines commonly used in SVDV propagation were examined by PCR, they both were found to express CAR RNA. Porcine CAR transcripts have also been detected recently in pig liver tissue (Fechner *et al.*, 1999). Porcine CAR seems a likely candidate receptor for SVDV in pigs, and future transfection studies with this molecule are indicated to resolve this concept. Moreover, interactions of CVBs with porcine CAR homologue could provide further insight into receptor interactions with these viruses. This may also be of some concern in xenotransplantation of porcine organs.

These studies provide evidence that SVDV also uses the CVB receptor molecule DAF. It was found that pretreatment of DAF positive HeLa cells with anti-DAF monoclonal antibody decreased SVDV infection by 75% in plaque assays, suggesting that DAF may be a co-receptor molecule. Binding studies such as those done with CVB (Bergelson *et al.*, 1995; Martino *et al.*, 1998; Shafren *et al.*, 1995) would help define the nature of this receptor interaction, and confirm that there are specific interactions and that the blocking is not just due to steric hindrance. DAF receptor usage may also be a characteristic retained from the evolution of CVB5 into SVDV, although it is not known whether a homologue of human DAF is expressed in pigs. The SVDV UK/27/72 strain used for this study was collected in 1972, and was one of the first isolates reported. It

would be interesting to determine whether DAF receptor usage is also retained in more recent SVDV strains.

These experiments confirm that all CVBs share a common host cell receptor. The fact that clinical isolates use CAR as a receptor suggest that CAR may function as a receptor in the host, and that it may play an important role in the pathogenesis of coxsackievirus disease. The role in pathogenesis of possible co-receptors such as DAF remains poorly understood, and the relation between virus tropism and tissue-specific receptor expression remains to be defined. Since it appears that CAR is the major receptor for CVB, structural studies of virus-CAR interaction could be important in the development of new antiviral agents. Also, soluble CAR decreases virus infection of host cells, possibly opening new avenues for treating CVB and SVDV-induced disease.

H. STUDY #3

THE CVB3 VIRION RECEPTOR BINDING SITES

Purpose:

3A). To identify sites on the CVB3 virion which interact with virus receptors.

3B). To establish a model system which can be used to confirm these interactions.

Based on the publications -

Martino, T., K. Aitken, L. Chow, C. Gauntt, L. Bartosik, A. Bugeja, L. Weinrib, J. Ko, J. Martino, M. Shin, M. Sole, M. Petric, and P. Liu. (1997). Identification of capsid mutations in common myocarditic strains of coxsackievirus B3 by nucleotide sequencing; Implications for virus -receptor, -immune, and -autoimmune interactions. *Keystone Symposia.*

Martino, T. A., R. Tellier, M. Petric, D. M. Irwin, A. Afshar, and P. P. Liu. (1999). The complete consensus sequence of coxsackievirus B6 and generation of infectious clones by long RT-PCR. *Virus Research* **64**, 77-86.

Figures 21, 25, and 26 by T. A. Martino. Figures 23, 24 and long RT-PCR by Dr. Raymond Tellier. Figures 22, 27 by Dr. David Irwin. Thanks to Dr. Charles Gauntt at the University of Texas and Dr. Jodi Muckelbauer at Purdue University for the amino acid map of the CVB3-CG protomer unit (Figure 20). Also, thanks to Barbara Kellam and the summer students Luke Bartosik, Anne Bugeja, Laura Weinrib, James Ko, Jessica Martino, and Michael Shin for their help with sequencing.

H.1. Experimental Overview

In the first part of this study, the CVB3 variants from studies #1 and #2 were examined for the sites on the virion that may be important for DAF receptor binding. Because only some of the variants use DAF as a receptor molecule, the nucleic acid sequence which corresponded to the receptor binding capsid regions was determined to identify sites that differed between the DAF binding and non DAF-binding virus variants. In the second part of this study, a model system is described which can be applied to confirm that the identified sites are important for virus-DAF binding. Techniques were used which would allow for rapid cloning of the full-length genome of all the CVB3 variants, for use in further studies. As an example, the technical feasibility of such a study was demonstrated using only one virus genome, namely coxsackievirus B6 (CVB6). CVB6 was chosen because of the opportunity to clone and sequence the only remaining uncharacterized CVB serotype. This model system was successful in that full length infectious clones of virus were rapidly and reliably generated.

STUDY 3A. IDENTIFICATION OF CAPSID MUTATIONS IN CARDIOVIRULENT CVB3

H.2. Abstract

The CVB3-CG, -SH, -Ø, -RD, -VR30, -N, -NR, and -20 virus variants all use the coxsackie-adenovirus receptor (CAR) to infect susceptible cells. However, they differ in their ability to use the decay accelerating factor (DAF) receptor. Since CVBs bind to receptor molecules via their coat proteins, a physical basis for the receptor binding differences were determined by analyzing the coat proteins of each of the virus variants. To do this, the coat protein (VP1-4) region of the genome for the virus variants was sequenced, and the infered amino acid sequences were compared. A total of eighteen differences in amino acid residues were found. There were six changes in the VP1 coat protein region, five in the VP2 region, six in the VP3 region, and one in VP4 region. Six of the differing residues mapped to the virion surface. Four of the residues were located at or near the canyon region of the virion, a major surface depression predicted to be a primary receptor binding site. Two sites were located on the "VP2 puff" region and one on the "VP3 knob", which are major surface protrusions containing immunogenic domains. Phylogenetic analysis of the capsid regions of the genomes yielded 2 groupings, consistent with the DAF receptor binding profiles of these viruses.

H.3. Introduction

Group B coxsackieviruses (CVB) serotypes 1-6 are members of the enterovirus group, and the family *Picornaviridae* (reviewed in Melnick, 1990). CVB virions contain a single strand of positive sense RNA ~ 7.5 Kb in length. It is organized into 5'-end and 3'-end nontranslated regions, and a large open reading frame encoding structural and nonstructural gene products which are posttranslationally cleaved to yield the virus proteins. The virion capsid is encoded by genes in the P1 region of the genome (see Fig. 1). The capsid is an icosahedral shaped particle ~30 nm in size, and is comprised of the external coat proteins VP1, VP2, and VP3, and the internal coat protein VP4.

Structural features of the CVB capsid are believed to be important for virus virulence and infectivity. For example, protrusions out from the virion surface contain immunogenic sites which are important for virus neutralization, and for eliciting immune and autoimmune reactions in hosts (Gauntt 1997; Knowlton et al., 1996; Lee et al., 1997a). Deep depressions in the virion surface are hypothesized to contain sites for virus binding to cell surface receptors, for initiation of virus entry into susceptible host cells (see sections A, E, and Muckelbauer et al., 1995; Muckelbauer and Rossmann, 1997).

To date, two CVB receptors have been identified and well characterized. They are the coxsackie-adenovirus receptor (Bergelson et al., 1997a; Carson et al., 1997; Tomko et al., 1997) and the decay accelerating factor (DAF) receptor (Bergelson et al., 1995; Shafren et al., 1995). All CVBs use CAR to infect susceptible host cells (Study #2 and Martino et al., 2000), however, only some virus variants use DAF as a receptor molecule (Study #1 and Bergelson et al., 1995, 1997b; Lindberg et al., 1992; Martino et al., 1998; Mohanty and Crowell, 1993; Reagan et al., 1984; Shafren et al., 1995).

In this study, it was postulated that virus capsid residues important for binding to DAF could be identified by comparing the capsid proteins of DAF-binding and non DAF-

binding CVB3 variants. The receptor binding capsid region was sequenced for eight CVB3 variants with known receptor binding phenotypes. The CVB3 variants termed CVB3-CG, CVB3-SH, CVB3-Ø, and CVB3-RD use the DAF receptor, while the CVB3-VR30, CVB3-N, CVB3-NR, and CVB3-20 variants do not. Multiple alignments and phylogenetic analyses were performed to identify differences in the VP1-4 capsid sequences obtained. Since the 3-D structure of CVB3 has been solved (Muckelbauer et al., 1995; Figure 20), it was possible to map the amino acid differences onto the virion. In the following study (study 3B), an approach was developed which could allow for a validation that these amino acid differences are important.

Figure 20

Accessibility of CVB3 residues.

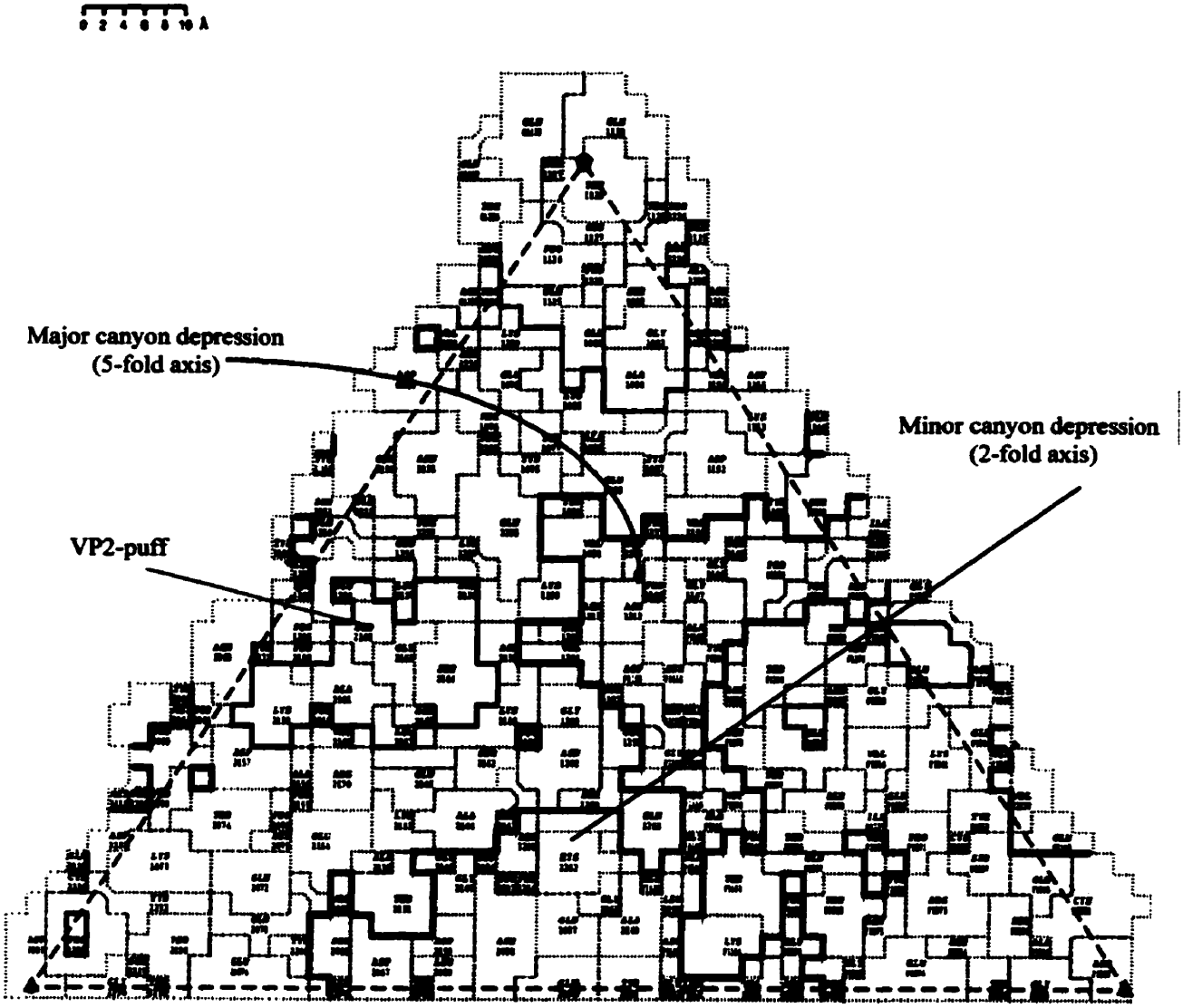


Figure 20. The protomer map of CVB3. Amino acids in the viral coat proteins VP1, VP2, and VP3 found on the surface of the CVB3 virion. Residues in the canyon depressions at the 5-fold and 2-fold axes of symmetry, and external on the "VP2-puff" region, are also denoted. Based on the x-ray crystallography analysis of CVB3-CG (Muckelbauer et al., 1995).

H.4. Materials and Methods

I). PCR amplification of CVB3 RNA. The CVB3 virus variants - CG, -SH, -Ø, -VR30, -N, -NR, and -20 were maintained as described in study #1. Viral RNA was purified from 10^8 PFU of virus using TRIzol reagent, according to the manufacturer's directions (Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada). First strand cDNA synthesis of the viral genome was performed using the techniques described in study #2, except that the primer used was oligo dT (Pharmacia Biotech Inc., Baie d'Urfe, Quebec, Canada). For PCR, a 4 µl aliquot of this reaction mixture was added to a 46 µl volume containing 1 x reaction buffer (Pharmacia), 0.2 mM each of dNTP (Pharmacia) and 2 µM each of upstream and downstream primers as described below. The preparation was denatured for 10 minutes at 94^0C, then 2.5 U of Taq polymerase (Pharmacia) and 50 µl of light mineral oil were added. PCR was performed on a DNA Thermal Cycler (Perkin Elmer Cetus) for 39 cycles of annealing 55^0C, 2 min; extension 72^0C, 3 min; denaturation 94^0C, 1 min.

