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DETERMINANTS OF HOST SUSCEPTIBILITY TO COXSACKIEVIRAL MYOCARDITIS

by

Mary Anne Opavsky

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy, Graduate Department of Institute of Medical Sciences, in the University of Toronto

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0-612-63730-1
ABSTRACT

Determinants of Host Susceptibility to Coxsackieviral Myocarditis
by Mary Anne Opavsky

For the degree of Doctor of Philosophy, Graduate Department of Institute of Medical Sciences, University of Toronto, 2001

Viral myocarditis is an important cause of heart failure and dilated cardiomyopathy. Group B coxsackieviruses (CVBs) are commonly identified as the infectious agent responsible for acute myocarditis. This thesis examined the role of the T cell, the coxsackie-adenovirus receptor (CAR), and downstream signal transduction pathways in host susceptibility to CVB3 myocarditis. The first study, using gene-targeted T cell knockout mice, demonstrated that both CD4+ and CD8+ T cells contribute to the pathogenesis of myocarditis. The severity of myocardial damage and associated mortality depended upon the predominant T cell subset available to respond to CVB3 infection. Distinct cytokine patterns were found to be one mechanism by which CD4+ and CD8+ T cell subsets influence the pathogenesis of the disease. In the second study, it was hypothesized that the extracellular signal-regulated kinases (ERKs), which are stimulated following T cell receptor (TCR) engagement and Lck activation, influence host susceptibility to CVB3 infection and disease. In Jurkat T cells the ERK1/2 cascade is rapidly activated in an Lck-dependent manner at the time of initial virus-host cell interaction. Moreover, inhibition of the ERK1/2 cascade attenuates CVB3 replication in both T cells and cardiomyocytes. Myocarditis-susceptible A/J mice infected with CVB3 showed exuberant activation of the ERK1/2 signalling pathway. In contrast, activation was weaker and transient in C57BL/6 mice, which are resistant to myocarditis. The ERK1/2 signalling cascade is triggered by CVB3 infection and correlated with severity of viral
myocarditis. The third study explored the viral receptor as a determinant of myocarditis susceptibility. CAR expression was highest in the hearts of myocarditis-susceptible A/J mice, which were found to have high cardiac CVB3 titres following infection. A truncated CAR isoform was identified, which bound virus, but did not mediate productive infection. Tissue abundance of CAR and differential expression of receptor isoforms may determine myocarditis susceptibility. Thus, the initial virus-receptor interaction, the T cell response, cytokine activation, and downstream signal transduction pathways in both the T lymphocyte and the heart, appear to affect host cell function and viral replication, influencing host susceptibility to myocarditis.
ACKNOWLEDGEMENTS

I deeply appreciate the opportunity to work with Dr. Peter Liu, my supervisor. The confidence he has placed in my work, and the example he has set have inspired me in my pursuit of an academic career. I know I will never meet another individual who brings such bountiful enthusiasm and dedication to both medicine and science.

I am grateful that I have had the guidance of a stellar group of scientists during the course of my studies. Dr. Marlene Rabinovitch, Dr. Martin Petric, Dr. Josef Penninger and Dr. Chris Richardson, as my program advisory committee, provided me with generous advice and guidance. The friendship of my colleagues and labmates, Dr. Tami Martino, Cathy Trinidad, Christie Lee, Dr. Fayez Dawood, Dr. Wen-Hu Wen, and Dr. Mei Sun, has been invaluable .... thank you all. The organization formerly known as the Medical Research Council of Canada (now the CIHR) provided support for this endeavor with a fellowship award.

It takes a very special family to unquestioningly support the spirit, the education and the dreams of one person. My parents, Mary and Frank Opavsky, grandparents, sister, brothers, beyond generous brother-in-law, and especially all my nieces and nephews (who don't really have a clue what Auntie Anne actually does) are in my thoughts as I complete this thesis.
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<tr>
<td>APC</td>
<td>altered particle</td>
</tr>
<tr>
<td>ACE</td>
<td>antigen presenting cell</td>
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<tr>
<td>BCG</td>
<td>angiotensin-converting enzyme</td>
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<tr>
<td>BRS</td>
<td>Bacille Calmette-Guérin</td>
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<td>BRDU</td>
<td>5-bromo-2-deoxyuridine</td>
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<tr>
<td>CAR</td>
<td>coxsackie-adenovirus receptor</td>
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<tr>
<td>CAV</td>
<td>group A coxsackievirus</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<td>CCR</td>
<td>chemokine receptor</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<td>CHOP</td>
<td>polyoma virus transformed CHO cells</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<tr>
<td>CREB</td>
<td>cAMP-regulated response element binding protein</td>
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<td>CSF-1</td>
<td>colony stimulating factor-1</td>
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<td>Csk</td>
<td>c-Src kinase</td>
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<td>CT-1</td>
<td>cardiotropin-1</td>
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<td>CVB</td>
<td>group B coxsackievirus</td>
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<td>CVB3</td>
<td>CVB type 3</td>
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<tr>
<td>DAF</td>
<td>decay accelerating factor</td>
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<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>encephalomyocarditis virus</td>
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<td>erythropoietin</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>expressed sequence tag</td>
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<td>EV</td>
<td>echovirus</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>Fyn</td>
<td>p59fyn protein tyrosine kinase</td>
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<td>GAM</td>
<td>goat anti-mouse</td>
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<td>GAR</td>
<td>goat anti-rabbit</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>GPI</td>
<td>glycoporphophoinositol</td>
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<tr>
<td>HAVcr-1</td>
<td>hepatitis A virus receptor</td>
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<td>hCAR</td>
<td>human CAR</td>
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<td>HeLa</td>
<td>human cervical carcinoma cell line</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HLA</td>
<td>human leukocyte antigens</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HRV</td>
<td>human rhinovirus</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
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<td>poliovirus</td>
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<tr>
<td>PVR</td>
<td>poliovirus receptor</td>
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<tr>
<td>RD cells</td>
<td>human rhabdomyosarcoma cells</td>
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<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid tripeptide</td>
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<tr>
<td>RmcB</td>
<td>anti-CAR monoclonal antibody</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>Rp-a</td>
<td>CAR receptor protein</td>
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<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>SA-HRP</td>
<td>streptavidin-horseradish peroxidase conjugate</td>
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<tr>
<td>sCAR</td>
<td>soluble CAR</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeat</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>Src</td>
<td>sarcoma</td>
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<td>Src homology</td>
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<td>SRE</td>
<td>serum response element</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>TBST</td>
<td>TBS with 0.05% Tween</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TPO</td>
<td>thrombopoietin</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>tyrosine</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<tr>
<td>vCAR</td>
<td>Vero cell CAR</td>
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<tr>
<td>VLA-2</td>
<td>very late antigen-2</td>
</tr>
<tr>
<td>VP</td>
<td>viral coat protein</td>
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<td>VRC</td>
<td>virus-receptor complex</td>
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DISSEMINATION OF THESIS CONTENT


CHAPTER 1

INTRODUCTION

Myocarditis is an inflammatory disorder of the myocardium (Richardson 1996). It is defined by evidence of myocyte destruction with an adjacent inflammatory cell infiltrate (Aretz 1986) While the etiology of myocarditis may be quite varied, infection is a common cause of this disease. Group B coxsackieviruses (CVBs) are often identified as the infectious agent associated with acute myocarditis (Woodruff 1980). The impact of myocarditis is significant. Clinical presentation varies from mildly symptomatic to fulminant heart failure to the development of dilated cardiomyopathy (DCM), requiring chronic supportive therapy or ultimately heart transplantation. Over 50% of patients with biopsy-proven myocarditis do not survive for 5 years following diagnosis (Mason 1995).

Considerable advances have been made in our understanding of the pathogenesis of viral myocarditis. Currently evidence exists in support of two major mechanisms of myocardial injury: a direct involvement of the viral infection leading to death or dysfunction of the myocyte; and, damage to the myocardium by a virus-induced immune response.

The pathogenicity of specific coxsackieviruses may depend on genomic mutations which facilitate cardiovirulence (Tracy 1996; Lee 1997). In addition, RNA from specific coxsackievirus strains may persist in myocytes, which could alter normal cell function (Wessely 1998b). Lastly, coxsackieviruses encode proteases which have been shown to target host proteins, such as the structural protein dystrophin (Badorff 1999), and may be important in the evolution of DCM following myocarditis.

The T lymphocyte has a central role in the pathogenesis of myocarditis, however, the contribution of the immune system to the development of cardiac disease
has now been extended. Detrimental and beneficial effects of various proinflammatory and antiviral cytokines have been examined, as has the involvement of nitric oxide in the disease process. Overall, multiple elements of the immune response have been shown to impact of the development of CVB myocarditis.

The recent identification of coxsackie-adenovirus receptor (CAR) (Bergelson 1997a) and decay accelerating factor (DAF) (Bergelson 1995; Shafren 1995) as the cell surface receptors for CVBs has allowed for the development of a framework for the study of the viral receptors as determinants of host susceptibility to myocarditis. Particularly intriguing is the shared affinity of CAR with adenovirus, also a causative agent of viral myocarditis.

Advances in molecular diagnosis and antiviral therapy have contributed to clinical management of viral myocarditis. Important findings which focus on the prevention of CVB disease include the development of DNA vaccines for single or multiple CVBs, which are now undergoing testing in animal models.

However, identifying those at risk for coxsackieviral myocarditis remains a challenge common to many infectious agents, as a wide difference exists in the individual host response. Several immunodeficiencies have been shown to make an individual more vulnerable to infection, but recent findings have identified specific mutations that also affect susceptibility to specific infections. Two important genes, the interferon-\(\gamma\) receptor (IRF-1) gene, and the natural-resistance-associated macrophage 1 protein gene (NRAMP1) are determinants of individual susceptibility to infection with atypical mycobacteria and the Bacille Calmette-Guérin (BCG) vaccine strain (Newport 1996; Jouanguy 1999). A mutation in the Toll-like receptor-4 may contribute to variability in responses to lipopolysaccharide (endotoxin) (Arbour 2000). Resistance to human immunodeficiency virus (HIV) infection is conferred by a deletion in chemokine receptor (CCR)-5, a coreceptor of macrophage-trophic isolates of the virus (Liu 1996b; Samson 1996). The exploration of specific host determinants of susceptibility to CVB
myocarditis is therefore extremely relevant and timely. The key potential factors targeted in the studies presented in this thesis are the T lymphocyte, intracellular signal transduction and the expression of CAR.
A. MYOCARDITIS

1. CLASSIFICATION

Gore and Saphir et al (Gore 1947), presented an early classification of the etiology of myocarditis, which included various viral, bacterial, rickettsial, spirochetal and fungal diseases, toxic substances and hypersensitivity reactions. At that time 10% of myocarditis was rheumatic carditis, and 10% was due to diphtheria (Gore 1947). Approximately 8% of patients had histories consistent with a viral etiology. A more recent etiologic classification is presented in Table 1, with infectious, noninfectious and idiopathic headings providing a basis for a clinical approach to diagnosis (Opavsky 1998). Lymphocytic myocarditis and post-viral myocarditis are often used interchangeably, referring to cases where the etiology is unknown, but a virus is suspected (Karjalainen 1993). Many cases of myocarditis remain labeled idiopathic.