II). Primers. The primers used to amplify CVB3 cDNA span across the capsid region of the virus genome. When paired, the primers were designed to amplify an approximately 500 bp segment, with a 250 bp overlap between segments. The primers were designated as follows;

TM5-5'-TGGATTGGCCATCCGGTG-3'	TM6-5'-GCAAGTTCACAGAACCAG-3'
TM7-5'-CTGGCCATACTCCATAGC-3'	TM8-5'-GCTATGGAGTATGGCCAG-3'
TM9-5'-ACTCTTTTGCCGTATCGC-3'	TM10-5'-GCGATACGGCAAAAGAGT-3'
TM11-5'-ACCCAGGGCAGTAATCTA-3'	TM12-5'-TAGATTACTGCCCTGGGT-3'
TM13-5'-TGGGACAACTGAGTCAAC-3'	TM14-5'-GTTGACTCAGTTGTCCCA-3'
TM15-5'-AGCACCTAGTGGTGAGTA-3'	TM16-5'-TACTCACCACTAGGTGCT-3'
TM17-5'-CCTTCAATAGCCTGACAG-3'	TM18-5'-CTGTCAGGCTATTGAAGG-3'
TM19-5'-CGCATGCTGACCTACATA-3'	TM20-5'-TATGTAGGTCAGCATGCG-3'
TM21-5'-TATCTGGTACAGGTCCAC-3'	TM22-5'-GTGGACCTGTACCAGATA-3'
TM23-5'-GTAGATTCTAATGGTGCT-3'	TM24-5'-AGGTCTCTGTTGTAACTT-3'

iii). Sequences of the CVB3 variants. PCR products were analyzed by electrophoresis on agarose gels stained with ethidium bromide. Bands corresponding to the ~500 bp segments for each virus variant were excised, and the cDNA was purified using Sephaglas reagent (Pharmacia) and subjected to sequencing in both directions using Thermosequenase (Amersham Canada Ltd., Oakville, Canada). The sequence of some of the virus variants has also been published by other groups, either prior to or during these studies. The published sequences CVB3-CG (Lee et al., 1997a), CVB3-SH (Knowlton et al., 1996), CVB3-Ø (Chapman et al., 1994; Tu et al., 1995), CVB3-VR30 (Lindberg et al., 1987), CVB3-N (Kandolf and Hofschneider, 1985; Klump et al., 1990), and CVB3-20 (Chapman et al., 1994; Tu et al., 1995) are identical to the sequences obtained in this study. CVB3-RD was not sequenced in this study, but was also analyzed using previously published data (Lindberg et al., 1992),

iv). Sequence analysis, alignments, and phylogeny. Nucleotide sequences and predicted amino acid sequences were generated and aligned using DNAsis PRO, Version 2.0. Since the structure of the CVB3 capsid has been solved (Muckelbauer et al., 1995), the location of the residues within the tertiary structure of the CVB3 virion could also be determined. A protomer map (Fig. 20) was used to position residues on the virion surface. Phylogenetic relationships between the different CVB3 variants were also examined, using the methods described in the following study.

H.5. Results and Discussion

In this study, the VP1-4 capsid region of the eight CVB3 variants termed CVB3-CG, -SH, -Ø, -RD, -VR30, -N, -NR, and -20 was sequenced and analyzed (Figure 21). The total length of the capsid region of the genome was 841 amino acids for all virus variants. Sequence alignments for all 8 viruses showed very high similarity. However, 18 deduced amino acid differences were noted and these were scattered throughout the capsid proteins. The differences found in each capsid protein are discussed in detail, below.

Six amino acid residues differed in the VP1 protein. The first difference was found at amino acid 23, and only in CVB3-VR30. The threonine residue changed to an asparagine residue. The site is not located within secondary structures, nor is it on the surface of the virion, however it still may be important for determining virus phenotype. For example, it has been shown for polioviruses that mutations at this site suppress temperature sensitivity, and can complement virion assembly defects (Minor et al., 1989). The second difference in the VP1 amino acid sequences for the CVB3 variants was found at residue 45. A glycine residue found in the sequence of most of the variants was changed to a serine for CVB3-CG and -SH. This is an internal site with no notable structural features. The third difference was found at amino acid 80, and it occurred in 4 out of 8 of the virus variants. There is an acidic glutamic acid residue in CVB3-CG, -SH, -Ø, and -VR30, and a basic lysine residue in CVB3-RD, -N, -NR, and -20. This site lies between the βB and βC strands of VP1 and is located on the surface of the virion. Interestingly, mutations at this site have been previously shown to modify CVB3 plaque phenotype (Zhang et al., 1995). The fourth difference occurs at amino acid 92, and is found in the CVB3-CG and -SH viruses. An aliphatic leucine residue changes to a isoleucine residue. This occurs just beyond the βC strand, as the protein

Figure 21

VP1

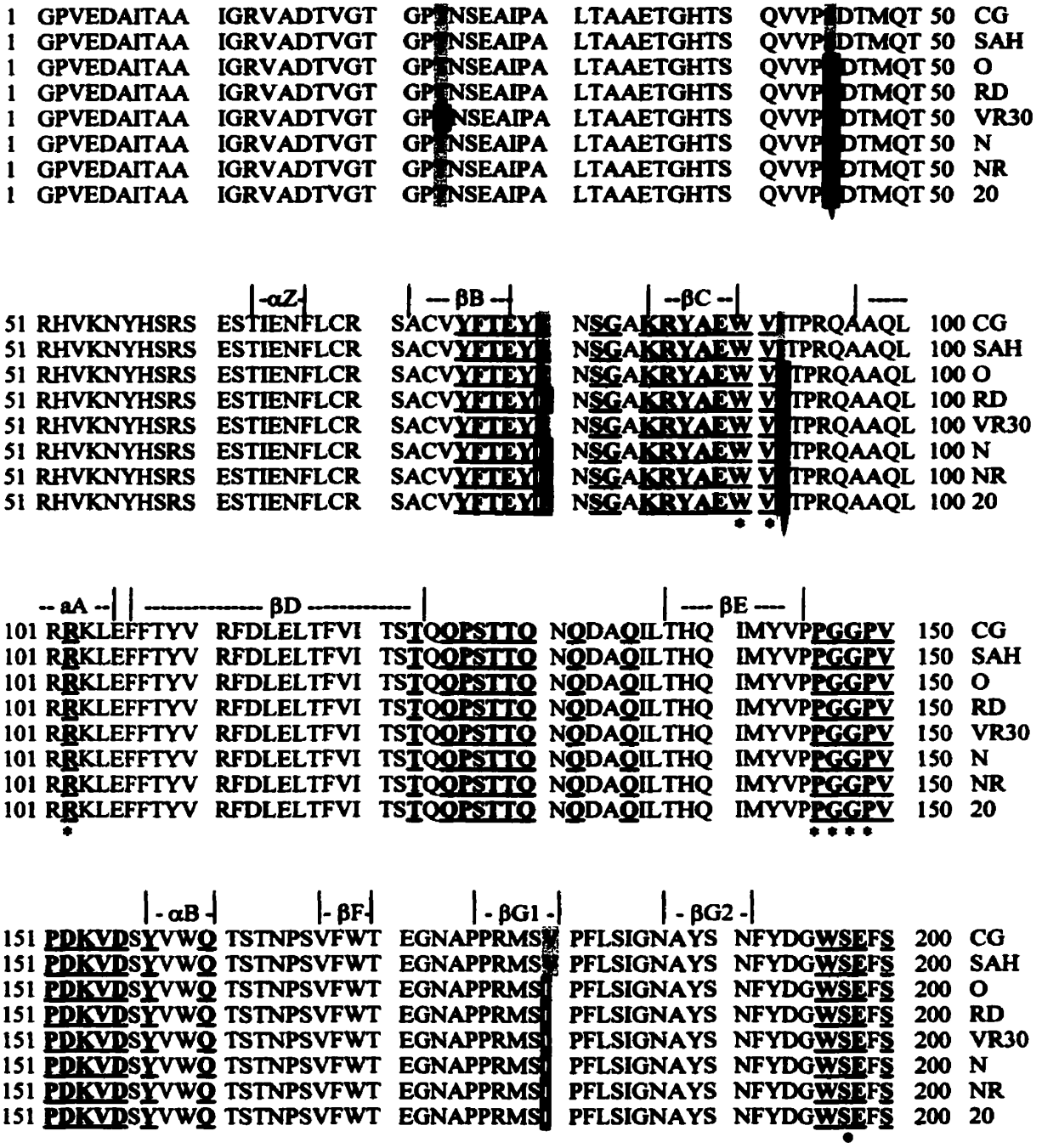


Figure 21. Sequence alignments of the capsid protiens (VP1-4) from CVB3 variants. Residues that differ between the virus variants are highlighted. Amino acids on the surface of the virion are in bold and underlined. • denotes residues in the canyon at the 5-fold axes of symmetry on the virion. + denotes residues in the depression at the 2-fold axes of the CVB3 virion. ^ denotes residues at the summit of the VP2 puff.

VP1 cont.

```
                          |-- βH --|                    |---------- βI ----------|
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 CG
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 SAH
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 O
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 RD
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 VR30
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 N
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 NR
201 RNGVYGINTL NNMGTLYARH VNAGSTGPIK STIRIYFKPK HVKAWIPRPP 250 20
    +   *   *  **   *  *
```

```
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 CG
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 SAH
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 O
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 RD
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 VR30
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 N
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 NR
251 RLCQYEKAKN VNF[redacted]PSGVTT TROSITTMTN T 281 20
     *                             *
```

VP2

```
                          |βA1|  |- βA2 -|
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 CG
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 SAH
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 O
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 RD
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 VR30
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 N
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 NR
1 SPTVEECGYS DR[redacted]RSITLGN STITTQECAN VVVGYGVWPD YLKDSEATAE 50 20
```

```
          |αZ|   |---- βB ---|          |- βC -|          |---- αA ----- |--βD --
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 CG
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 SAH
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 O
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 RD
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 VR30
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 N
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 NR
51 DQPTQPDVAT CRFYTLDSVQ WQKTSPGWWW KLPDALSNLG LFGQNMQYHY 100 20
                    +  +++                +   ++++      +    ++
```

VP2 cont.

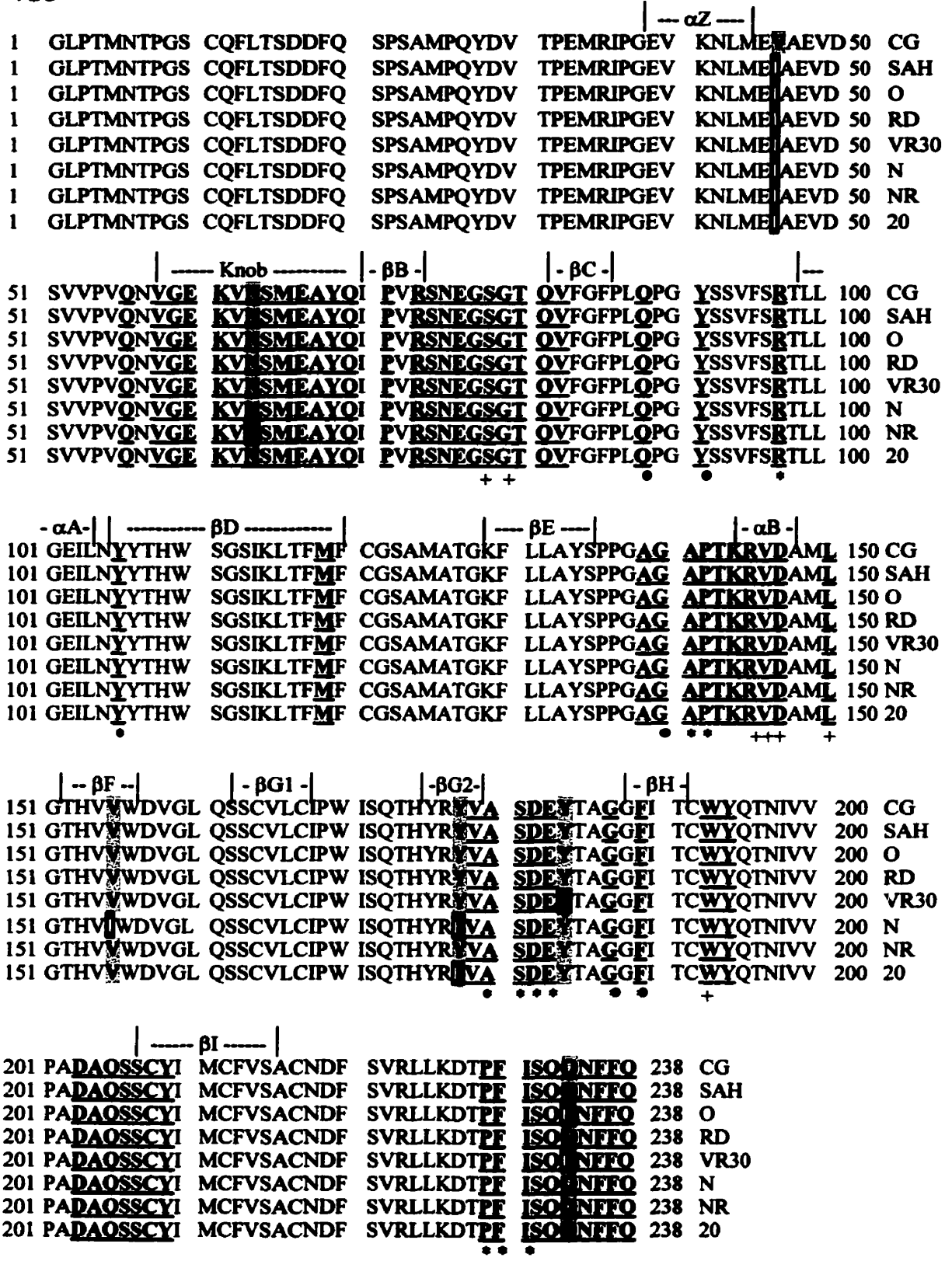
```
      ------ βD --------|        |------ βE ------|------------VP2 PUFF ----------------
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 CG
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 SAH
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 O
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 RD
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 VR30
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 N
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 NR
101 LGRTGYTIHV QCNASKFHQG CLLVVCVPEA EMGCATLINT PSSAELLGGD 150 20
                                                ^          +++

      ----------VP2 PUFF----------|- αB -|| --- βF ---|     |--
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 CG
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 SAH
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 O
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 RD
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 VR30
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 N
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 NR
151 AKEFADKPV ASGSNKLVQR VVYNAGMGVG VGNLTIFPHQ WINLRTNNSA 200 20
          ^    ^^^^^

   -βG1-|       |- βG2 -|      |-------- βH ---------|          |------ βI-----
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 CG
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 SAH
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 O
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 RD
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 VR30
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 N
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 NR
201 TIVMPYTNSV PMDNMFRHNN VTLMVIPFVP LDYCPGSTTY VPITITIAPM 250 20
                  +

   ----- βI -----|
251 CAEYNGLRLA GHQ 263 CG
251 CAEYNGLRLA GHQ 263 SAH
251 CAEYNGLRLA GHQ 263 O
251 CAEYNGLRLA GHQ 263 RD
251 CAEYNGLRLA GHQ 263 VR30
251 CAEYNGLRLA GHQ 263 N
251 CAEYNGLRLA GHQ 263 NR
251 CAEYNGLRLA GHQ 263 20
               ++ +++
```

VP3



VP4

1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	CG
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	SAH
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	O
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	RD
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	VR30
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	N
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	NR
1	MGAQVSTQKT	GAHET[redacted]LNAS	GNSIIHYTNI	NYYKDAASNS	ANRQDFTQDP	50	20

51	GKFTEPVKDI	MIKSLPALN	69	CG
51	GKFTEPVKDI	MIKSLPALN	69	SAH
51	GKFTEPVKDI	MIKSLPALN	69	O
51	GKFTEPVKDI	MIKSLPALN	69	RD
51	GKFTEPVKDI	MIKSLPALN	69	VR30
51	GKFTEPVKDI	MIKSLPALN	69	N
51	GKFTEPVKDI	MIKSLPALN	69	NR
51	GKFTEPVKDI	MIKSLPALN	69	20

folds back beneath the virion surface. For CVB3, mutations near this region modify the hydrophobic binding pocket below the canyon depression (Muckelbauer and Rossman, 1997). Also, mutations in this region for polioviruses lead to attenuation of virus virulence (Girard et al., 1990). A fifth difference between the CVB3 variants was found at amino acid 180, and only in the CVB3-CG and -SH viruses. In this case, the aliphatic isoleucine residue is changed to a valine residue. This occurs at the end of the βG1 strand, near a region that forms part of the hydrophobic binding pocket beneath the canyon floor (Muckelbauer and Rossmann, 1997). The sixth change occurs at residue 264, and is found only in CVB3-20. An acidic glutamine residue is changed to a basic arginine residue. This site is located on the virion surface.

Five amino acid differences were noted in the VP2 protein of the CVB3 variants. At residue 13, a valine is found in CVB3-CG, -SH, and -20, and an alanine in CVB3-Ø, -RD, -VR30, -N, and -NR. This site occurs internally, just before the βA1 strand. Interestingly, amino acid substitutions at this site in poliovirus type 3 suggest that it is important for establishing a persistent virus infection in Hep2c cells, possibly through modifications to the virion structure and changes in poliovirus-receptor interactions (Duncan et al., 1998). The second difference between CVB3 variants occurs at amino acid 108. An aliphatic isoleucine residue is in CVB3-CG, -SH, and -VR30, and a valine residue is in CVB3-Ø, -RD, -N, -NR, -20. This site resides internally, within the βD strand. It has been postulated that amino acid residue 108 is a DAF receptor binding site. This hypothesis is based on the observation that the residue differs between the DAF binding CVB3-RD variant (valine) and a non-DAF binding CVB3-VR30 parental virus strain (isoleucine) (Lindberg et al., 1992). Further support came from studies using chimeric viruses constructed from the CVB3-RD and -VR30 variants (Lindberg et al., 1992). However, findings of sequences reported here were not consistent with this hypothesis, since the valine residue was also found in CVB3-N, -NR, and -20 viruses, even though the viruses do not use DAF. Also, the isoleucine residue was found in CVB3-CG and CVB3-SH, even though these virus strains can use the DAF receptor.

Thus, additional sites are likely required to determine the DAF binding phenotype of CVB3 variants. A third amino acid difference in VP2 was found at amino acid 138. An asparagine residue in CVB3-CG and -SH is an aspartic acid in CVB3-Ø, -RD, -VR30, -N, -NR, and -20. This is a surface residue found at the summit of the "VP2 puff", a region containing receptor recognition sites and neutralizing immunogenic sites (Muckelbauer and Rossman, 1997; Muckelbauer et al., 1995; Wimmer et al., 1993). A fourth change is found at amino acid 151. A serine is present in CVB3-CG, -SH, -Ø, -RD, -NR, and -20, and a threonine is present in CVB3-VR30 and -N. This site is also located on the surface of the VP2 puff region. The serine residue has been postulated to be a DAF receptor binding site, by comparative sequence analyses between CVB3-RD and -VR30, and by the use of chimeric virus constructs (Lindberg et al., 1992). However this hypothesis is not supported by findings reported here, since the serine residue is also found in CVB3-NR and -20 variants, even though they do not use DAF as a receptor. A fifth difference between CVB3 variants in the VP2 protein is found at amino acid 245. An isoleucine residue is present in CVB3-CG and -SH, whereas a valine is found in the remaining virus types. This mutation occurs internally in the βI strand, near the carboxy terminus of the protein.

Six changes were observed in the VP3 protein region of the CVB3 variants. The first difference occurs at amino acid 46. An isoleucine normally found in the VP3 sequence is changed to a valine in CVB3-CG. This occurs internally, just after the αZ helix. The second difference occurs at amino acid 63. An amide asparagine residue found in all the other CVB3 variants is changed to an aromatic tyrosine residue in CVB3-NR. This is a surface residue found in the "VP3 knob", a major surface protrusion which contains neutralizing immunogenic sites (Muckelbauer and Rossmann, 1997; Muckelbauer et. al., 1995; Wimmer et al., 1993). A third change between CVB3 variants is found at amino acid 155. A isoleucine residue is found in CVB3-N, while a valine is found in all the other virus types. This is an internal residue on the βF strand. The fourth difference is at amino acid 178. A phenylalanine is in CVB3-N and CVB3-20,

and a tyrosine is in CVB3-CG, -SH, -Ø-, RD, -VR30, and -NR. This occurs on the βG2 strand just as it approaches the virion surface. A mutation at this site in polioviruses suppress temperature sensitivity and assembly mutations, in a similar manner to the mutation at VP1 residue 23 noted above (Minor et al., 1989). The fifth change occurs at amino acid 184. A cysteine is found in CVB3-VR30, while a tyrosine is found in all the other virus variants. This site occurs internally between the βG2 and βH strands, in a region of residues that make up part of the receptor binding canyon depression. The sixth difference is at amino acid 234. An aromatic phenylalanine residue is found in CVB3-Ø and -VR30, whereas the amide containing glutamine residue is found in the other virus variants. This is a surface residue adjacent to the virion canyon. In studies using polioviruses, this region has been implicated in temperature sensitivity, and is important for proper cleavage of the P1 and VP0 gene products.

One final amino acid difference between the CVB3 variants was found in the VP4 protein. At residue 16, a glutamine is found in CVB3-CG, -SH, -Ø, -RD, and -VR30, and an arginine is found in CVB3-N, -NR, and -20. The VP4 protein is completely internalized within the CVB virion, and it's structure has not been well defined. In studies using poliovirus, VP4 residues have been shown to play a role in virion assembly and in determining plaque phenotype (Wimmer et al., 1993).

Phylogenetic analysis of the entire VP1-4 capsid region for the CVB3 virus variants was performed (Figure 22). The evolutionary tree was constructed using neighbor-joining methods, and rooted to CVB3-VR30. This virus was chosen since it is the prototype virus, and because many of the variants, although not all, were derived from this strain. The analysis yielded two virus groupings, consistent with the DAF receptor binding phenotype of the virus variants. The first group contained the DAF-binding variants CVB3-CG, -SH, -Ø, and -RD. A second group contained the non DAF-binding variants CVB3-NR, -N, and -20.

In summary, amino acid residues in the capsid proteins which differ between CVB3 variants have been identified. Several of these residues are likely important for

interactions between the virion capsid and the DAF receptor molecule. However, further studies using techniques such as cloning and site-directed mutagenesis are likely required to confirm the relevance of these amino acid differences. To facilitate this work a model system has been developed which allows for rapid and reliable cloning of infectious CVB genomes. This is described in more detail in the next study, below.

Figure 22

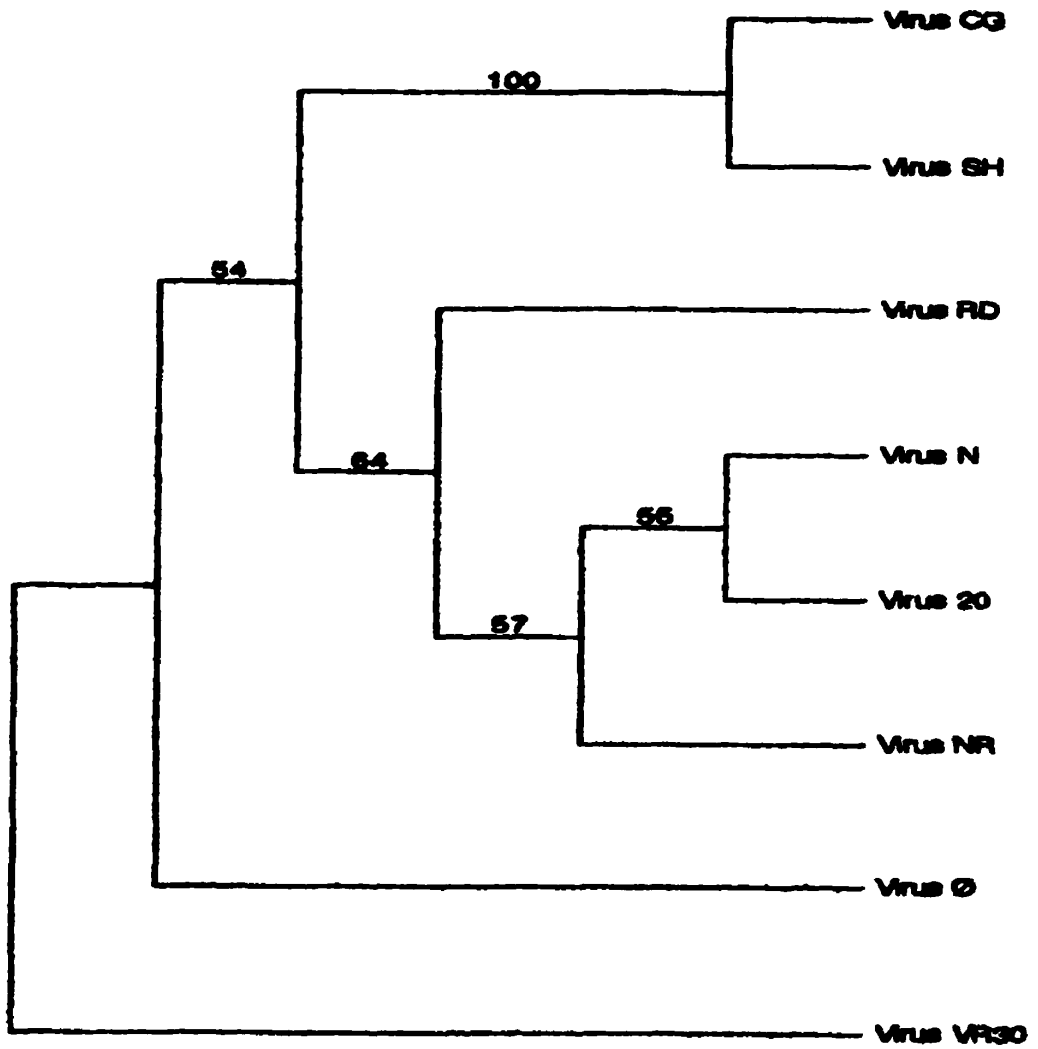


Figure 22. Phylogeny of CVB3 variants. The most parsimonious phylogenetic relationship of the VP1-4 coding region of the CVB3 variants is shown. CVB3-VR30 was used to root the tree since it is the prototype virus strain. Analysis by neighbor-joining distance method and bootstrapping provides ~54% support for these linkages. Branch lengths are proportionate to the percentage of amino acid changes occurring on each lineage.