Other classification systems are also relevant to the disease. Myocarditis can be classified according to histologic appearance. The pathologic diagnosis of myocarditis includes colocalization of myocyte injury with cellular infiltration, while borderline myocarditis may have either lymphocytic infiltration or myocyte necrosis alone (Aretz 1987). Classification based on clinical presentation may have important implications for management and prognosis. In a recent study, patients with fulminant myocarditis had a better outcome than those with an acute, but less severe clinical presentation (McCarthy 2000).

2. THE EPIDEMIOLOGY OF MYOCARDITIS

Establishing the true incidence of myocarditis in the general population is difficult due to its varied clinical presentation. In one autopsy series 1402 cases of
<table>
<thead>
<tr>
<th>Table 1: Etiology of Myocarditis</th>
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<tbody>
<tr>
<td><strong>Infectious</strong></td>
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<td><strong>Non-Infectious</strong></td>
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<td><strong>Idiopathic</strong></td>
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myocarditis from 40,000 autopsy specimens were identified, representing an overall prevalence of 3.5% (Gore 1947). Similarly, isolated focal myocarditis was identified in autopsies of 2.3% to 5% of accidental deaths (Stevens 1970; Bandt 1979). In a younger population the prevalence of myocarditis at autopsy was much higher following sudden non-accidental death, with 17 to 21% of children affected (Okuni 1975; Bandt 1979). Using stricter diagnostic criteria, the evaluation of 12,747 serial autopsy specimens in Malmo, Sweden over a 10 year period [1975-1984] revealed a prevalence of myocarditis of 1.06% (Gravanis 1991). Epidemiologic studies based on pathologic examination of endomyocardial biopsies early in the course of myocarditis may be more representative than autopsy series. Patients presenting with congestive heart failure have been assessed for myocarditis by endomyocardial biopsy in several studies. In fifteen such studies the reported incidence of biopsy-diagnosed myocarditis in patients with unexplained congestive heart failure of recent onset, ranged widely, from 0% to 67% in studies reviewed (Nippoldt 1982; Fenoglio 1983; Parrillo 1984; Rose 1984; Zee-Cheng 1984; Cassling 1985; Dec 1985; Hosenpud 1985; French 1986; Chow 1988; Popma 1989; Vasiljevic 1990; Lieberman 1991; Herskowitz 1993; Mason 1995). The wide range may relate to different inclusion criteria, demographics, viral epidemiology, and inherent differences in host susceptibility (Lie 1988).

3. EPIDEMIOLOGY OF INFECTIOUS MYOCARDITIS

Many infectious agents, including viral, bacterial, parasitic, and fungal pathogens, have the potential to produce myocardial inflammation and necrosis. The pathogens listed in Table 2 have been associated with infectious myocarditis in humans. The true incidence of infectious myocarditis is not known as mild or asymptomatic myocarditis may be missed, or in many instances cardiac dysfunction may be overlooked in the presence of a generalized illness (Friman 1995). Estimates
<table>
<thead>
<tr>
<th>GROUP</th>
<th>common</th>
<th>uncommon</th>
<th>rare</th>
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<tbody>
<tr>
<td>Viral</td>
<td>coxsackieviruses, group A</td>
<td>adenovirus</td>
<td>Dengue</td>
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<tr>
<td></td>
<td>coxsackieviruses, group B</td>
<td>CMV</td>
<td>Epstein-Barr virus</td>
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<td></td>
<td>echoviruses</td>
<td>HIV</td>
<td>hepatitis B &amp; C</td>
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<td></td>
<td>influenza A &amp; B</td>
<td>poliomyelitis</td>
<td>hemorrhagic fevers</td>
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<td></td>
<td></td>
<td>HSV</td>
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<td>mumps</td>
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<td>parainfluenza 2</td>
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<td>rubella</td>
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<td>vaccina &amp; variola</td>
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<td>varicella</td>
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<td>yellow fever</td>
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<tr>
<td>Bacterial</td>
<td>Corynebacterium diphtheriae&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chlamydia pneumonia</td>
<td>Actinomyces israelii</td>
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<tr>
<td></td>
<td>Lyme disease&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chlamydia psittaci</td>
<td>Borrelia recurrentis</td>
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<td></td>
<td>Mycoplasma pneumonia</td>
<td>Clostridium perfringens&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Brucella abortus&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td>Rocky Mountain spotted fever&lt;sup&gt;a&lt;/sup&gt;</td>
<td>leptospirosis&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>Coxiella brunetii</td>
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<td></td>
<td></td>
<td>Neisseria meningitidis&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Chlamydia trachomatis&lt;sup&gt;C&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>scrub typhus (R.tsutsugamushi)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Escherichia coli</td>
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<td>Streptococcus pyogenes</td>
<td>Hemophilus influenzae</td>
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<td>Legionella</td>
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<td>Listeria monocytogenes</td>
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<td>Mycobacterium tuberculosis</td>
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<td>Salmonella species</td>
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<td>Staphylococcus aureus</td>
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<td>Tropheryma whippelii</td>
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<td></td>
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<td>Yersinia enterocolitica</td>
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<tr>
<td>Fungi</td>
<td>aspergillus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Cryptococcus neoformans&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Coccioidioides immitis&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>candida&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>Parasites</td>
<td>Chagas' disease&lt;sup&gt;a&lt;/sup&gt;</td>
<td>African trypanosomiasis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Toxocara canis (visceral larva migrans)</td>
</tr>
<tr>
<td></td>
<td>Toxoplasma gondii&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Trichinella spiralis</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Geographic variation in occurrence; <sup>b</sup>Immunosuppressed patients more commonly affected; <sup>c</sup>Can occur as part of congenital infection; <sup>d</sup>More prevalent in severe systemic disease. Modified from Opavsky et al 1998.
based on clinical assessment in combination with non-invasive studies such as ECG, myocardial enzyme levels and echocardiography and epidemiologic features have suggested that up to 15% of patients with common infectious diseases have presumptive evidence of myocarditis (Bengtsson 1957; Bandt 1979; Woodruff 1980; Karjalainen 1993; Friman 1995).

Over the past 50 years it has been established that viruses are a primary cause of acute myocarditis (Woodruff 1980; O'Connell 1987). O'Connell has estimated that at least 70% of the population has come into contact with cardiotropic viruses at some point in their life (O'Connell 1987). Acute myocarditis, although not always clinically apparent, may occur in one half of those infected (O'Connell 1987). A recent history compatible with a viral illness is common in patients with myocarditis. Forty-two percent to 88% of patients with recent onset heart failure and evidence of myocarditis by myocardial biopsy were documented to have a prodrome consistent with a viral illness (Nippoldt 1982; Fenoglio 1983; Parrillo 1984; Dec 1985; Popma 1989). Electrocardiographic changes are found in up to 40% of patients with viral infections such as infectious mononucleosis, poliomyelitis, and measles (Befter 1947; Bradford 1950; Laake 1951; Ross 1952; Goldfield 1955; Fish 1958). These series support an extraordinary population of individuals with subclinical myocarditis, about which relatively little is known.

Global surveillance by the World Health Organization (WHO) from 1975 to 1985 revealed an incidence of 30 to 40 cardiovascular diagnoses per 1000 cases of documented viral infections were attributable to Group B coxsackieviruses (CVBs) (Grist 1993). Influenza A and B, unspecified picornaviruses, and Group A coxsackieviruses were also common. More recently, Fairley et al (1996) investigated the etiology of all cases of infectious myocarditis, pericarditis, or myopericarditis, collected by a national laboratory surveillance program in England and Wales from 1990 to 1993, to obtain an overall perspective on the disease in the developed world.
Enteroviruses were associated with 21% of the 81 cases of myocarditis (Fairley 1996). Of the 134 patients with myopericarditis, a remarkable 132 had evidence of CVB infection. Influenza A and B were associated with 21% and 14% of cases of myocarditis, respectively (Fairley 1996). *Mycoplasma pneumoniae* was also common, being diagnosed in 14% of patients with myocarditis (Fairley 1996). Myocarditis was most often the primary illness with viral, chlamydial or mycoplasma infection (Fairley 1996). In cases associated with bacterial pathogens, the primary diagnoses included pneumonia, endocarditis, and other focal sites of infection, with involvement of the myocardium being secondary (Fairley 1996).

Currently, the most predominant infectious agents associated with myocarditis vary according to geographic location. In North America and the developed world, viruses are most often responsible for myocarditis. However, in South and Central America *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is the principal cause of myocarditis (Peters 1991; Hagar 1995). Over 20 million people are infected with *T. cruzi* worldwide, and therefore at risk for myocarditis and dilated cardiomyopathy (Hagar 1991; Hagar 1995). Diptheria is now rare in developed countries, but continues to be endemic in developing countries and the former Soviet Union where it may lead to myocarditis (Halvadar 1992; World Health Organization April 1993). The tick-borne agent of Rocky Mountain spotted fever, is endemic throughout the United States (Wilfert 1984). Transmitted by a different tick vector, the agent of Lyme Disease, occurs in foci in the Northeast, the Midwest, and the West in the United States, and also occurs in Europe and Scandinavia, the former Soviet Union, China, Japan, and Australia (Steere 1979; Stewart 1982; Schmid 1985; Kawabata 1987; Dekonenko 1988; Ai 1990).

Clusters of increased prevalence of myocarditis often reflect specific viral seasonal epidemics (Grist 1974; Woodruff 1980). The first observations of a connection between infection and myocardial disease were related to epidemics of
mumps, influenza, poliovirus and enteroviruses (Woodruff 1980; See 1991). Some enteroviral infections, including CVB2, CVB3 and CVA9, are often endemic; while those with CVB5 are more likely to be epidemic in nature (See 1991). Approximately 5 to 10% of such patients may experience cardiac symptoms during epidemics due to coxsackie B viruses, poliovirus or influenza virus (Bandt 1979).

Martino et al (1995) summarized four studies (Helin 1968; Sainani 1968; Smith 1970; Sainani 1975) examining host age as a factor in viral myocarditis. Fifty-two percent of cases of myocarditis occur in young adults aged 20 to 39 years, and susceptibility declines with increasing age, with 14% of cases of myocarditis found in those aged 40 to 59 years and 5% in patients 60 years or older. Many of the viruses that are associated with myocarditis are very common during childhood, but children are not the most susceptible group. However, during the first 6 months of life children are particularly sensitive to enteroviral infection (Grist 1978; Baker 1980; Woodruff 1980; Kaplan 1983). In addition to age, factors which contribute to susceptibility to viral myocarditis include gender, pregnancy, nutritional status and exercise (Woodruff 1980; Lyden 1984; Reyes 1985; Lyden 1987; Beck 1994).

4. THE ROLE OF ENTEROVIRUSES IN MYOCARDITIS

Among the cardiotropic viruses, enteroviruses are thought to be the leading cause of myocarditis, with Group B coxsackieviruses involved in about 50% of cases (Smith 1970; Grist 1974; Woodruff 1980; McManus 1986). Initial evidence from studies using primarily serological diagnosis and viral culture is now supported by molecular detection of viral RNA from cardiac biopsy/autopsy specimens.

In infants, CVBs 1-5 may produce a severe systemic illness characterized by myocarditis, meningoencephalitis and hepatitis, which is often fatal (Javett 1956; Kibrick 1958; Jennings 1966). Beyond infancy, myocarditis has been associated with
group A coxsackieviruses (serotypes 4, 14, and 16) (Bell 1968; Melnick 1996a) CVBs (serotypes 1 to 5) (Bell 1968; Helin 1968; Sainani 1968; Smith 1970; Melnick 1996a) and echoviruses (serotypes 1, 6, 9, 19 and 22) (Russell 1970; Bell 1971; Monif 1976; Melnick 1996a). The recent findings of Fairley et al (1996) show that 63% of all cases of myocarditis and myopericarditis were associated with CVB infection. Conversely, in individuals with CVB infections, the incidence of cardiac involvement is on average about 5%, (Woodruff 1980) primarily based on documented seroconversion.

Until recently most of the evidence for the etiologic role of enteroviruses in myocarditis came from the correlation between elevated virus specific antibody titre and viral isolation from sites other than the myocardium (Bell 1968; Sainani 1968; Smith 1970; Schmidt 1973; Sainani 1975; Toshima 1979). Enteroviruses also have been isolated infrequently directly from myocardial tissue (Crevel 1956; Kibrick 1958; Sutton 1963; Sutton 1967; Longson 1969; Monif 1976). Serologic evaluation of the link between enteroviral infection and myocarditis has indicated an overall association of approximately 33%, and was based on the demonstration of virus specific IgM antibody (El-Hagrassy 1980; McCartney 1986; Muir 1989; Tracy 1990a), elevated neutralizing antibody titres (Helin 1968; Smith 1970; Grist 1974; McCartney 1986; Tracy 1990a), or a documented 4-fold change in antibody titre in paired sera (Smith 1970; Sainani 1975), in association with the illness. Also, elevated enteroviral antibody titres have been documented more commonly in patients with congestive heart failure due to myocarditis, compared to patients with other causes of heart failure (Cambridge 1979; Kitaura 1981; Lau 1982).

The identification of enteroviral RNA in the hearts of patients with myocarditis and dilated cardiomyopathy strongly supports an etiologic role for enteroviruses in myocarditis as initially shown by Bowles et al (1986) using slot blot probe hybridization (Bowles 1986; Bowles 1989; Archard 1991). Subsequently, several studies using PCR or ISH have documented a prevalence of enteroviral RNA in 18% to 83% of
myocardial biopsies from patients with myocarditis (Table 3) (Easton 1988; Jin 1990; Tracy 1990b; Weiss 1991; Hilton 1993; Kandolf 1993; Giacca 1994; Martin 1994; Satoh 1994; Nicholson 1995; Ueno 1995; Fujioka 1996; Pauschinger 1997; Grumbach 1999). Limitations of these studies include: different patient populations; different definitions of myocarditis; the focal nature of myocardial involvement and limitations of the biopsy technique; variable sensitivity of molecular detection methods; and seasonal or geographical distribution of infectious agents.

It is important to distinguish between active viral replication at a level below that detectable by routine viral culture techniques and the persistence of viral genome when enteroviral RNA is detected in the myocardium (Hohenadl 1991). Because enteroviruses have single-stranded positive sense RNA genome, negative strand RNA is produced only during replication (Hohenadl 1991). Detection of minus-strand RNA by PCR indicates the presence of replicating virus and has been suggested to be of prognostic importance (Pauschinger 1997; Pauschinger 1998).

5. CLINICAL PRESENTATION AND DIAGNOSIS

i) Clinical presentation

Clinical suspicion of myocarditis, based on both cardiac-specific and non-specific findings, is the most important step in ensuring an early diagnosis. In most patients, mild myocarditis is not clinically apparent, and as a consequence often goes unrecognized (Abelman 1971). Children often present acutely, with chest pain, congestive heart failure, shock, arrhythmias, or sudden death (Okuni 1975; Bandt 1979; Karjalainen 1993; Gowrishankar 1994). Pain usually reflects associated pericarditis, and is typically sharp and pleuritic in nature, but may also be mistaken for acute myocardial infarction (Dec 1992; Karjalainen 1993; Narula 1993). Fatigue, dyspnea, and orthopnea, characteristic of heart failure, or palpitations and syncope
<table>
<thead>
<tr>
<th>Detection method</th>
<th>% positive (# of patients)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40% (2/5)</td>
<td>Jin et al, 1990</td>
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<tr>
<td></td>
<td>20% (1/5)</td>
<td>Weiss et al, 1991</td>
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<tr>
<td></td>
<td>21% (7/34)</td>
<td>Martin et al, 1994</td>
</tr>
<tr>
<td></td>
<td>33% (1/3)</td>
<td>Giacca et al, 1994</td>
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<td></td>
<td>33% (12/36)</td>
<td>Satoh et al, 1994</td>
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<tr>
<td></td>
<td>80% (4/5)</td>
<td>Ueno et al, 1995</td>
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<td></td>
<td>83% (5/6)</td>
<td>Nicholson et al, 1995</td>
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<td></td>
<td>18% (5/28)</td>
<td>Fujioka et al, 1996</td>
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<td></td>
<td>41% (21/51)</td>
<td>Pauschinger et al 1997</td>
</tr>
<tr>
<td></td>
<td>20% (2/10)</td>
<td>Grumbach et al, 1999</td>
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<tr>
<td><strong>ISH</strong></td>
<td></td>
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<td></td>
<td>46% (6/13)</td>
<td>Easton &amp; Eglin, 1988</td>
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<td></td>
<td>18% (3/17)</td>
<td>Tracy et al, 1990</td>
</tr>
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<td></td>
<td>20% (2/10)</td>
<td>Hilton et al, 1993</td>
</tr>
<tr>
<td></td>
<td>24% (23/95)</td>
<td>Kandolf et al, 1993</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction; ISH = *in situ* hybridization

representing ventricular arrhythmias also occur (Karjalainen 1993). Ventricular tachycardia and fibrillation are seen early in the course of acute myocarditis (Karjalainen 1984) and are likely responsible for cases of sudden death. Atrioventricular block is serious but uncommon, except in association with Lyme disease, when it is reversible (Steere 1980; McAlister 1989; Cox 1991; van der Linde 1991; Nagi 1996).

On physical examination evidence of heart failure may include tachycardia, hypotension, elevated jugular venous pressure, ventricular gallop, rales on chest auscultation, and peripheral edema (Peters 1991; Karjalainen 1993). A pericardial friction rub may be found in myopericarditis. The physical exam may be completely normal in mild cases of myocarditis.

Symptoms and signs of the systemic infection or underlying disease may accompany cardiac-specific findings. For example, fever, skin lesions or pulmonary disease may provide clues to the responsible etiologic agent. If cardiac involvement is mild, cardiac symptoms may be overshadowed by systemic manifestations (Abelman 1971). In the neonatal period, enteroviral infection is a severe multisystem disease affecting the central nervous system, the heart and the liver (Baker 1980; Woodruff 1980; Kaplan 1983).

ii) The natural history of myocarditis

Many patients with symptomatic myocarditis, in particular those with fulminant disease, recover quickly and completely with general supportive care and heart-specific therapy (McCarthy 2000). Both symptomatic and subclinical cases can evolve into DCM and chronic heart failure ultimately necessitating organ transplantation or culminating in death (Quigley 1987; Levi 1988). In the NIH myocarditis trial patients with biopsy confirmed myocarditis by the Dallas criteria (Aretz 1986) had a mortality of 20% at one year and 56% at 4.3 years (Mason 1995). Thus, although myocarditis does
resolve spontaneously in many patients, significant early and late morbidity exists, including heart failure and death.

6. THE LINK BETWEEN VIRAL MYOCARDITIS AND DILATED CARDIOMYOPATHY

Dilated cardiomyopathy is characterized by dilated and poorly functioning ventricles (Abelman 1984; Abelman 1988; Goodwin 1992; Richardson 1996). DCM is believed to be a common end point of many processes, including infection, toxins, alcohol and genetic cardiac disease. Seven to 10 cases of DCM occur per 100,000 inhabitants per year, and it has been suggested that a significant proportion, up to 20%, are a consequence of antecedent infection of the heart (Quigley 1987; Levi 1988; Sole 1993). Dilated cardiomyopathy is commonly a cause of congestive heart failure and an indication for heart transplantation. With heart failure reaching epidemic proportions at present, DCM has a significant impact on public health worldwide (Torp 1978; Gillum 1986; Rakar 1997; Liu 2001).

Several investigators have contributed to the establishment of the relationship between viral myocarditis and DCM. As with myocarditis, the concept for this association arose from a history compatible with a viral infection in DCM patients and was subsequently confirmed by serological testing (Cambridge 1979; El-Hagrassy 1980; McCartney 1986; Muir 1989). The localization of virus by ISH in areas of myofiber degeneration, hypertrophy and fibrosis has provided more definitive evidence for the link between DCM and myocarditis of viral etiology (Easton 1988; Kandolf 1991; Martino 1994). In 20 studies the detection of enterovirus RNA in the myocardium by RT-PCR in patients with acute, chronic or end-stage DCM, ranged from 0 to 58%, with a mean detection rate of 20% (Jin 1990; Weiss 1991; Grasso 1992; Keeling 1992; Koide 1992; Weiss 1992; Zoll 1992; Liljeqvist 1993; Schwaiger 1993). Not surprisingly, other viruses have been detected by nucleic acid probes and PCR in
the heart tissue of patients with DCM, including CMV (Maisch 1993; Schonian 1995), adenovirus (Griffin 1995; Hufnagel 1997; Grumbach 1999), and hepatitis C (Matsumori 1995). Viral detection may be of prognostic value, since enterovirus-positive myocardium in DCM patients was correlated with advanced disease, a requirement for cardiac transplantation (Bowles 1989), and a higher mortality (Archard 1991).