STUDY 3B.

SEQUENCE OF COXSACKIEVIRUS B6 AND GENERATION OF INFECTIOUS CLONES BY LONG RT-PCR

H.6. Abstract

The full length sequence for the human pathogen coxsackievirus B6 (CVB6, Schmitt strain) has been determined. Long-RT-PCR was used to generate full length DNA amplicon of CVB6, and the amplicons were directly sequenced. One-step cloning of the full-length amplicon generated an infectious clone of CVB6. RNA generated from CVB6 amplicon DNA or CVB6 clones, by transcription with T7 RNA polymerase, was demonstrated to be infectious upon transfection into HeLa cells *in vitro*. The CVB6 genome is characteristic of enteroviruses, with a 5'-nontranslated region (743 nucleotides) followed by an open reading frame (encoding a 2184 amino acid polyprotein) and a 3'-nontranslated region (100 nucleotides) and polyadenylated tail. The predicted amino acid sequence of CVB6 clustered with the other CVB serotypes and swine vesicular disease virus (SVDV).

H.7. Introduction

Coxsackievirus group B (CVB) is etiologically linked with serious human diseases including myocarditis, meningitis, and encephalitis (Grist et. al., 1978; Woodruff 1980; Melnick 1990; Martino et. al., 1994a,b, 1995b; Rotbart 1995). CVB serotypes 1-6 are members of the genus enterovirus within the family *Picornaviridae* (Rueckert 1990). The enterovirus genus also includes the group A coxsackieviruses, echoviruses, polioviruses, and the newer numbered enteroviruses. Sequencing of enterovirus genomes has improved our understanding of the biology of these agents, which impacts on our understanding of their pathogenesis. The enterovirus genome is a single stranded positive sense RNA approximately 7500 bp in length, whose 5'-terminus is covalently linked to VPg and whose 3'-end is polyadenylated. The RNA sequence has untranslated regions at the 5' and 3' ends and contains a single large open reading frame encoding a polyprotein of approximately 250 kDa in size. The polyprotein is postranslationally cleaved to yield the structural coat proteins termed VP1-4, the VPg peptide, and nonstructural proteins including proteases and polymerases.

To date, the entire genetic sequences of CVB1 (Iizuka et. al., 1987), CVB2-Ohio1 (Polacek et. al., 1999), CVB3-Nancy (Lindberg et. al., 1987), CVB4-Benschoten (Jenkins et. al., 1987) and CVB5 (Zhang et. al., 1993) have been reported, but not the full-length genome for CVB6. Many phenotypic CVB variants have also been sequenced in part or in whole. Phylogenetic analyses indicate that most enterovirus genomes are closely related, although several serotypes do not fall within predicted enterovirus groupings and some (such as hepatitis A) have required reclassification within the *Picornaviridae* family (Pöyry et. al., 1996; Oberste et. al., 1998; Oberste et. al., 1999).

In this study, the complete sequence for the reference strain of CVB6 (Schmitt strain) was determined. In addition, phylogenetic analysis for comparison with other

enterovirus sequences was performed. Finally, an infectious clone that will be useful as a reference reagent as well as a tool for mutagenesis studies was constructed.

H.8. Materials and Methods

i). Virus, cells, and antibodies. The reference strain of CVB6 (Schmitt) was obtained from the American Type Culture Collection (ATCC) as an infected cell culture supernatant, and was used directly without passage for these experiments. The cell line HeLa (CCL-2) was obtained from ATCC. It was grown in RPMI 1640 media supplemented with $NaHCO_3$ to pH 7.5%, 0.5% penicillin and streptomycin, and 10% fetal calf serum (FCS). All media and supplements were from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada. Anti-CVB6 monoclonal antibody MAb3309 was obtained from Chemicon International Inc., Temecula, California, USA.

ii). Primers. Primers EEAT7 and MLOT were used for PCR of full-length amplicons of CVB6. EEAT7 (5'-CTACGGCCGAGCTAATACGACTCACTATAGTTAAA ACAGCCTGTGGGTTG-3') consists of an Eag I restriction enzyme site (underlined), the core sequence of the T7 promoter (dot underlined) (Dunn and Studier, 1983; Van der Werf et. al., 1986; Milligan et. al., 1987), a single guanosine nucleotide, and the first 20 nucleotides of CVB3 sequence. MLOT primer (5'-GTACGCGTTTTTTTTTTTTTTCCG CACCGAATGCGGAGAATTTA-3') consists of an Mlu 1 restriction site (double underlined) followed by a stretch of 15 thymidine nucleotides and 23 nucleotides which are complementary to the CVB3 sequence upstream of the poly A tail. The following CVB6 specific primers were used in determining the sequences at the 5'-end and 3'-ends of the genome; R2 (5'-TGTCGTAATGGGCAACTC), R4 (5'-CACCGCATTCCCCACAGG-3'), R5 (5'-GCTCCTGTTTTCTGTGTT-3'), F4 (5'-TGACTCTACCTGCGTTCT-3'). Additional primers used for 5'-end and 3'-end analysis as described below include anchor primer AAP (5'-GGCCACGCGTCGACTAGTACGG

GIIGGGIIGGGIIG-3') (Gibco/BRL) which contains deoxyinosine residues to facilitate priming from homopolymeric tailed cDNA, and primer AUAP (5'-GGCCACGCGTCGACTAGTAC-3') (Gibco/BRL) and oligo $dT_{(12-18)}$ (Pharmacia).

iii). Generation of CVB6 amplicons by RT-long-PCR. CVB6 RNA was purified from 100 µl of virus stock using TRIzol reagent, according to manufacturers directions (Gibco BRL). The RNA pellet was resuspended in 10 mM dithiothreitol (DTT) and 5% (vol/vol) RNasin (Promega) and stored at -70^{0}C. Reverse transcription was carried out as described previously (Tellier et. al. 1996). Briefly, a 10 µl aliquot of RNA was heated for 5 min at 65^{0}C and put on ice; then 10 µl of master mix was added containing 1 x Superscript II reverse transcription buffer (Gibco/BRL), 0.5 µl RNasin (Promega), 1 µl of 100 mM DTT (Promega), 1 µl of 10 mM dNTP mixture (Pharmacia), 2.5 µl of 10 µM downstream primer MLOT and 1 µl of Superscript II reverse transcriptase (Gibco/BRL) and incubated at 42^{0}C for 1 h. This was followed by the addition of 1 µl of RNase H (1-4 U/µl) (Gibco/BRL) and 1 µl of RNase T1 (900-3000 U/ml) (Gibco/BRL) and incubation at 37^{0}C for 20 min. Long PCR amplification (Tellier et. al. 1996) of the complete viral genome was set up in thin-wall tubes (Stratagene) in a final volume of 50 µl in 1xKlenTaq PCR reaction buffer (Clontech) with 250 µM each dNTP (Pharmacia), 10 pmol of each primer (EEAT7 and MLOT), and 1 µl of Advantage KlenTaq Polymerase Mix (Clontech). Exactly 40 µl of light mineral oil (Sigma) overlay was added to the PCR mixture, then a 2 µl aliquot of the RT reaction was added under the oil. PCR was performed using a Robocycler 40 thermal cycler (Stratagene) for 35 cycles; denaturation 99^{0}C, 35 sec; annealing 67^{0}C, 30 sec; elongation 68^{0}C for 9 min 45 sec for the first 15 cycles, 11 min for the next 10 cycles, and 13 min for the last 10 cycles.

iv). Sequencing of the CVB6 amplicons. PCR products were analyzed by electrophoresis on agarose gels stained with ethidium bromide. Bands corresponding to the ~7.5 Kb genome of CVB6 were cut out and the cDNA was purified using the Jetsorb kit (Genomed, Frederick, Maryland), and subjected to sequential sequencing in both directions using Thermosequenase (Amersham Canada Ltd., Oakville, Canada).

v). Determination of 5'- and 3'- end sequences. Since CVB6 amplicons were generated with primers deduced from alignments, the original sequences at the very ends of the virus genome were also determined. To determine the sequence at the 5'-end, a 5'-RACE kit (Gibco/BRL) was used, in accordance with the manufacturers directions. Briefly, first strand cDNA synthesis was generated using the CVB6 specific primer termed R5. The first stand cDNA was purified from unincorporated dNTPs and primers by purification on GlassMAX spin cartridges (Gibco/BRL). TdT was then used to add homopolymeric tails to the 3'-end of the cDNA. Tailed cDNA was amplified by PCR using CVB6 specific antisense primer R2 and a complementary homopolymeric anchor primer termed AAP. Nested PCR was performed using CVB6 specific antisense primer R4, and a complementary homopolymeric adapter primer termed AUAP. PCR products were ligated into TA cloning vector (Invitrogen) and cloned into *E.coli*, according to manufacturers directions. Three clones were sequenced using Thermosequenase (Amersham). Sequences at the 3'-end of the virus genome were determined using RT-PCR, with first strand cDNA synthesis carried out using oligo dT primer, thus taking advantage of the poly(A) tail in CVB RNA genomes as a priming site. PCR was performed using CVB6 specific primer F4 and also oligo $dT_{(12-18)}$. The 3'-end PCR products were cloned and sequenced as described above.

vi). Generation of CVB6 clones and transcription of viral RNA. CVB6 amplicons and the plasmid Litmus 39 (New England Biolab) were digested with the enzymes Mlu I and Eag 1 (New England Biolab). Digested insert and vector were electrophoresed into 0.8% ethidium-bromide agarose gels and purified as described (Tellier et. al. 1996). Ligation was performed using the Ligation Express kit (Clontech) according to the manufacturer's directions, and products were used to transform competent DH5α cells (Gibco BRL). Colonies grown on LB agar containing X-Gal IPTG and ampicillin were screened for the presence of a full-length insert. Candidate clones were digested with Mlu 1 and Eag 1 restriction enzymes and purified using Geneclean (Geneclean II kit, BIO 101). These were used as templates for T7 transcription, as were purified amplicons. T7 transcription was performed using the Ampliscribe T7 transcription kit (Epicentre), according to manufacturers directions.

vii). Viral RNA transfection into HeLa cells. Transfection of CVB6 RNA into HeLa cells was performed using Lipofectin reagent (Gibco/BRL) according to manufacturers directions. Briefly, a 150 µl aliquot of transfection mixture containing 10 µl Lipofectin reagent and 10 µl of CVB6 RNA (or untranscribed amplicon or lipid only as control) was prepared. Serial 2-fold dilutions of the mixture were added to HeLa cell monolayers, and cells were incubated at 37^0C for 5 hours. The transfection mixture was removed, the cells were washed, then further incubated in regular media at 37^0C for 3 days, and monitored for CPE.

CVB6 progeny production was also monitored. Supernatant from transfection experiments was added to HeLa cells plated on 8-chamber glass tissue culture slides (Becton Dickinson, Franklin Lakes, NJ. USA), and incubated at 37^0C for 45 min. The supernatant was removed, the cells were washed, then further incubated in growth media at 37^0C for 8 h. Monolayers were fixed with acetone and stained with anti-CVB6

MAb3309 (Chemicon) according to manufacturers directions. CVB6 antigens were detected by fluorescence microscopy on a Nikon Microphot FXA microscope (Nikon Canada Inc., Mississauga, Ontario, Canada).

viii). Cross reactivity of CVB6. To determine the relationship between CVB6 and other enteroviruses, nucleic acid alignments were identified on GenBank using BLASTN (Altschul et. al. 1990). Initial comparisons based on sequences from the first half of the virus genome indicated that CVB6 was most closely aligned with swine vesicular disease virus (SVDV). In light of this observation, antisera to CVB6 and to SVDV were tested for their ability to cross-neutralize these viruses. In a similar light, it had previously been demonstrated that CVB5 and SVDV share high genetic alignments (Zhang et. al. 1993), and they could be cross-neutralized with antisera (Graves 1973).

For these studies, a commercially available neutralizing antibody against CVB6 (Chemicon) was used, as well as neutralizing polyclonal anti-SVDV serum (Newman et. al., 1973). For controls a commercially available neutralizing antibody against CVB5 (Chemicon International Inc., Temecula, California, USA), and a polyclonal neutralizing serum against poliovirus type 1 (a gift from Connaught Laboratories, Toronto) were used. Neutralization assays were performed essentially as described (Snyder et. al. 1974) except that Vero cells were used for CVB6 neutralization assays, and swine testis cells were used for SVDV. In addition, CVB6 was tested (at a titer of $1x10^{7.6}$ TCID50/ml) in a double antibody sandwich ELISA capture assay for SVDV (Dulac et al. 1993).

ix). Alignments and Phylogeny. The relationship of CVB6 to other CVBs and SVDV was examined. For comparison purposes, one or more enterovirus sequences from the other enterovirus groups (e.g. CAV, EV, PV, numbered enteroviruses (ENT)) was chosen. The following enterovirus sequences were used in the analysis; CVB1,

accession number M16560 (Iizuka et. al. 1987); CVB2 Ohio-1 reference strain, accession number AF085363 (Polacek et. al. 1999); CVB3 Nancy reference strain, accession number M16572 (Lindberg et. al. 1987); CVB4 Benschoten reference strain, accession number X05690 (Jenkins et. al. 1987); CVB5, accession number X67706 (Zhang et. al. 1993); echovirus 6 (EV6), accession number U16283 (Gratsch et. al. 1994); echovirus 9 (EV9), accession number X84981 (Zimmermann et. al. 1995); echovirus 11 (EV11), accession number X80059 (Dahllund et. al. 1995); echovirus 12 (EV12), accession number X79047 (Kraus et. al. 1995); coxsackievirus A9 (CAV9), accession number D00627 (Chang et. al. 1989); poliovirus type 1 (PV1), accession number V01149 (Racaniello and Baltimore, 1981); enterovirus 71 (ENT71), accession number U22522 (Brown and Pallansch, 1995); swine vesicular disease virus (SVDV), accession number X54521 (Inoue et. al. 1989).

Amino acid and DNA sequences were aligned by the multiple sequence alignment program Clustal (Higgins and Sharp, 1989) as implemented in MacDNASIS version 3.2 (Hitachi, San Bruno, CA). Phylogenies of the sequences were estimated by parsimony and the neighbor-joining (distance) method using the computer packages PAUP* 4.0b1 (Swofford, 1998) and PHYLIP (Felsenstein, 1995). For neighbor-joining, distances between sequences were estimated using PROTDIST with PAM001 distances (Felsenstein, 1995).

H.9. Results and Discussion

The sequence for the reference strain of CVB6 (Schmitt) was determined. Full length CVB6 DNA amplicons were obtained for sequencing, using the technique of long-RT-PCR (Tellier et. al. 1996, Lindberg et. al. 1997). Amplicons of ~7500bp (consistent with the size of enterovirus genomes) were consistently obtained, as determined by ethidium-bromide agarose gel electrophoresis (Fig. 23). Nucleotide and predicted amino acid sequences for CVB6 were determined and deposited in GenBank (Accession #AF105342). To confirm that the virus genome was amplified and cloned functionally intact, CVB6 RNA from amplicon DNA and full-length clones was transcribed (Fig. 24), and the viral RNA was transfected into HeLa cells. RNA transcribed from amplicon DNA and from 3 out of 6 clones tested was infectious, as determined by cell cytopathic effect (Fig. 25). It was also confirmed that the progeny of the transfection experiments were CVB6, by indirect immunofluorescence using a monoclonal antibody to CVB6 (Fig. 26). A high frequency of error by the DNA polymerase used in the PCR could introduce stop codons in the polyprotein coding region at high frequency and render the genome defective. However, the fact that a large proportion of clones are functionally intact reflects favorably on the fidelity of the PCR process under these conditions.

The entire nucleotide sequence of CVB6-Schmitt (Accession #AF105342) is 7398 bp in length, (compared with CVB1, 7389; CVB2, 7411; CVB3, 7396; CVB4, 7395; CVB5, 7402), excluding the polyadenylated tail. The sequence is characteristic of enteroviruses, with a 5' nontranslated region (743 bp), a polyprotein sequence (6555 nucleotides or 2184 triplet codons), and a 3'-nontranslated region (100 bp).

The 5'-NTR of CVB6 contains nine AUG codons. This is similar to all the other enterovirus sequences characterized to date, which have multiple AUG codons in the 5'-

Figure 23

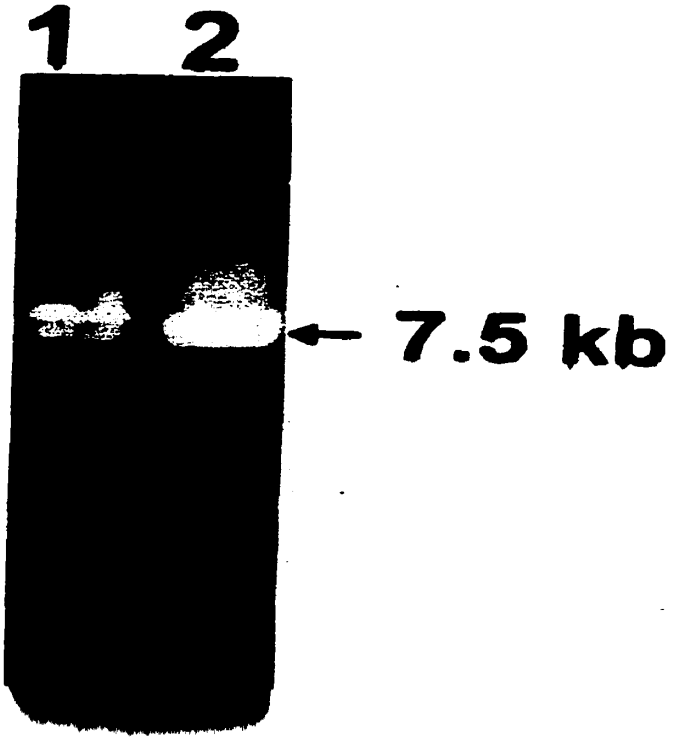


Figure 23. Amplification of the CVB6 genome by long RT-PCR. PCR products were electrophoresed on 0.8% EtBr agarose gels. Lane 1 contains the DNA marker lambda-*HindIII* digest. Marker sizes are 23.13 kb, 9.416 kb, 6.557 kb, 4.361 kb, 2.322 kb, 2.027 kb, and 0.564 kb. The 4.361 kb marker does not show since the sample was not heated prior to loading. The small 0.56 kb fragment is not visible. Lane 2 contains a 10 µl aliquot of the PCR reaction mix for CVB6. The major band at approximately 7.5 Kb is the anticipated size of the full length CVB6 amplicon.

Figure 24

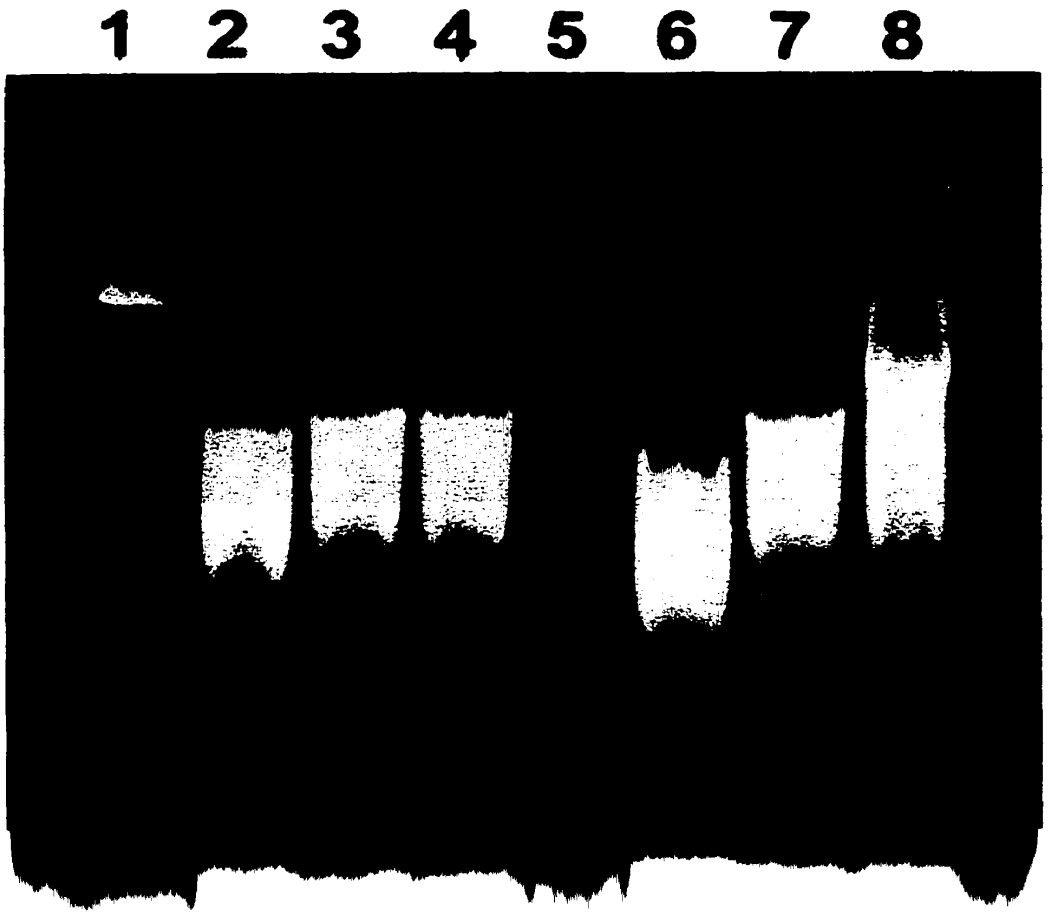


Figure 24. Transcription of CVB6 RNA. Aliquots of T7 transcription reactions were electrophoresed on non-denaturing agarose gels. Lane 1, marker lambda-*Hind*III digest. Lanes 2-7, reactions from clones 2, 4, 5, 6, 7, and 8, respectively, showing RNA as well as full length inserts and linearized plasmid (digests with Eag1 tend to be partial); the migration relative to the markers is aberrant, because of different buffers and loading dyes. Lane 8, transcription from amplicon DNA.

Figure 25

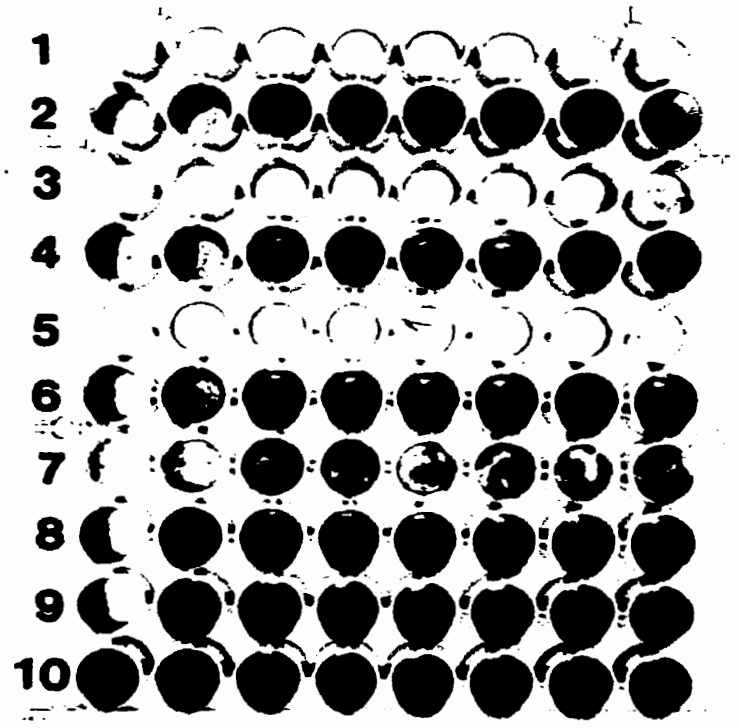


Figure 25. Infectivity of transcribed viral RNA. Monolayers of HeLa cells in 96-well plates were transfected with serial 2-fold dilutions of viral RNA (transcribed as described in Fig.24), using Lipofectin reagent. Transfected cell monolayers were incubated at 37C for 3 days. To monitor for cell cytopathic effect (CPE), monolayers were fixed in 2% formalin and stained with 0.1% crystal violet dye. Rows 1-6, RNA transcribed from clones 2, 4, 5, 6, 7, and 8, respectively. Row 7, transcribed amplicon DNA. Row 8, untranscribed amplicon DNA. Row 9, lipid only control. Row 10, untransfected HeLa cells.