7. THE TREATMENT AND PREVENTION OF VIRAL MYOCARDITIS

Currently, acute myocarditis is treated by supportive therapy which includes the conventional approaches to the treatment of heart failure and managing complications as they occur (Liu 2001). Angiotensin-converting enzyme (ACE) inhibitors and diuretics, with or without digoxin are indicated for patients in heart failure with myocarditis and DCM (Cohn 1991; The SOLVD Investigators 1991; Liu 1992). Early in the course of myocarditis, antiarrhythmics, such as amiodarone, may also be required to control severe ventricular arrhythmias. More aggressive therapy, including vigorous diuresis, inotropic agents and transvenous pacing may be required in fulminant presentations (Liu 1996a). Extracorporeal membrane oxygenation (ECMO) and left ventricular assist devices (LVAD) may be required to support circulation until recovery from acute myocarditis (Nagai 1996). If supported aggressively, patients with severe haemodynamic compromise have an optimistic prognosis (McCarthy 2000; Liu 2001). Heart transplant is a viable option for patients with acute viral myocarditis in cardiogenic shock or patients with end-stage DCM, unresponsive to aggressive medical management.

Several uncontrolled and/or nonrandomized studies have investigated whether immunosuppressive therapy has a role in myocarditis. Overall, it appears that some therapeutic benefit exists; however, the effects of such therapy ranged widely in different studies (Liu 1992). In most studies corticosteroids, azathioprine, and
cyclosporine, alone or in combination were evaluated (Mason 1980; Edwards 1982; Fenoglio 1983; Vignola 1984; Zee-Cheng 1984; Dec 1985; Hosenpud 1985; Anderson 1987; Salvi 1989; Chan 1991; Jones 1991; Gagliardi 1993; Kuhl 1994; Maisch 1994; Kleinert 1997). In two preliminary studies in children the use of IVIG was evaluated, using similar protocols to those employed in Kawasaki's disease. Therapy appeared to improve left ventricular function and tended to increase survival (Drucker 1994; McNamara 1997). The NIH Myocarditis Treatment Trial was designed to evaluate the effect of immunosuppression on active myocarditis diagnosed using the Dallas criteria (Mason 1995). Immunosuppressive therapy, either prednisone and azathioprine or prednisone and cyclosporine, did not improve ventricular function or survival as compared to controls receiving conventional therapy (Mason 1995). This trial did not establish a definitive role for immunosuppressive treatment. However, the outcomes of patients presenting in acute viral, autoimmune, and chronic phases of disease were pooled in this study. As well, diverse etiologic agents may have been responsible for the clinical picture of myocardial disease in these patients. Improved cardiac function was associated with increased evidence of immune activation in patients of both groups (Mason 1995; Liu 2001), suggesting that at least some aspects of the host immune response are important in myocardial protection. Immunosuppression should be avoided in at least two subgroups of patients with myocarditis, namely in those with no inflammatory infiltrate on biopsy, ideally confirmed by immunohistological staining (Kuhl 1992), and in patients early in the course of disease with active replicating virus present. With immunosuppression, viral proliferation can be enhanced resulting in more severe infection. This suggests that myocardial biopsies may be indicated despite their limitations. Further identification of specific elements of the host immune response that increase host susceptibility to viral myocarditis is expected to lead to maximal effective targeted therapy.
Specific antiviral agents are now being developed for use in a wide range of infectious diseases. With an optimized diagnostic strategy for determining viral etiology, the use of specific antiviral agents in the therapy of myocarditis will become a plausible therapeutic approach. A distinct class of antiviral agents, isoxazoles, with activity against enteroviruses and other picornaviruses, has been undergoing animal and clinical evaluation (McKinlay 1993). Isoxazoles bind to a hydrophobic pocket in the bottom of the "canyon" on the surface of the enterovirus particle, altering virion stability and blocking the release of the genomic RNA after attachment and penetration of the host cell membrane, thereby preventing viral replication (McKinlay 1993). WIN 54954, a member of this antiviral family, tested in a murine CVB3 model reduced mortality but not myocardial inflammation (Fohlman 1996). Another anti-picornaviral compound, VP 63843 or Pleconaril was reported to reduce the duration and severity of symptoms in patients with enteroviral meningitis (Weiner 1997).

Active vaccination against Group B coxsackieviruses has been effective at preventing both myocarditis and mortality in animal models (Matsumori 1987b). Live attenuated (Fohlman 1993; Zhang 1997b), inactivated whole virus vaccine (Fohlman 1993), and subunit vaccines (Fohlman 1990; Fohlman 1993), have all been assessed. Both inactivated virus and virus subunit preparations seem to induce at least partial protection against virus challenge and are safer than live attenuated vaccines (Fohlman 1993). Recently, immunization with a monovalent anti-CVB3 DNA vaccine (Henke 2000) and a polyvalent DNA vaccine developed against the viral capsid of 2 strains of CVB3 and one strain of CVB4 have provided protection from CVB3 myocarditis in animal models (Lee 2000a). Individuals at risk of myocarditis and DCM, perhaps based on an identifiable susceptibility, would be an important target population for vaccination.
B. ENTEOVIRAL MYOCARDITIS: PATHOGENESIS AND HOST SUSCEPTIBILITY

Considerable advances have been made in our understanding of the pathogenesis of viral myocarditis. Evidence has accumulated in support of two major mechanisms of myocardial injury: a direct involvement of the viral RNA leading to cell death or myocyte dysfunction; and, a virus-induced immune response targeting the heart. An early anti-viral immune response contributes to acute myocarditis, however, much later autoimmune phenomena may come into play in chronic myocarditis or DCM. The virus may be directly involved in both acute and chronic phases of disease. In this section evidence for both mechanisms will be presented, as well as a perspective on how elements of the immune response determine host susceptibility to coxsackieviral myocarditis.

1. THE GROUP B COXSACKIEVIRUS MURINE MODEL OF ENTEOVIRAL HEART DISEASE

Our understanding of the pathogenesis of viral myocarditis has been greatly advanced by the study of virus-induced disease in animal models (O'Connell 1985). Murine models, in particular, have contributed greatly to the understanding of the involvement of the virus and the immune response in the pathogenesis of viral myocarditis and to its relationship with DCM. The infection of mice with Group B coxsackieviruses produces a myocarditis which closely resembles human disease, resulting in a useful model to study the virally-induced heart disease (Martino 1995). More intriguing is the observation that the severity of acute and chronic viral myocarditis, and specific host responses to CVB infection, can vary among different mouse strains, and with different viruses (Gauntt 1984; Wolfgram 1986; Buie 1987; Lodge 1987; Klingel 1993).
The progression of CVB myocarditis in mice follows a well characterized pattern. Following intraperitoneal inoculation with CVB3, virus was detectable in the pancreas, liver and feces as early as 12 hours postinfection (pi), in the absence of detectable viremia. All tissues were infected by day 2 to 4 (Mena 2000). Within two days after intraperitoneal injection of mice with cardiotropic virus, myocardial damage becomes detectable, with evidence on microscopic examination of coarse granularity, pallor and multivesicular vacuolation of myocytes (Wilson 1969; McManus 1993). Groups of myocytes become affected by days 3 to 5 post-infection, followed by necrosis and the development of calcification (McManus 1993). Altered expression patterns of structural and nonstructural genes, such as GTPase, a subunit of NADH dehydrogenase, mouse β-globin and cAMP-regulated response element binding protein (CREB) binding protein (CBP) occurs 4 days post CVB3 infection (Yang 1999a).

Clearance of virus from the myocardium begins by day 4 post-infection (Woodruff 1974; Wong 1977; Martino 1994) coincident with the appearance of neutralizing antibody (Mena 2000). Viral replication in the heart is limited by the elevation of neutralizing antibody titres (Rager-Zisman 1973; Cho 1982), infiltrating macrophages (Bell 1971; Woodruff 1980), natural killer cells (Godeny 1986; Godeny 1987) and interferon (Kandolf 1985; Lutton 1985; Matsumori 1987a; Heim 1992). Subsequently, infiltrating T lymphocytes appear, primarily CD8+ cytotoxic T cells up to day 6, after which CD4+ helper T cells become more dominant (Godeny 1986; Klingel 1992). Classically, CD8+ T cells lyse infected host cells via perforin (Young 1990; Seko 1991; Seko 1993b), while CD4+ T cells activate effector cells via cytokines and chemokines (Mak 1994; Abbas 1996). Myocyte expression of major histocompatibility complex (MHC) class I antigen, intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and the costimulatory molecule CD40 are upregulated on CVB3 infected myocardial tissue (Seko 1993a; Seko 1996; Seko
From days 7 to 14 multiple foci of inflammatory cells and necrotic myocytes develop, giving the histologic appearance of acute myocarditis (Woodruff 1980; Lodge 1987; Klingel 1992; McManus 1993). Spasm of coronary microvasculature is found in the myocardium of infected mice (Silver 1989; Dong 1992). Infectious virus is completely cleared in the majority of animals 10 days after viral inoculation (Woodruff 1970; Woodruff 1980).

Extensive myocardial changes continue beyond day 14 in those murine strains which develop chronic myocarditis (Martino 1995). As the inflammatory response diminishes, replacement fibrosis, further calcification and myocyte hypertrophy become evident (Wilson 1969; Lodge 1987; Leslie 1990; Schnitt 1993). Eventually a condition similar to DCM in humans develops, with tissue scarring, cardiac dilation with little if any inflammation apparent (Reyes 1981; Matsumori 1982; Wee 1992). Although infectious virus disappears early, enteroviral RNA persists in the myocardium with the development of chronic disease (Klingel 1992; Wee 1992).

2. THE DIRECT ROLE OF THE VIRUS IN THE PATHOGENESIS OF CVB3 MYOCARDITIS

Current evidence in murine models suggests that the initial phase of CVB myocarditis is virally mediated (Kishimoto 1990; Mall 1991; Chow 1992; McManus 1993). Enterovirus infections are generally cytolytic in nature. Early in the course of disease, by day 3, necrotic myofibers are detected before any inflammatory cell infiltrate is apparent (Mall 1991; McManus 1993). In mice with severe combined immunodeficiency (SCID) (Chow 1993a) and nude athymic mice (Kishimoto 1990) extensive myocyte necrosis occurs without accompanying inflammatory infiltration, providing evidence for the early role for virus infection in myocyte damage. Viral RNA, detected by in situ hybridization (ISH) is apparent in a focal distribution in mouse
hearts early in the course of infection (Hofschneider 1990; Klingel 1993; Ukimura 1997; Klingel 1998).

CVB replicates in cultured human fetal and murine neonatal cardiac myocytes (Yoneda 1979; Kandolf 1985). Myocytes lose their ability to contract by 2 days pi, indicating significant damage to cell homeostasis. Classic cytopathic effect with myofibril and myofilament destruction and the development of cytoplasmic vacuolization is evident with electron microscopy.

Evidence is also accumulating for a role for virus in the pathogenesis of chronic myocarditis and DCM. Enteroviral RNA has been localized to myocarditic foci as long as 30 days following infection of mice with CVB3 (Klingel 1992), and in the encephalomyocarditis virus (EMCV) model viral RNA persists up to 3 months following infection (Kyu 1992; Wee 1992), suggesting a direct role in the evolution of DCM (Martino 1994). Viral RNA may continue to be replicated at low levels, and the presence of both positive and negative strands of viral RNA is indicative of such ongoing replication (Hohenadl 1991; Klingel 1992; Klingel 1996; Andréoletti 1997; Pauschinger 1998). Persistent extracardiac reservoirs of CVB3, such as the spleen and lymph nodes, may also be relevant (Anderson 1996; Klingel 1996). This persistence beyond the acute phase of myocarditis may provide a target for ongoing immune activation, perpetuating the disease state (Klingel 1992; Kandolf 1993). Furthermore, the expression of replication deficient enteroviral genome in transgenic mice impairs myocardial function and causes dilated cardiomyopathy (Wessely 1998a; Wessely 1998b). Viral nucleic acids may also alter myocardial function directly, by triggering myocyte dropout via necrosis or apoptosis, or by inhibiting normal cellular function, contributing to a failing heart. Viral proteins are also implicated in the evolution of chronic heart disease. Proteolytic cleavage of the cytoskeletal protein dystrophin by enteroviral protease 2A may contribute to the evolution of post-viral dilated cardiomyopathy (Badorff 2000; Lee 2000b).
3. THE ROLE OF THE IMMUNE RESPONSE IN THE DEVELOPMENT OF CVB3 MYOCARDITIS

While numerous components of the immune response offer protection from viral damage via inhibition of viral replication and clearance of virus-infected cells, they may also promote host tissue damage through excess activation of cytokines and cellular components of the immune system, as illustrated in Figure 1. The genetics of both the virus and the host may also influence the balance between protective and destructive elements of the immune response. The innate and specific arms of the immune response that participate in the response to viral infection.

i) The host immune response to viral infection

The host immune response to viral infection is composed of innate or nonspecific responses and adaptive or specific responses. Both innate and specific components, and the effector mechanisms they direct, are critical to the control of viral infections and impact on resulting pathology (Figure 2).

Cells of the innate immune system include monocytes and macrophages, natural killer (NK) cells, dendritic cells, and polymorphonuclear leukocytes (PMNs). NK cells and PMNs are distributed in the circulation and the spleen, but respond rapidly when challenged and migrate to the involved tissue. Monocytes, macrophages and dendritic cells are located in the lymph nodes as well. From the spleen and lymph nodes these cells process and present antigens to activate the adaptive immune responses. Lymphocytes are the cell mediators of specific immunity. Their role is in the discrimination of self versus nonself. Cytokines not only participate in innate immune responses, but also mediate the effects of specific cellular responses.

Innate responses are the host's first line of resistance in the face of viral infection, until specific responses develop (Fearon 1996; Mak 1998). Within a day
Figure 1: Elements of the immune response which may determine host susceptibility to viral myocarditis. Some elements of the immune reaction are protective and attenuate viral replication with minimal myocardial damage. Others can promote a destructive inflammatory response, including tumour necrosis factor-α and T lymphocytes. The outcome of viral infection of the heart likely depends upon the balance between the protective and destructive responses. In addition, host and viral genetics both play significant roles in susceptibility. Modified from Opavsky et al, 1998.
Figure 2: The immune response to viral infection. Both the innate and adaptive arms of the immune response are activated by viral infection. Components of the innate response are activated early. Interferon-α/β (IFN-α/β) induce activation and cytotoxicity of natural killer (NK) cells. Interleukin-12 (IL-12) is a potent activator of NK cells as well. Interferon-γ (IFN-γ) activates macrophages (MAC), which produce IL-12 and tumour necrosis factor-α (TNF-α). Adaptive responses to viral infections include T cell (CD4+ and CD8+) and B cell activation, with cytotoxicity, antiviral and proinflammatory cytokine production, and apoptosis of infected cells. IL-2 is a potent T cell growth factor. Modified from Ahmed and Biron, 1999.
Following infection innate responses are activated, and provide protection via induction of type 1 interferons (IFN-α and -β), inducible nitric oxide synthase (iNOS;NOS2) dependent mechanisms, and NK cell-mediated cell death. Interferon-α/β have direct antiviral effects and stimulate NK cell cytotoxicity (Biron 1994; Berke 1995). INOS, produced by activated macrophages, catalyzes the production of nitric oxide (NO), which can disrupt several viral and host mechanisms needed for viral replication (Karupiah 1993; Bi 1995; MacMicking 1997; Tay 1997; Saura 1999). Macrophages also participate in host defense by producing IL-12, a potent stimulator of IFN-γ production by NK cells, and TNF-α (Orange 1995; Orange 1996). IFN-γ induces macrophages to produce NO, IL-12 and tumor necrosis factor-α (TNF-α) (MacMicking 1997; Coffman 1999).

The two major antigen-specific elements of the adaptive immune response are T lymphocytes and antibodies. Two different combinations of the chains of the T cell receptor (TCR) participate in antigen recognition, TCRα/β and TCRγ/δ (Abbas 1996; Kaufmann 1996). The α/β+T cells express either CD4 or CD8 coreceptors, and recognize antigen in association with major histocompatibility complex (MHC) II or MHC I, respectively (Zinkernagel 1974; Kaufmann 1996). The interaction of the T cell receptor (TCR) with the MHC-peptide complex triggers T cell differentiation and proliferation (Abbas 1996). The T cell receptor is a multisubunit complex which is fully functional in association with accessory molecules. Following activation during viral infection, CD8+ T cells destroy virus-infected cells by two mechanisms: release of cytoplasmic granules containing perforin which lyses target cell membranes and granzymes which trigger apoptosis by stimulation of caspases (Kägi 1994); and, the induction of apoptosis via FAS/FAS ligand (Berke 1995). They also elaborate IFN-γ and TNF-α which interfere with viral replication, and activate macrophages. CD4+ T cells release IFN-γ and TNF-α, activate macrophages, and provide help for cytotoxic CD8+ T cell responses and the activation of B cells and subsequent antiviral antibody
production (Clark 1994; Abbas 1996). Free virus is recognized by its specific antibodies which inhibit ongoing viral replication by neutralization of virions and by killing infected cells by complement-mediated cytotoxicity or ADCC.

CD4+ T cells express two phenotypic cytokine profiles, designated Th1 and Th2 (Rocken 1992; Fitch 1993). Th1 responses are involved in the defense of viral infection, while Th2 cells respond in allergic reactions or to parasitic infections (Abbas 1996). Th1 type cells typically express IFN-γ and IL-2, while Th2 cells produce IL-4, and IL-5 (Abbas 1996). Susceptibility to a variety of infectious diseases has been linked to the balance between these two functional T cell subsets (Abbas 1996). The γ/δ+T cells are in the minority and are able to recognize proteins directly in an unrestricted fashion (Kaufmann 1996). They typically express a Th1 cytokine profile and display cytolytic activities (Kaufmann 1996). Recently a unifying role for γ/δ+T cells has been proposed in linking the innate and acquired arms of the immune response as cytokines elaborated by this T cell subset can influence T cell responses (Groh 1998; Mak 1998; Mallick-Wood 1998; Zuany-Amorim 1998).

ii) The role of the T cell in enteroviral myocarditis

Although classically considered to be protective in the context of viral infection, T lymphocytes are implicated in myocardial damage in murine models of CVB3 myocarditis. Evidence for this comes from several studies. Cardiac inflammation and myocyte necrosis are prevented by the depletion of T lymphocytes by thymectomy, irradiation and bone marrow reconstitution; or, by injection of antithymocyte serum (Woodruff 1974). In addition, depletion of CD4+ or CD8+ T cells by administration of specific monoclonal antibodies therapy can prevent myocardial injury in CVB3 infected mice, implicating specific T cell subsets in immune-mediated myocyte damage (Lodge 1987; Kishimoto 1989). A series of immune knockout models provide clear evidence for the role of T cells in pathogenesis of CVB3 myocarditis. Gene-
targeted knockout of both CD4+ and CD8+ T cells or all TCR β+ T cells protects the heart in CVB3 infected mice (Opavsky 1999). Minimal myocarditis develops in β2 microglobulin knockout mice, which are unable to process antigen via the MHC I pathway, impairing the cytotoxic lymphocyte response (Henke 1995). With knockout of the T cell specific tyrosine kinase, p56lk, which is essential to T cell activation, survival of CVB3 infected mice is 100% and myocarditis does not develop (Liu 2000). Disruption of exon 6 of the CD45 gene, coding for a protein tyrosine phosphatase important to T cell activation, also results in 100% survival with little cardiac disease apparent (Irie-Sasaki 2001). These studies support the general hypothesis that T cell activation plays a central role in the pathogenesis of CVB3 myocarditis.

iii) Natural killer cells

NK cells appear to have a protective role in CVB3 myocarditis, as they do in most viral infections. Activation of NK cells occurs as early as day 3 after CVB3 infection (Godeny 1986). Following depletion of NK cells with anti-asialo GM1 antiserum, CVB3 titres increased in the heart and myocarditis was more severe than in untreated infected animals (Godeny 1986). The transfusion of asialo-GM1+ splenic cells (presumed NK cells) into NK cell-depleted mice provided protection form CVB3 infection (Godeny 1987). In CD4−/CD8−/ mice protection from CVB3 myocarditis was associated with early NK cell infiltration and increased myocardial IFNγ expression as compared to controls (Opavsky 1999). NK cells can mediate protection via several mechanisms, as discussed in Section 3i above.

iv) Macrophages

Five to ten days following CVB inoculation, macrophages are detected in the myocardium of infected mice. Opposing roles for the macrophage in the development of CVB myocarditis have been reported. Genetic knockout of the chemokine macrophage inflammatory protein -1α (MIP-1α) protected mice from the development
of myocarditis indicating a detrimental role for the macrophage (Cook 1995). Other studies have supported a protective role for macrophage in host defense to viral myocarditis (Woodruff 1979). Macrophage function can be impaired by the uptake of injected silica particles. This treatment was found to increase mortality of mice following CVB3 infection, whereas the transfusion of activated macrophages protected mice from CVB3 disease, indicating a protective role for the macrophage in myocarditis (Rager-Zisman 1973). In addition, macrophage colony-stimulating factor protects mice from viral myocarditis, in part due to increased production of endogenous interferon from activated macrophages (Hiraoka 1995).

v) Cytokines

The proinflammatory cytokines, TNF-α, IL-1β, IL-6 and lymphotoxin-α (LT-α; TNF-β), and the interferons (IFNs) α, β, γ, with antiviral effects, are important participants in both innate and adaptive responses (Curfs 1997; Ahmed 1999). A role for many cytokines in the pathogenesis of myocarditis has been explored using the mouse model. The Th1 phenotype of CD4+ T cells has been associated with significant myocardial disease in CVB3 myocarditis, and may be gender-related (Huber 1994a; Seko 1997) CVB3 infection of the heart induces TNF-α, and IL-6 RNA expression in the heart by day 7 pi (Freeman 1998). Expression of these proinflammatory cytokines was associated with impaired cardiac function (Freeman 1998). Inducible NOS was detected in association with cytokine expression, and was found to be persistently elevated in susceptible mice. Myocardial expression of TNF-α, IL-1-α and β, IL-6 were elevated as early as 1 to 2 days pi (Seko 1997). The detrimental effects of TNF-α, including extensive fibrosis and inflammation, on the heart were demonstrated by myocyte-targeted expression of the cytokine (Bryant 1998). IL-1, also a proinflammatory cytokine promotes CVB3 myocarditis (Lane 1992; Huber 1994b). IL-2 has been reported to have opposing effects on the development of
CVB3 myocarditis (Huber 1994b). Moreover, host resistance to CVB3 myocarditis can be overcome by co-treatment with IL-1 or TNF-α (Lane 1992).