Figure 26

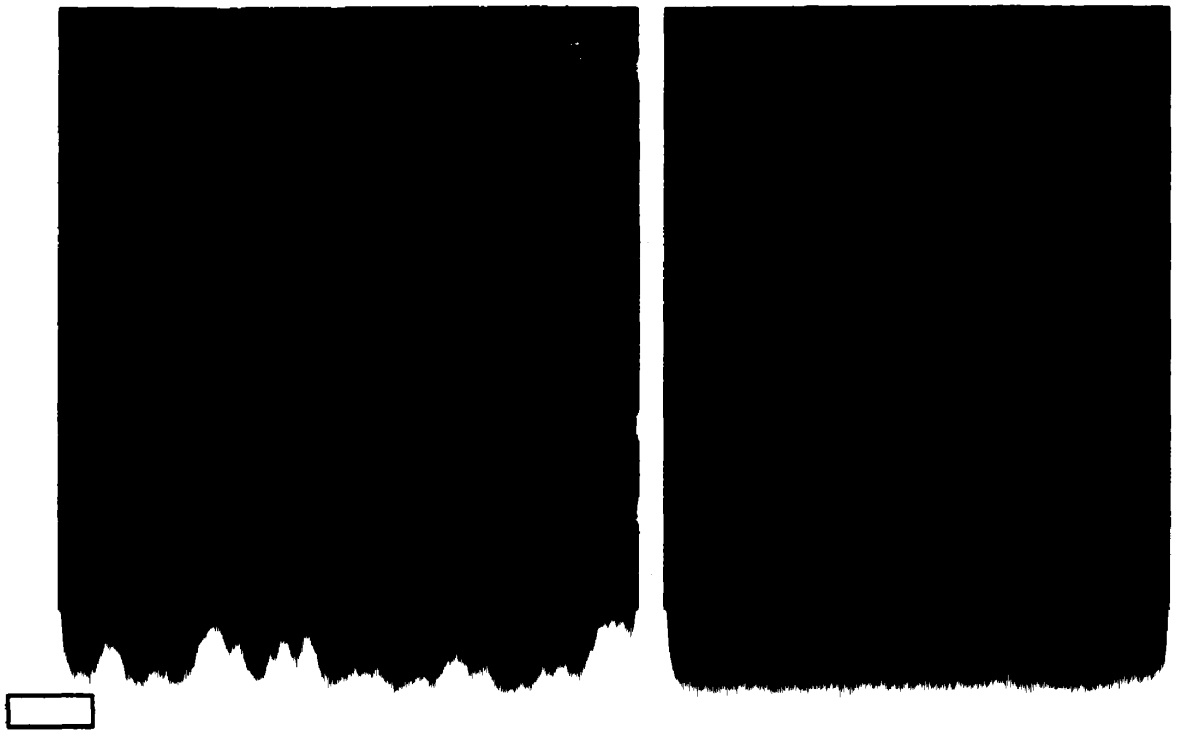


Figure 26. CVB6 progeny production in transfected HeLa cells, as detected by indirect immunofluorescence. Virus progeny were detected using a monoclonal antibody to CVB6. HeLa cells are stained red by Evans blue solution, and virus particles are depicted by the fluorescent green/yellow staining within the cells. Infectious virus were readily recovered from clones as well as from the transcribed amplicon.

NTR. However it appears unlikely that the AUG codons upstream of the AUG proximal to the polyprotein coding region are used for translation initiation. This has been clearly demonstrated for poliovirus (Pelletier et. al. 1988). The 5'NTR of CVB6 also contains numerous sequence motifs which can form complex secondary structures (not shown), which may be involved in enteroviral RNA transcription, protein synthesis, and virus virulence (Chang et. al., 1989; Skinner et. al. 1989; Andino et. al., 1990a; Pöyry et. al., 1992; Pöyry et. al., 1996).

Comparative protein sequence alignments of the VP1-3 capsid proteins of CVB6 with all CVB1-5 serotypes and SVDV were homologous, though some regions displayed more sequence differences (not shown). Variability within VP1-3 sequences is expected, since these proteins comprise the virion surface and have been implicated in enterovirus antigenicity, tropism and virulence (Minor et. al., 1986; Page et. al., 1988; Minor 1990; Muckelbauer et. al., 1995; Pöyry et. al., 1996; Oberste et. al., 1998; Oberste et. al., 1999). In contrast, the protein sequence of the internal coat protein VP4 is more conserved.

Comparative alignments were also used to predict the cleavage sites for the CVB6 capsid proteins. Predicted cleavage sites for CVB6 proteins were identical to those noted for CVBs and SVDV at most sites, but interesting differences were noted at two junction points. The VP3/VP1 junction of CVB6 was predicted to fall between glutamine and serine residues. This is in contrast to the site noted for the CVB1,3,4,5 serotypes and SVDV, which falls between glutamine and glycine residues. The change results from a single base pair mutation at nucleotide 2448 (G-A), which was confirmed by sequencing of CVB6 amplicon DNA in both directions, though not by direct peptide sequencing of CVB6 proteins. This cleavage site is also found in the sequence of CVB2 (Polacek et al. 1999) and echovirus 9 (Zimmermann et. al. 1995). Similarly, the P2-B/P2-C junction of CVB6 is defined by glutamine and serine residues, whereas the site noted for all other CVBs and SVDV falls between glutamine and asparagine residues. Although this cleavage site is not present in other CVB serotypes or in SVDV, it is also

found in the sequence of enterovirus 71 (Brown and Pallansch 1995), echovirus 12 (Kraus et. al. 1995), and bovine enterovirus (Earle et. al. 1988).

The CVB6 sequence was also compared with an unpublished partial sequence of CVB6 recently deposited in the GenBank database (accession number AB017151) (Sato et. al., 1998). The authors reported 3411-nt from the 5'-end of the genome. Several differences between that sequence and the one reported here. The most notable difference occurred in VP2, resulting in numerous nucleotide and amino acid changes. This region is referred to as the "VP2 puff region", and it is particularly significant that the changes occurred here because major neutralizing immunogenic sites occur in this region (Minor et. al., 1986; Page et al., 1988; Minor 1990). To confirm the sequence reported here, the following experiments were performed. 1) The region was resequenced using full-length CVB6 amplicons generated from the same RNA template and primers but different PCR reactions, and they were identical to the sequence reported. 2) The region was sequenced using 3 infectious clones of CVB6 (clones #2,5,7), and results were consistent with the sequence reported. 3) Restriction enzyme digestion of amplicon DNA was performed using ApaLI and FspI enzymes, whose predicted restriction patterns differed for the two sequences. Restriction patterns were consistent with the sequence data reported here (data not shown). Furthermore, the digestion was complete, suggesting that there was no significant genetic heterogeneity of the virus population at these sites. In this study, CVB6 was obtained directly from ATCC and used for amplification reactions without passage through cell culture. Genetic integrity of the virus was retained, as evidenced by the infectivity of transcribed viral RNA *in vitro*. Hence, the alternative sequence reported by Sato et. al. (1998) could be a different variant of CVB6, or from virus passaged in a different cell line.

Initial analysis performed on the first half of the virus sequence with BLAST had indicated that CVB6 was most closely aligned with SVDV, but cross-neutralization could not be demonstrated, and phylogenetic analysis using neighbour-joining methods failed

to support a strong relationship. Instead, evolutionary trees constructed from entire polyprotein sequences using neighbor-joining methods indicated that CVB6 clustered with the other CVB1-5 serotypes and SVDV (Fig. 27). CVB6 was more closely related to CVB2 and CVB4 serotypes than to the other CVBs. CVB2 and CVB4 were most closely related to each other, with CVB6 falling just outside this group. Interestingly, it has been previously reported that reference strains of CVB also tend to divide along these lines in their ability to interact with the decay accelerating factor (DAF or CD55) coreceptor molecule (Shafren et. al., 1995; Bergelson et al. 1995) although there are clinical and laboratory variants with different receptor specificity's (Bergelson et. al. 1997b).

Even though CVB6 is clearly a coxsackievirus, as further demonstrated by the phylogenetic tree, its epidemiology is peculiar since it appears essentially absent from North America. Although reasons for this are unclear, it could be that CVB6 is less virulent than other CVBs (isolation is usually attempted from patients who are symptomatic). Alternatively, CVB6 may have been diagnosed less often in the laboratory due to a lack of cost-effective or specific reagents. The consensus sequence of CVB6 has now been determined, which completes the genetic database for the group B coxsackieviruses. This information will prove helpful for the design of diagnostic tests using molecular techniques, and comparison with other CVBs may shed light on the pathogenesis of these viruses. The availability of an infectious clone will permit the study of specific mutations.

Figure 27

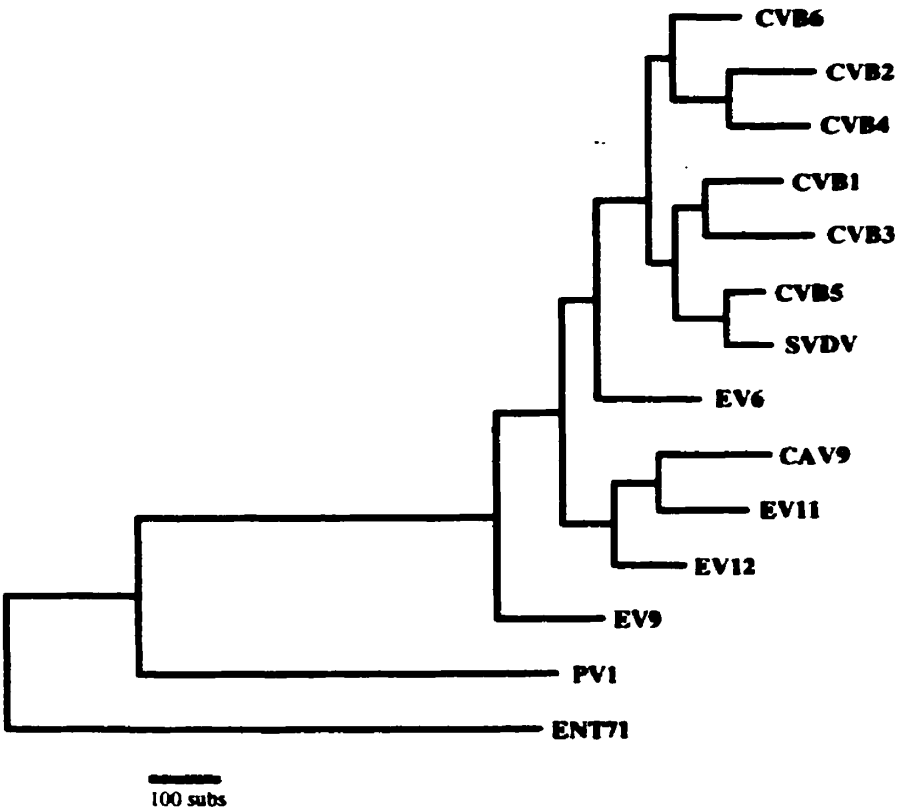


Figure 27. Phylogeny of CVB6 and related enteroviruses. The most parsimonious phylogenetic relationship of the coding region of CVB6 and related viruses is shown. ENT71 was used as an outgroup to root the tree, as it was the least similar. Branch lengths are proportionate to the number of estimated nucleotide replacements (causing amino acid changes) that occurred on each lineage. Similar phylogenies were estimated by the neighbor-joining distance method. The scale bar at the bottom is for 100 nucleotide replacements. Accession numbers and references for the virus strains used are as described in the Materials and Methods section.

I. SUMMARY AND MAJOR CONCLUSIONS

I.1. Study #1

In this study, interactions between cardiovirulent coxsackieviruses (CVB) and the decay accelerating factor (DAF) receptor were examined. The findings demonstrate that the CVB3 variants -CG, -CGP1V, -SH and -Ø interact strongly with the DAF receptor. Anti-DAF MAbs can block these viruses from binding to and infecting susceptible cells. Also, virus binding is reduced following pretreatment of cells with PIPLC enzyme, which renders them DAF deficient. In contrast, the CVB3 variants -VR30, -N, -NR, and -20 do not use DAF as a receptor molecule, in the same assay systems. It was also demonstrated that CVB3 binding to cells leads to DAF downregulation at the cell surface, as monitored by flow cytometry analysis. In addition, it was found that DAF may co-localize with additional receptor molecules such as CAR, on the cell surface. This was indicated through the use of competitive binding assays between coxsackieviruses and adenoviruses. Finally, it was shown that CVB3-DAF interactions might be important pathogenically through the activation of signaling cascades. DAF associates with the T-cell activation molecule $p56^{lck}$. However, T-cell activation, which is a hallmark feature of myocarditis, does not occur in knockout mice lacking $p56^{lck}$. These mice are resistant to CVB3-induced heart disease.

An additional study following from this work could be to examine DAF downregulation on CVB infected cells using confocal microscopy. The ability of infected cells with downregulated DAF to protect against complement can be monitored through complement and chromium release assays. The interaction of nonhuman DAF homologues with CVB also remains to be investigated. The ability of CVB3 binding to DAF to activate signaling pathways through $p56^{lck}$ can be further examined using *in vitro* assays. For example, CVB3-DAF-$p56^{lck}$ complexes can be purified from cell membranes by immunoprecipitation, and analyzed by Western blot analysis. Tyrosine

kinase activation stemming from virus-DAF-lck interactions can be examined by staining the blots with anti-phosphotyrosine antibodies. CVB-DAF interactions leading to T cell activation can be monitored by using bioassays for protein markers of T cell activation such as interleukin-2. The ability of protein tyrosine kinase inhibitors to protect against CVB-DAF mediated T cell activation could also represent a fruitful area of study.