IFNs can inhibit CVB replication in cultured human myocardial fibroblasts (Heim 1995) and fetal cardiomyocytes (Kandolf 1985). Protection from CVB myocarditis by interferons has been repeatedly demonstrated in several studies, in association with decreased levels of virus in the heart (Lutton 1985; Kishimoto 1988; Matsumori 1988a). Interestingly, blocking IFN-β action using monoclonal antibodies exacerbated CVB3 myocarditis when administered 3 days post-viral inoculation (Lutton 1985). Expression of IFN-γ in the pancreas protected mice from CVB3 myocarditis, and resulted in reduced viral titres in both the pancreas and the heart (Horwitz 2000).

Given the interplay between cytokines and other effectors of the immune response, and their overlapping immunomodulatory roles, their influence on myocarditis is likely complex and dependent on the specific virus-host environment. In CD4⁻/⁻CD8⁻/⁻ mice, reduced myocardial pathology was associated with a cytokine profile of elevated IFN-γ and reduced TNF-α (Opavsky 1999). Patterns of cytokine expression may reflect the nature of the innate and specific cellular responses which predominant in different mouse strains. By subsequent interaction with their cell surface receptors, cytokines activate cell signal transduction pathways, directing proinflammatory, or antiviral host cell responses, thereby influencing susceptibility to myocardial inflammation and dysfunction.

vi) Nitric oxide

Nitric oxide is important in the host defense to infectious diseases (Griffith 1995; Hare 1995; Mikami 1996) and the pathogenesis of many cardiac disease states (Kelly 1996; Lowenstein 1996). A variety of cell types including cardiac myocytes, cardiac endothelial cells, and macrophages express iNOS when stimulated by TNF-α, the IFNs, and IL-1 (Kelly 1996). In in vitro myocyte systems and murine models of
coxsackieviral myocarditis, NO appears to have an antiviral effect (Hiraoka 1996; Lowenstein 1996; Mikami 1996; Zaragoza 1997). Expression of iNOS is upregulated in the heart in response to CVB3 infection (Lowenstein 1996; Mikami 1996). Genetic knockout of iNOS resulted in fulminant CVB3 myocarditis (Zaragoza 1998), suggesting a protective role for NO in the disease process. In addition, recent work by Knowlton's group has shown that NO inhibits the viral proteolytic cleavage of dystrophin (Badorff 2000). Dilated cardiomyopathy has been associated with this action of enteroviral protease, suggesting a possible protective mechanism for NO in the pathogenesis of viral heart disease. But, the effect of inhibiting NO by N-nitro-L-arginine methyl ester (L-NAME) appears to be dose-dependent, (Lowenstein 1996; Mikami 1996), with low doses of L-NAME protecting susceptible mice from severe viral myocarditis (Mikami 1997). Differences in murine strain used in the model may influence the outcome. Regardless, nitric oxide appears to have an important immunomodulatory role in CVB3 myocarditis.

vii) Extracellular matrix

Components of the extracellular matrix (ECM), such as elastin, participate in the myocardial response to injury. Serine elastase, produced by inflammatory cells, and potentially by myocytes, can degrade the ECM and thus promote myocyte loss and differentiation into myofibroblasts (Zaidi 2000). As well, these enzymes can liberate matrix peptides which are highly chemotactic, promote inflammatory cell infiltration and stimulated expression of adhesion molecules. Elafin is a 6 kDa protein which interacts with the active site of serine elastases and inhibits their action (Tsunemi 1996; Zaidi 2000). Treatment with elafin, and overexpression of elafin targeted to cardiovascular tissue, protects mice from EMCV myocarditis (Lee 1998; Zaidi 2000). Interestingly, a link between NO and serine elastase activity has been demonstrated (Mitani 2000). NO inhibits elastase via suppression of the ERK signalling cascade, a
known upstream activator of AML1B, a transcription factor for elastase. These findings suggest an additional mechanism by which NO may have a protective role in the pathogenesis of viral myocarditis and dilated cardiomyopathy.

viii) Autoimmunity

Autoimmunity has also been implicated in the pathogenesis of myocarditis (Rose 1988; Craighead 1990; Liu 2001). Heart-specific autoantibodies have been postulated to develop throughout the production of anti-idiotypic antibodies, antigenic mimicry or the triggering of an immune response to cardiac self-antigens during the course of acute viral myocarditis (Erlanger 1986; Paque 1989; Weremeichik 1991; Gauntt 1995). Autoantibodies to a variety of cellular proteins, including myosin, the ADP/ATP translocator protein, other mitochondrial proteins, fibronectin and cytoskeletal components may have a pathogenic role in myocarditis (Schulze 1989; Craighead 1990; Cunningham 1992; Takemoto 1993). The establishment of an etiologic role for autoantibodies in human viral myocarditis is the subject of ongoing investigation, however the association may simply be a byproduct, rather than the cause, of the underlying immune activation. A unique model of myocarditis has been developed in which a normal mouse heart is heterotopically transplanted into a second mouse with post-CVB3 chronic myocarditis (Nakamura 1996). The transplanted heart developed myocarditis, which may be consistent with a role for autoimmunity in persistent disease.
C. THE BIOLOGY OF ENTEROVIRUSES

The biology of the virus is central to any discussion regarding the pathogenesis of disease. The host has evolved specific defense mechanisms, and the virus has developed ways to circumvent them. Thus, in considering determinants of host susceptibility to coxsackieviral infection, a clear understanding of the virus is important. In this section, structural and functional characteristics of enteroviruses, as well as key elements of viral-host interaction will be reviewed.

1. DISCOVERY

In 1908 polioviruses were the first enteroviruses to be discovered. They were identified as filterable agents that cause poliomyelitis. Landsteiner and Popper induced spinal cord lesions typical of poliomyelitis by injecting monkeys with infected human neuronal tissue (Landsteiner 1908). Intense study of poliovirus was inspired by the devastating epidemics of the early 20th century, forming a basis for the study of all enteroviruses (Rotbart 1992). In 1949 Enders, Weller and Robbins revolutionized viral research by propagating poliovirus in tissue culture, for which they received the Nobel Prize in 1954 (Enders 1949). Dalldorf and Sickles first isolated coxsackieviruses from the feces of 2 children while investigating a poliomyelitis epidemic in Coxsackie, New York (Dalldorf 1948; Dalldorf 1965; Melnick 1996b). Subsequently, additional related viruses were isolated, including the first of the group B coxsackieviruses (Dalldorf 1949; Melnick 1949; Dalldorf 1950). Newborn mice were found to develop paralysis and skeletal muscle lesions following intracerebral inoculation of virus isolates (Dalldorf 1948). It became apparent as more distinct isolates were described, that distinct viral syndromes existed (Mahy 1988). The typical patterns of disease produced in newborn mice formed the basis for the division of coxsackieviruses into two groups.
Group A coxsackieviruses produce widespread myositis of skeletal muscle and accompanying flaccid paralysis. In contrast, newborn mice infected with group B coxsackieviruses develop encephalitis and spastic paralysis, in association with involvement of the heart, pancreas, liver and necrosis of fat tissue (Godman 1952). Echoviruses, also identified in the course of poliovirus research, were first isolated from healthy individuals (Fenner 1976; Melnick 1996a; Melnick 1996b). These viruses generally are nonpathogenic in suckling mice, but do produce cytopathic changes in cell culture (Committee on the ECHO viruses 1955; Mahy 1988) 'ECHO' represents the acronym 'Enteric Cytopathogenic Human Orphan', because initially these viruses were considered "orphans" in search of a disease (Gelfand 1961).

2. CLASSIFICATION

Enteroviruses are members of the Picornaviridae, a family of viruses responsible for a wide spectrum of diseases in humans and animals (Melnick 1996a). In 1957 poliovirus, coxsackieviruses and echoviruses were grouped together as enteroviruses (Melnick 1957). Enteroviruses pathogenic in humans are polioviruses (serotypes 1-3), group A coxsackieviruses (serotypes 1-22 and 24), group B coxsackieviruses (serotypes 1-6), echoviruses (serotypes 1-7, 9, and 11-33), and more recently identified serotypes, which have been designated numerically (enteroviruses 68-71). Several characteristics are common to all enteroviruses, including their small size (20-30nm), resistance to ether and mild acid, cationic stabilization to thermal inactivation and enteric route of infection. Growth of enteroviruses in cell culture is associated with a typical cytopathic effect. Cells of the monolayers round up, shrink and develop nuclear pyknosis and degenerate and detach from the monolayer. Under agar overlay, spread of the virus is limited to that of cell-to-cell and a pattern of dying
cells or plaques forms, providing the basis for the plaque formation assay used in virus quantification (Koch 1966).

Enteroviruses target a wide range of cell types, tissues and hosts. The alimentary tract is the portal of entry for enteroviruses. Following primary viral replication in the lymphatic tissue of the pharynx and gut viremia may occur, leading to the dissemination of the virus to specific target organs where it can mediate tissue damage with the participation of the immune response. Enteroviruses are the etiologic agents of a wide range of diseases, and as a group show a predilection for the central nervous system and the skin. The heart, pancreas and reticuloendothelial organs are also targets of enteroviruses.