I.2. Study #2

In this study, interactions between CVB and the coxsackie-adenovirus (CAR) receptor were characterized. The findings showed that CAR is a functional receptor molecule for reference strains and clinical isolates representing all six serotypes of CVB. The virus strains infect and replicate in hamster cells expressing human CAR, but not in control cells lacking the CAR receptor molecule. Furthermore, CAR was shown to be a functional receptor for swine vesicular disease virus (SVDV), using this assay system. SVDV is a porcine enterovirus that is antigenically and genetically related to CVBs. Finally, pretreatment of CVB or SVDV with soluble CAR specifically inhibits virus infection of susceptible cells.

In the future, the native biological function of CAR could be examined by using CAR knockout animal models. The role of CAR in CVB heart disease could also be further studied using the knockout model systems. Specific interactions between CVB3 and CAR may be further characterized by determining binding sites on the CVB3 virion and the CAR molecule, and by examining the receptor binding affinities of different virus variants and serotypes. The distribution and regulation of CAR expression in the human heart and in other tissues could also be investigated. Studies on soluble CAR treatment in CVB heart disease *in vivo* could also be of interest.

I.3. Study #3

In the first part of this study, sites were identified on the CVB3 virion which may be important for virus-receptor binding. This was done by comparing the deduced amino acid sequences of the capsid binding regions of 8 virus variants with known receptor binding phenotypes. Eighteen amino acid differences were found in total, scattered amongst the four viral coat proteins. Several residues mapped onto or near the virion surface, including some within the receptor binding canyon depression and some on immunogenic domains which protrude from the virion surface. Phylogenetic analysis of the capsid sequences yielded two virus groupings, based on their ability to use DAF as a receptor molecule. The second part of this study involved the development of techniques which could be used to clone CVB genomes for further study. These techniques allowed for the rapid and reliable cloning of CVB, and in doing so a phylogenetic tree for group B coxsackieviruses was completed.

In the future these techniques can be used to determine which specific amino acid residues in the virion capsid proteins are important for virus-receptor binding. This can be done by amplifying and cloning the cardiovirulent CVB3 genomes, and then subjecting them to site directed mutagenesis. Mutated progeny viruses can then be examined for their receptor binding phenotypes. The techniques of long-RT-PCR and full-length genome cloning also have additional applications. For example, they can be used to investigate the integrity of the viral RNA which persists in heart tissue beyond the acute stage of the disease process. Amplification, cloning, and sequencing of other virus variants obtained clinically or experimentally could also shed new light on the disease processes that occur.

J. GENERAL DISCUSSION AND FUTURE DIRECTIONS

In this thesis, findings are presented which characterize interactions between CVB and two cell surface receptors, namely the decay accelerating factor (DAF) receptor, and the coxsackie-adenovirus receptor (CAR). However, an important question for future consideration is how do these virus-receptor interactions play a role in the pathogenesis of CVB-induced heart disease? One possibility, as indicated from these findings, is that CVB receptors are determinants of viral cardiotropism. Furthermore, virus-receptor binding may trigger signaling pathways which lead to pathogenic consequences.

J.1. Receptors as determinants of virus tropism.

It has long been postulated that enterovirus receptors are determinants of virus tropism. In a pioneering study by Dr. John Holland, it was demonstrated that enteroviruses bind to homogenates made from the tissues in which they normally propagate *in vivo*, presumably through interactions with 'receptor' molecules on the surface of the cells (Holland, 1961). Subsequent support for a relationship between receptors and enterovirus tropism came from studies using polioviruses. For example, poliovirus infection is naturally restricted to humans, and humans express the PVR from a single gene copy on human chromosome 19 (Koike et al., 1990). Also, a homologue is encoded by nonhuman primates, and they are susceptible to poliovirus infection when the virus is inoculated into the CNS (Koike et al., 1992). Moreover, poliovirus can replicate and cause paralysis in transgenic mice expressing the PVR gene, but not in nontransgenic mice lacking the receptor gene (Ren et al., 1990). And intramuscularly inoculated poliovirus spreads to neural pathways in the transgenic mice in a PVR dependent manner (Ohka et al., 1998). Finally, poliovirus infection of cells can be

blocked by pretreatment of virus with soluble receptor (Kaplan et al., 1990a), although mutants resistant to neutralization by soluble receptor do occur (Kaplan et al., 1990b).

It is tempting to speculate that the coxsackie receptor CAR is also a determinant of viral tropism. In support of this hypothesis, CAR receptor mRNA has been detected in human heart, brain and pancreas tissue by Northern blot analysis (Bergelson et al., 1997a; Tomko et al., 1997), consistent with the tissue tropism and disease manifestations seen in CVB infections in humans. Moreover, CAR mRNA homologues are found in murine heart and liver (Bergelson et al., 1998), organ sites that are infected with CVB when the virus is injected intraperitoneally into mice. Lastly, it was demonstrated in these studies that CVB infection of susceptible cells can be blocked by pretreating the virus with soluble CAR receptor (see study #2 and Martino et al., 2000).

It seems likely that CAR is a determinant of CVB tropism, however, additional factors are also important in determining virus tropism. This was indicated from studies which showed that CAR mRNA is expressed not only in tissues that support virus replication, but also some tissues in which the virus is not found to propagate *in vivo* (Bergelson et al., 1997a, 1998; Tomko et al., 1997). This is not a novel concept, as it has also been shown previously for polioviruses that PVR mRNA is expressed in tissues and cells in which the virus fails to replicate (Freistadt, 1994; Mendelsohn et al., 1989; Shiroki et al., 1997; Zhang and Racaniello, 1997).

One additional factor which is likely important for determining CVB tropism is the presence of co-receptor molecules on host cells. The use of co-receptor molecules has been extensively studied for other viruses, such as human immunodeficiency virus (HIV) which uses both CD4 and a chemokine co-receptor molecule to infect cells (reviewed in Chan and Kim, 1998; Dimitrov, 1997; Littman, 1998; Wyatt and Sodroski, 1998). A well characterized co-receptor molecule for CVBs is DAF (Study #1; Bergelson et al., 1995, 1997b; Martino et al., 1998; Shafren et al., 1995). Other putative co-receptors for CVBs which are not well characterized to date include a 100 kDa nucleolin-like protein (Raab de Verdugo et al., 1995), the fibronectin receptor integrin $\alpha v\beta 6$ (Agrez et al., 1997), and

cell surface molecules found on newborn mouse brain cells (Xu and Crowell, 1996) and hamster CHO cells (Kramer et al., 1997).

Co-receptor utilization may also help to explain why both CVB and adenoviruses (Ad) can use CAR as a receptor, yet exhibit different viral tropisms. For CVBs, CAR is functional receptor molecule which allows for productive virus infection of cells, with DAF functioning as an attachment or co-receptor molecule. For adenovirus, however, CAR is only a binding protein with which fiber proteins interact (Bergelson et al., 1997a; Roelvink et al., 1998; Tomko et al., 1997), and the presence of additional co-receptors such as αvβ3 or αvβ5 integrin which bind the penton base protein are required for virus internalization (Wickham et al., 1993). Human MHC class I α2 domain can also be used as an attachment receptor by adenovirus fiber protein (Davison et al., 1999; Hong et al., 1997). These differing co-receptor profiles likely influence the differing host ranges and tissue distributions and disease phenotypes observed during CVB and adenovirus infections *in vivo*.

In addition to functioning as determinants of viral tropism, CVB co-receptor usage may also be important for enhancing virus virulence, through sequestration of virus at the cell surface. This hypothetical mechanism is illustrated in Figure 28. In this scenario, a host cell expresses both CAR and DAF co-receptor molecules, and the DAF receptor is more accessible at the cell surface (as indicated from Study #1). A DAF binding virus binds first to DAF, and is then brought into proximity to CAR for binding to this receptor molecule. The dual use of receptor molecules leads to rapid infection of the host cell. However, some virus variants do not use DAF as a receptor, presumably due to mutations in the virion capsid proteins which affect virus-receptor binding phenotype (as indicated in Study #3). For these non-DAF binding viruses, infection proceeds more slowly because they must navigate the cell surface alone until they encounter CAR, and steric hindrances from other cell surface molecules may occur.

Figure 28

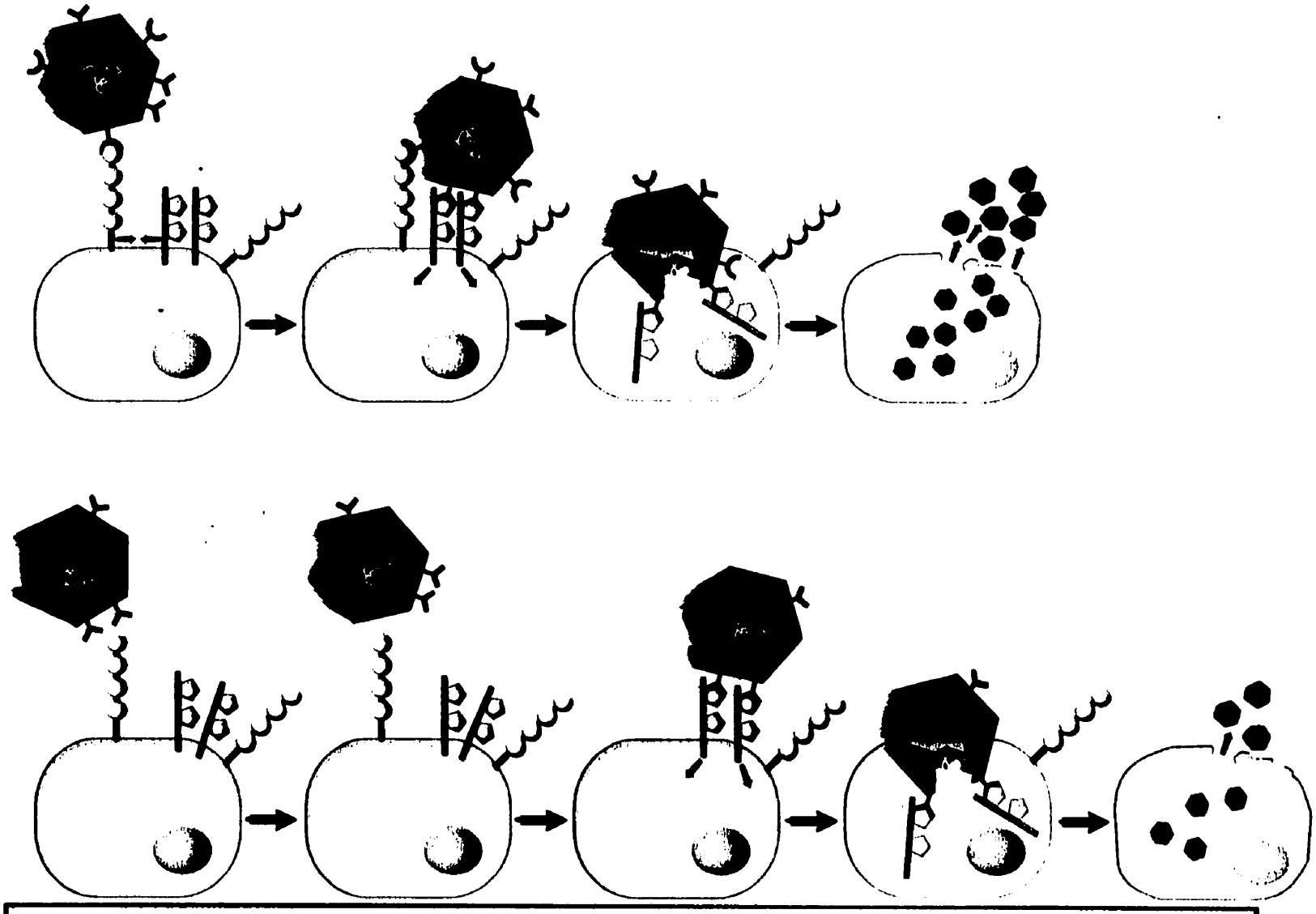


Figure 28. DAF binding facilitates CAR dependent endocytosis of virus. Theoretical mechanism by which co-receptor use promotes CVB infectivity. Top panel. A host cell expresses both DAF (pink) and CAR (purple), and DAF is more accessible at the cell surface. A DAF binding virus attaches first to DAF, and is then brought into proximity to CAR. Virus binds CAR and viral RNA is released, leading to progeny virus production. Bottom panel. A non-DAF binding virus strain navigates the cell surface until it reaches CAR and infects the cell. Steric hindrances reduce the speed at which virus can locate and bind CAR, possibly reducing virus virulence. Illustration by David Hou.