3. THE GENOME

The complete genetic sequences of CVB1 (lizuka 1987), CVB2-Ohio1 (Polacek 1999), CVB3-Nancy (Lindberg 1987), CVB4-Benschoten (Jenkins 1987), CVB5 (Zhang 1993) and CVB6-Schmitt (Martino 1999) have been reported. Many phenotypic variants have also been partially or entirely sequenced (Martino 1999). Enteroviruses have a positive single-stranded RNA genome, approximately 7400 nucleotides in length, encoding a large open reading frame (ORF) flanked on either side by non-translated regions. Properties of the poliovirus genome are representative of all enteroviruses. The enteroviral genome is monocistronic, with the open reading frame (ORF) encoding for a single polyprotein of about 250 kDa, which is post-translationally processed to produce capsid proteins, proteases and polymerases (Jacobson 1968; Wimmer 1993; Hellen 1995b). The viral RNA is covalently linked at the 5' end to a small virus-encoded protein, VPg (protein 3B) (Wimmer 1993). The organization of the enteroviral genome and corresponding polyprotein cleavage sites are illustrated in Figure 3.
i) The 5'-non-translated region

The noncoding 5'NTR contains secondary structures which are important in the initiation of positive strand RNA synthesis and translation by binding viral and cellular proteins (Jackson 1990; Rueckert 1996). There are 2 highly conserved regions within the 5'NTR (Skinner 1989; Rohll 1994) (Figure 3). The initial 88 nucleotides of the 5'NTR form a cloverleaf structure which plays a role in viral plus-strand synthesis (Andino 1990a; Andino 1990b; Wimmer 1993; Hellen 1995b). The cloverleaf structure forms a ribonucleoprotein complex with the viral protease-polymerase 3CDpro and host cell factors or the viral polypeptide 3AB (Andino 1990a; Andino 1990b; Wimmer 1993; Harris 1994b; Blyn 1996). The second conserved structure, from nucleotides 134 to 556 in the poliovirus genome, has several stem loop structures and functions as an internal ribosome entry site (IRES) (Jang 1988; Pelletier 1988; Trono 1988a; Trono 1988b; Jang 1989; Pelletier 1989). Mutational analysis of the CVB3 genome indicates that the IRES segment is located closer to the initiation codon than found in poliovirus (Liu 1999). Antisense oligonucleotides designed to target the core sequence of the internal ribosome entry site, the translation initiation codon region, the 5'end of the 5'NTR or the 3' end of the 3'NTR block viral protein production and viral replication (Wang 2001). Folding of the genome may form a replication complex of the 5' and 3' ends with host proteins. The IRES has binding sites for four host proteins: eIF-2α, p50, p52 and p57, and is responsible for directing binding of 40S ribosomal subunits to initiate cap-independent viral protein translation (Hambidge 1992; Pilipenko 1992a; Wimmer 1993; Hellen 1994a). The type 1 IRES of enteroviruses and rhinoviruses, and the type 2 IRES of cardioviruses and aphthoviruses are clearly distinguishable, but can be functionally interchangeable (Wimmer 1993; Alexander 1994). While the AUG codon at the 3' border of type 2 IRES is the initiation codon, in type 1 IRES initiation occurs at the second downstream AUG triplet (Pelletier 1988; Wimmer 1993; Hellen 1994b)
Figure 3: Characteristics of the enteroviruses. (A) The arrangement of the picornaviral capsid proteins. Sixty copies of each coat protein, VP1, VP2 and VP3 are assembled. Depressions surrounding the peak at the 5-fold (5X) axes of symmetry form the canyon region. This is the hypothesized site of coxsackieviral receptor binding. (B) The secondary structures of the 5'-nontranslated regions of poliovirus. The boundaries of the cloverleaf structure (bold) and the IRES are outlined. The initiating AUG codon is indicated by an open box. (C) The enteroviral polyprotein and protein cleavage sites. The precursor P1 region contains capsid proteins, while the non-capsid region contains the nonstructural P2 and P3 proteins. The P2 section is cleaved into 2Apr0, 2B, 2C and the P3 section is cleaved into 3AB and 3CDpro intermediates, and 3A, 3B (VPg), 3Cpro, and 3Dpol. Figures A is from Racaniello, 1995, Figure B is from Hellen and Wimmer, 1995a and based on previous models (Andino et al, 1990, Hellen et al, 1989, Pilipenko et al, 1989, Skinner et al, 1989, and Wimmer et al, 1989), and Figure C is from Hellen and Wimmer, 1995b.
ii) The polyprotein and its cleavage products

The viral RNA directs the synthesis of a single polyprotein which is processed by a regulated series of cleavage reactions by virally encoded proteases 2Apro, 3Cpro and 3CD pro (Palmenberg 1979; Toyoda 1986). Processing is complex, with the production of intermediates and final products which are all important to replication of the viral genome. The P1 region of the genome encodes the structural capsid proteins (Wimmer 1993). Nomenclature for the polyprotein element P1 designates the capsid proteins as 1A, 1B, 1C, and 1D, which correspond to the cleaved VP4, VP2, VP3 and VP1 proteins, respectively (Wimmer 1993). VP2 and VP4 are not separated by cleavage until virus maturation, and are designated as VP0. Sixty copies of each structural protein are organized into the icosahedral picornaviral capsid, shown in figure 3A. After the myristoylated P1 precursor protein is cleaved by 3CDpro encapsidation begins with the assembly of single copies of VP0, VP1 and VP3 into 5S protomers (Hogle 1990; Wimmer 1993). These associate to first form 14S pentamers, and possibly 75S empty capsids. Provirions are formed when a single copy of genomic RNA with VPg covalently linked to the 5' end enters the viral capsids. Infectious and stable mature 160S virions may be produced by autocatalytic cleavage of VP0 to VP4 and VP2 (Wimmer 1993).

A canyon 24 Å deep and 12 to 30 Å wide separates a peak formed by VP1 molecules at the fivefold axis of symmetry. Based on the crystal structure of rhinovirus 14 (Arnold 1990) and the interaction between rhinovirus 16 and it's receptor intracellular adhesion molecule-1 (ICAM-1) (Olson 1993) the canyon was proposed as the site of attachment of virus to receptor. Mutagenesis studies of canyon sites brought functional relevance to structural observations, demonstrating that binding was altered in rhinoviruses (Colonno 1988) and polioviruses (Colston 1994; Harber 1995). Recent crystal structure analysis of poliovirus complexed with soluble receptor revealed receptor binding in the canyon, supporting this hypothesis (Belnap 2000; He 2000).
Several other potential enteroviral binding sites on the viral capsid have been described. These include: a depression at the twofold axes (Muckelbauer 1995), a "pocket factor" below the canyon floor within the β-strands of VP1 which is exposed by virus receptor binding and antiviral compounds (Muckelbauer 1997) and numerous amino acid residues of the capsid proteins (Ramsingh 1992; Ansardi 1993; Wimmer 1993; Ansardi 1994; Lee 1997; Zhang 1997a; Rezapkin 1999).

The P2 region of the genome encodes 2Apro, 2B and 2C proteins, and the stable intermediate 2BC. Protease 2Apro is multifunctional. In the life cycle of the virus, it catalyzes cleavage at the P1/P2 junction of the polyprotein (Toyoda 1986; Lee 1988; Hellen 1992), can activate IRES-dependent translation (Hambidge 1992) and is involved in RNA synthesis (Molla 1993). The 2Apro also cleaves a number of host proteins. The 220 kDa component of the eukaryotic initiation factor eIF-4γ is cleaved, preventing capped cellular mRNA translation (Ehrenfeld 1982; Kräusslich 1987; Lamphear 1993; Sommergruber 1994). Translation of viral RNA is not affected because it is initiated via a cap-independent binding of ribosomes at the IRES (Pelletier 1988; Jang 1989). Poly(A)-binding protein (PABP), important in the translation of host mRNA, is also cleaved (Joachims 1999; Kerekatte 1999). Protease 2A can have a direct effect on myocyte integrity by cleaving dystrophin in infected cardiac myocytes. This may contribute to the pathogenesis of dilated cardiomyopathy following viral infection (Badorff 1999; Badorff 2000; Lee 2000b).

Protein 2B and its precursor 2BC can inhibit cellular protein secretion (Doedens 1995) and permeabilize the plasma membrane (Carrasco 1978), possible contributing to virally induced host protein synthesis shut off and inducing cell lysis (van Kuppeveld 1997). Protein 2C has three conserved segments: an amino-terminal amphipathic helix, a nucleoside triphosphate-binding site, and a zinc finger motif (Hellen 1995b). The polypeptide has nucleotriphosphatase activity which is important for virus viability.
Polypeptides 3A, 3B, 3Cpro, 3Dpol and the stable intermediates 3AB and 3CDpro are encoded by the P3 segment of the enterovirus genome. Protein 3A and its precursor 3AB are associated with membrane-associated replication complexes, perhaps participating in the formation of viral replication initiation complexes (Bernstein 1988; Giachetti 1991; Giachetti 1992). Following cleavage, protein 3B (VPg) is linked to the 5' end of both positive and negative strand enteroviral RNA. The uridylinated VPg is the primer for the RNA polymerase 3Dpol in the initiation of viral RNA synthesis (Nomoto 1977; Paul 1998). These two primary viral proteins, 3B and 3Dpol, are responsible for the replication of enteroviral RNA (Agol 1999). The 3CDpro functions as a proteinase to cleave P1 into VP0, VP3 and VP1 (Jore 1988; Ypma-Wong 1988), and interacts with the cloverleaf region as an RNA-binding protein during replication. The catalytic section is 3Cpro (Hanecak 1982), while 3Dpol has RNA polymerase activity (van Dyke 1980). Following cleavage 3Cpro can inactivate nuclear transcription factors such as transcription factor IIIC and cleave the cellular TATA box binding protein (Clark 1991; Das 1993). Subsequently, 3Cpro can shut down host protein synthesis and contribute to viral-mediated cell death. Nitric oxide inhibits virus replication by nitrosylating the cysteine residue in coxsackieviral 3Cpro, indicating a potentially significant innate host defense mechanism (Saura 1999).

iii) The 3' nontranslated region

The 3'NTR of enteroviruses is 70 to 100 nucleotides in length and forms a tRNA-like structure (Iizuka 1987; Pilipenko 1992b; Jacobson 1993). Secondary and tertiary interactions form the multi-domain, three-dimensional origin of replication (oriR) region, which serves as the template for VPg-dependent initiation of negative strand RNA synthesis (Pilipenko 1992b; Melchers 1997). Viral replication may be influenced
by interactions between the 3'NTR and 3AB, 3CD and a host cell factor (Harris 1994a; Todd 1995; Mellits 1998). The viral RNA is polyadenylated at the 3' end, and as a component of the oriR, participates in complementary viral RNA replication.