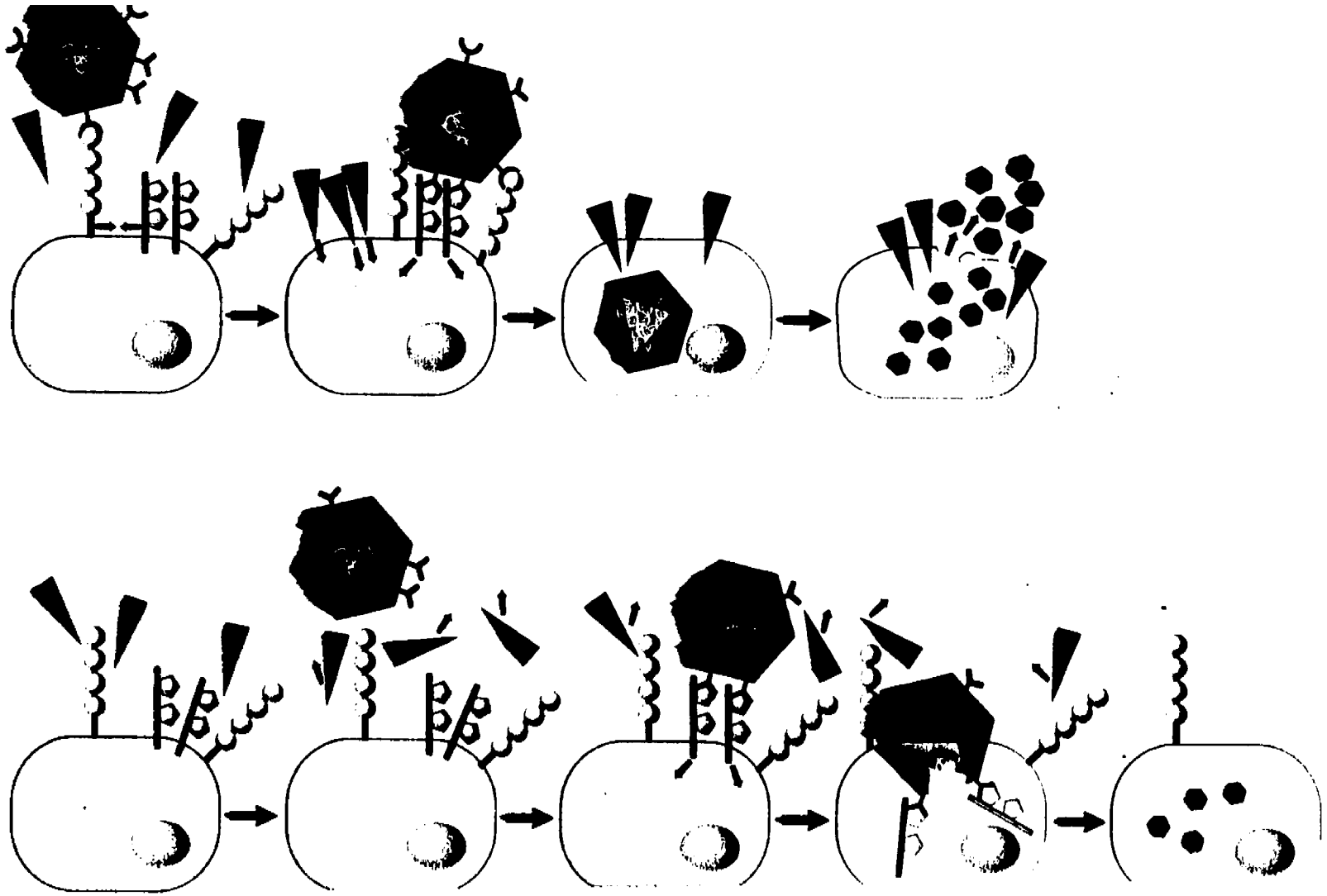


Figure 29. Complement assisted lysis and accelerated virus progeny release mediated by CVB3 binding to DAF. Theoretical mechanism for enhancing virus virulence. Top panel. A host cell expresses DAF (pink) and CAR (purple). A DAF binding virus attaches first to DAF, then to CAR, and is internalized into the cell along with the receptor molecules. DAF is now reduced on the cell surface and the cells are more susceptible to complement mediated lysis (red triangles). This facilitates the rapid release of progeny virus, thus increasing virus virulence. Bottom panel. A non-DAF binding virus strain must navigate the cell surface until it binds to CAR. DAF remains on the cell surface. Progeny virus are released from infected cells at a slower rate in the absence of complement assisted lysis. Illustration by David Hou.

The complement regulatory functions of the DAF co-receptor molecule could also be important for modulating virus virulence. A hypothetical mechanism by which this could occur is shown in Figure 29. In this scenario, when DAF binding viruses infect cells, DAF is downregulated at the cell surface (as indicated from Study #1). Loss of DAF from the cell surface renders the infected cells more susceptible to complement-mediated lysis, which in turn facilitates the rapid release of progeny virus particles. In contrast, when non-DAF binding viruses infect cells, DAF remains at the cell surface and the progeny virus particles are released more slowly from the infected cell.

CVB tropism may be influenced not only by the presence of its receptors on host cells, but also by the manner in which the receptor proteins are expressed on the cell membrane. For example, CAR is absent from the apical surface of airway epithelial cells but is expressed on the basolateral surface (Walters et al., 1999), and thus it may be unavailable for binding to CVB at this site. Moreover, different isoforms of CAR have been isolated from tissues, and they may differ in their ability to facilitate CVB infection of host cells (A. Opavsky, personal communication). Changes to the level of receptor expression on host cells could also influence virus tissue tropism and viral dissemination. For example, CAR expression is downregulated during skeletal muscle maturation (Nalbantoglu et al., 1999), consistent with the lack of CVB infection observed at this site. DAF expression on cells is also influenced by biological factors, including plasminogen activator inhibitor type 2 (Shafren et al., 1999), IL-4, and TNF-α (Moutabarrik et al., 1993).

It is important to note that even though CVB may bind to receptors on susceptible cells, the cells may still not be permissive to viral infection. Factors other than receptor expression also necessary to determine viral tropism. For example, it has been shown for polioviruses that some cell types expressing the poliovirus receptor do not produce progeny virus, because they lack cellular factors which are necessary for viral RNA synthesis and/or protein translation (Agol et al., 1989; Dorner et al., 1984; La Monica and Racaniello, 1989). In a similar light, it was observed in these studies that only some

CVB viruses replicate in neonatal myocytes *in vitro*, despite the ability of all of these viruses to interact with CAR, and despite the presence of CAR receptor mRNA in the myocyte cells (unpublished observations). Also, CVB may localize in cells and tissues even if they lack receptor molecules. For example, CVB3 retention and localization in murine spleen is dependent in part on the complement protein C3 (Anderson et al., 1997). Moreover, polioviruses (and presumably other enteroviruses) are taken up by the phagocytotic M cells in the lumen of the intestine, for delivery to the lymphatic tissues (Sicinski et al., 1990). Furthermore, poliovirus has been shown to permeate the blood-brain barrier and enter the CNS, without the aid of the poliovirus receptor (Yang et al., 1997).

In summary, it is hypothesized that CVB tropism is influenced by the receptor molecules CAR and DAF. Future studies on viral tropism are important for our understanding of the pathogenesis of CVB diseases. This information may also prove useful in the treatment of CVB infections in a clinical setting. For example, CVB may be prevented from binding to and infecting susceptible cells in tissues such as the heart by treatment with soluble receptors proteins which can inactivate the virus particles. Also, viral dissemination may be limited by treatments which can downregulate receptor expression within susceptible tissue sites.

J.2. Receptors as triggers of signaling pathways.

DAF can play a role in the activation of T-cells (see section C and below). In this thesis, it was further postulated that CVB binding to DAF can trigger the requisite pathways leading to T-cell activation, and that this contributes to the development of CVB-induced myocarditis (Study #1). CVB binding to CAR may also have biological consequences, however, this is more difficult to speculate on because the native function(s) of CAR have not been elucidated to date. A hypothesis on how CVB-DAF

binding might be important for T-cell activation and for determining disease phenotype is therefore proposed.

The CVB receptor DAF (CD55) is anchored to the cell membrane by a glycophosphatidylinositol (GPI) linkage (see Figure 3 and section C). Several other GPI anchored proteins identified to date include the human leukocyte markers CD14, CD16, CDw17, CD24, CD48, CD58, CD59, CD66, CD67, and CD73, the mouse antigens Thy-1, Ly-6, J11d, and Qa-2, and rat RT6, as well as a fibronectin receptor, LAM-1, and cell anchored ectoenzymes such as acetyl-cholinesterase and alkaline phosphatase (Cinek and Horejsi, 1992 and references therein; Stefanova et al., 1991).

Binding of natural ligands or antibodies to GPI-linked proteins can trigger leukocyte activation. For example, cross-linking antibodies to DAF on the surface of human T-cells and stimulating with phorbol esters induces proliferation of the cells *in vitro* (Davis et al., 1988). Also, cross-linking experiments using murine EL-4 T-cell transfectants expressing human DAF leads to the secretion of IL-2 (Shenoy-Scaria et al., 1992), which is considered a late stage event in T cell activation. Also, treatment of human Jurkat T-cell lines with anti-DAF MAb and a calcium ionophore stimulates cells to produce IL-2 (Tosello et al., 1998). MAbs must be directed against the SCR3 domain of DAF to trigger T-cell activation, and interestingly, this is the same domain which controls complement regulation, indicating that it is a physiologically relevant site (Coyne et al., 1992; Lublin et al., 1991).

The GPI anchor of DAF is also critical for T cell activation. This was demonstrated in a study in which EL-4 transfectants expressing GPI-DAF could be activated by MAb cross-linking, but cells expressing the transmembrane form of DAF were not activated (Shenoy-Scaria et al., 1992). But this raises the question as to how activation signals can be transduced to the cell when the GPI-DAF molecules are devoid of intracellular domains. The observation that DAF and other GPI anchored molecules may exist in very large noncovalent complexes sheds some light on the phenomena. These complexes contain the GPI-anchored proteins along with

(glyco)lipids and several intracellular components including protein tyrosine kinases such as $p56^{lck}$ (Cinek and Horejsi, 1992; Stefanova et al., 1991). One can imagine that cross-linking of GPI proteins within these complexes induces changes in the intracellular components such as the protein tyrosine kinases, resulting in phosphorylation and T cell activation. Experimental support for this hypothesis stems from the observations that both DAF and $p56^{lck}$ can be co-immunoprecipitated from T cells (Shenoy-Scaria et al., 1992; Tosello et al., 1998). Furthermore, MAb cross-linking of GPI-DAF on EL-4 cells triggers tyrosine phosphorylation of cellular proteins including $p56^{lck}$ (Shenoy-Scaria et al., 1992). That $p56^{lck}$ plays a crucial role in IL-2 secretion and T-cell activation (Abraham et al., 1991; Hatakeyama et al., 1991; Straus and Weiss, 1992) provides further support for this hypothesis.

It is tempting to speculate that since DAF is also a receptor for CVB, it may play a role in viral myocarditis through the triggering of these pathways. The aberrant T-cell responses that occur in viral myocarditis may result from DAF-mediated activation of T-cells, instead of the normal MHC-mediated processes. Indirect support for this hypothesis comes from the observations that human peripheral blood lymphocytes and human T cell lines express DAF on the cell surface, and can bind to radiolabeled CVB3 (Vuorinen et al., 1994, 1999; A. Opavsky, personal communication). Also, CVB3 binds to DAF at the SCR3 domain (Study #1 and Bergelson et al., 1995; Martino et al., 1998; Shafren et al., 1995), which is the same domain used by anti-DAF MAbs to trigger T-cell activation. There is also evidence that CVB binding to DAF can trigger phosphorylation events. CVB infection of DAF-positive HeLa cells in culture leads to tyrosine phosphorylation of cellular proteins, as detected by antiphosphotyrosine Western blot analysis (Huber et al., 1997). Furthermore, evidence that $p56^{lck}$ is critical for development of viral heart disease comes from studies in this thesis. Transgenic mice lacking $p56^{lck}$ fail to generate T-cell responses to CVB3 infection and are resistant CVB3-induced myocarditis, whereas immunologically intact mice are susceptible to the disease (Study #1).

There may also be other mechanisms by which CVB binding to DAF/p56^{lck} complexes contributes to the development of myocarditis. For example, p56^{lck} may be important for virus replication. In support of this hypothesis, it has been shown that CVB3 can infect and replicate to high titer in cultured human Jurkat T cells expressing both DAF and p56^{lck}, but not Jurkat cells lacking the p56^{lck} molecule (Liu et al., 2000). This is thought to occur through activation of the ERK1/2 mitogen-activated protein kinase (MAPK) pathway (Opavsky et al., 2000). Further evidence for a role for p56^{lck} in virus replication comes from studies in this thesis. Lower virus titers were observed in the hearts of p56^{lck} knockout mice, as compared to heterozygote mice carrying one functional copy of the gene (Study #1). However, virus replication still occurred in the hearts of the knockout mice, indicating that the virus can also replicate in some cell types that do not naturally express the p56^{lck} gene (Liu et al., 2000). Finally, DAF also associates with other signaling molecules such as fyn. Investigating a role for this pathway in the context of CVB3 myocarditis may also be a fruitful area of investigation.

In summary, viruses use cellular molecules to infect and replicate in cells. These molecules have native biological functions which are often critical to the functioning of the host cell. It is postulated that when CVB attaches to its DAF receptor, then the biological ability of DAF to activate T cells through p56^{lck} is commuted, with pathogenic consequences. Interactions between DAF and p56^{lck} may also be important for virus replication in infected cells. Further studies on the signaling pathways associated with CVB receptors are currently being pursued, and represent an exciting new field of study.

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