4. MUTATIONS OF THE ENTEROVIRAL GENOME AND VIRULENCE

Enterovirus virulence can be altered by mutations of the viral genome. Poliovirus vaccine strains each have mutations in the 5'NTR, in association with other mutations throughout the genome (Evans 1985; Omata 1986; Kawamura 1989; Westrop 1989; Ren 1991). A number of mutations in the genomes of coxsackieviruses have been implicated as determinants of virulence. A single mutation in the region encoding an external loop of VP1 of coxsackievirus B4 confers increased virulence in a murine model of pancreatitis (Caggana 1993). Cardiovirulence of CVB3 correlates with mutations in the 5'NTR (Tu 1995) and in other areas of the genome (Lee 1997). The VP2 region of the coxsackievirus capsid has a protruding loop structure termed the puff region. A mutation within the genome encoding the puff region of VP2 attenuates the myocarditic phenotype of a cardiovirulent coxsackievirus B3 variant, despite cardiac viral titres being comparable to wildtype infection (Knowlton 1996). The attenuated variant does not induce a strong inflammatory response or significant myocardial disease in association with an inability to stimulate cytokine expression.

The RD variant of CVB3 (Nancy) has acquired the ability to replicate in the human rhabdomyosarcoma cell line RD (Lindberg 1992). The RD mutation has been documented to allow the virus to interact with the decay accelerating factor (DAF) coreceptor (Hsu 1990). The two mutations responsible for this interaction are in a region which encodes coxsackieviral VP2, an area comparable to the puff region of poliovirus and rhinovirus 14 (Lindberg 1992). Thus, mutations in the enteroviral
genome may alter virulence by affecting viral replication, receptor affinity or the host response.

5. REPLICATION OF ENTEROVIRUSES

Enteroviruses enter the cell following interaction with specific cell surface receptors. Entry may be through the endocytotic pathway or by the formation of infectosomes in the host cell surface (Rueckert 1996). Binding to the viral receptor can result in destabilization of the capsid structure and may contribute to uncoating (Crowell 1983; Racaniello 1992; Wimmer 1993). Following the presumed removal of VPg by a cellular enzyme, genomic RNA is released into the cytoplasm to function as mRNA (Wimmer 1982). Translation is initiated through the IRES leading to the synthesis and processing of the precursor polyprotein (Harris 1989; Harris 1990). Viral RNA replication occurs in association with membraneous replication complexes. However, while a role for certain viral proteins and possibly some host proteins are implicated in the regulation process, many elements remain hypothetical (Wimmer 1993). The genomic viral RNA (positive) serves as a template for the replication of a complementary strand RNA (negative), which may transiently exist as a double stranded RNA replicative intermediate (Hellen 1995b; Agol 1999). Subsequently, additional positive strand enteroviral RNA is produced, and is transcribed, translated, or encapsidated. The generation of free negative strands is thought to be unlikely (Wimmer 1993).

During replication of enteroviral RNA high rates of mutation have been demonstrated. Enterovirus infections with a single serotype commonly reflect a heterogeneous population of viruses with slightly divergent nucleotide sequences, referred to as a quasispecies. In a single infected individual, this genetic variability provides the opportunity for the selection of new genetic strains (Domingo 1985).
mutation rates result because RNA viruses cannot eliminate errors with cellular proofreading or editing functions (Holland 1982). A high rate of mutation was demonstrated in naturally occurring clinical isolates of CVB4 replication (Prabhakar 1982). The frequency of mutation was calculated to be as high as $10^{-4}$ using monoclonal antibodies as the selection agent. Variable clinical presentation within one viral serotype may reflect the quasispecies nature of the virus. Tissue tropism likely reflects high affinity viral-receptor interactions.
D. ENTEROVIRAL RECEPTORS

The attachment and entry of viruses into specific host cells via interaction with specific cell surface receptors is a principal determinant of host range and cell tropism. The investigation of virus-receptor interactions has contributed to our understanding of viral pathogenesis and directed new therapeutic strategies. Four decades ago the concept that viral receptors were important determinants of enteroviral infection was first proposed (Holland 1959; Holland 1961; Quersin-Thiry 1961; Philipson 1962). Although much information about the nature of enterovirus receptors was obtained from biochemical and functional analysis (Zajac 1965b; Zajac 1965a; McLaren 1968; Crowell 1978), it was 25 years before the use of molecular biology facilitated the cloning and characterization of the poliovirus receptor (Mendelsohn 1989). A growing number of enteroviral receptors have now been discovered (Table 4 and Figure 4), including coxsackie-adenovirus receptor (CAR) and decay accelerating factor (DAF), the receptors for Group B coxsackieviruses (CVBs) (White 1989; Bergelson 1993a). Identification of these receptors has contributed to our understanding of the cardiotropism of CVB and provides the potential to explain differences in host susceptibility to viral infection.

1. THE POLIOVIRUS-RECEPTOR INTERACTION

The cell receptor for poliovirus has been well described and provides an important model for the understanding of the initial steps in enteroviral infection. Poliovirus and group B coxsackievirus binding components were initially identified in HeLa cells by Krah and Crowell (Krah 1982). The poliovirus receptor was eventually identified using monoclonal antibodies to identify mouse L cells, which are not permissive to infection, that became susceptible to poliovirus infection after
Table 4: Cellular receptors for enteroviruses

<table>
<thead>
<tr>
<th>virus</th>
<th>receptor(^a)</th>
<th>sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus 1-3</td>
<td>PVR</td>
<td>(Mendelsohn 1989)</td>
</tr>
<tr>
<td>Echovirus 1 and 8</td>
<td>VLA-2</td>
<td>(Bergelson 1992; Bergelson 1993)</td>
</tr>
<tr>
<td>Echo 6,11,12,21,7,13,21,29,33,3,6,11-13,19,21,24,25,29,30,33</td>
<td>DAF</td>
<td>(Bergelson 1994; Ward 1994; Powell 1998)</td>
</tr>
<tr>
<td>Echovirus 22</td>
<td>αvβ(_1) integrin</td>
<td>(Pulli 1997)</td>
</tr>
<tr>
<td>Coxsackievirus A13,18,21</td>
<td>ICAM-1</td>
<td>(Greve 1989; Tomassini 1989; Staunton 1990)</td>
</tr>
<tr>
<td>Coxsackievirus A21</td>
<td>DAF</td>
<td>(Shafren 1997)</td>
</tr>
<tr>
<td>Coxsackievirus A9</td>
<td>Integrin αvβ(_3)</td>
<td>(Roivainen 1994)</td>
</tr>
<tr>
<td>Coxsackievirus B1-6</td>
<td>CAR</td>
<td>(Bergelson 1997)</td>
</tr>
<tr>
<td>Coxsackievirus B1, 3, 5</td>
<td>DAF</td>
<td>(Bergelson 1994; Shafren 1995)</td>
</tr>
<tr>
<td>Enterovirus 70</td>
<td>DAF</td>
<td>(Karnauchow 1996)</td>
</tr>
</tbody>
</table>

\(^a\)PVR = poliovirus receptor; VLA-2 = very late antigen-2 = α2β1 integrin; DAF = decay accelerating factor; ICAM-1 = intercellular adhesion molecule-1; CAR = coxsackie-adenovirus receptor
Figure 4: Picornavirus cell surface receptors. Coxsackie-adenovirus receptor (Car), poliovirus (Pvr), intracellular adhesion molecule 1 (Icam-1), a receptor for rhinoviruses and vascular adhesion molecule 1 (Vcam-1), a receptor for encephalomyocarditis virus, have immunoglobulin-like domains. CD55, or decay accelerating factor has four short consensus repeat (SCR) domains. The hepatitis A virus receptor (HAVcr-1) has an N-terminal Ig domain. The low-density lipoprotein receptor (Ldlr) binds minor group rhinoviruses. The integrins α2β1 and αVβ3 are enteroviral receptors. (Reprinted from Trends in Microbiology, Vol. 6, Evans DJ and Almond JW, Cell receptors for picornaviruses as determinants of cell tropism and pathogenesis, pp 198-202, 1998, with permission from Elsevier Science)
transformation with HeLa cell DNA (Mendelsohn 1986; Mendelsohn 1989). Human genomic DNA clones were then isolated from DNA libraries constructed from the transformed susceptible mouse L cells. Subsequently, these clones were used to screen cDNA libraries, yielding full length cDNA clones which directed the synthesis of functional poliovirus receptors.

The poliovirus receptor is a member of the immunoglobulin superfamily, with an N-terminal sequence, three extracellular immunoglobulin(lg)-like domains, a transmembrane domain and a cytoplasmic tail. The Ig-like domains are stabilized by intrachain disulfide bonds and eight N-glycosylation sites have been identified. The first Ig-like domain of PVR contains the binding site for poliovirus (Koike 1991; Selinka 1992; Bernhardt 1994b). While this single domain is adequate for infection, chimeras with other cell surface receptors (ie CD4 and ICAM-1) are not as efficient in promoting infection as full length PVR (Koike 1991; Selinka 1991; Selinka 1992; Bernhardt 1994b). Alternative splicing results in two mRNAs that encode PVRα and PVRδ that have different cytoplasmic domain lengths (Koike 1990). Two soluble PVR isoforms, PVRβ and PVRγ lack the transmembrane domain (Mendelsohn 1989; Koike 1990). Glycosylation of the PVR increases the predicted molecular size from 43 kDa (PVRα) to 45 kDa (PVRδ) to up to 80kDa with full glycosylation (Bernhardt 1994a). It is speculated that the PVR plays a role in cell adhesion, consistent with the other members of the Ig superfamily (Bernhardt 1994b).

After binding of the virus to receptor, a role for the receptor in the release of the RNA genome into the cell has been postulated. Poliovirus binding to free or cell-bound receptors at 37°C produces conformationally altered virus (135S A particles) (Fenwick 1962; Guttman 1977; Fricks 1990; Kaplan 1990). Although the 135S A particles contain infectious RNA they can no longer bind to susceptible cells. The hydrophobic N terminus of VP1 becomes irreversibly exposed on the surface of the A particle and VP4 is lost. The development of the A particle may reflect events occurring during
poliovirus entry. Antiviral WIN compounds bind the hydrophobic pocket in the canyon region, allowing virus binding to its receptor, but preventing the formation of A particles and productive infection (Fox 1986). But, cold-adapted mutant viruses can infect susceptible cells without A particle formation (Dove 1997). The exposed hydrophobic VP1 region of the A particle may form a pore for entry of viral RNA by insertion into the cell membrane (Kirkegaard 1990; Racaniello 1995; Rueckert 1996; Flint 2000). This uncoating event may occur at the cell membrane or from within an endosome following receptor-mediated endocytosis (Racaniello 1995).

2. THE COXSACKIE-ADENOVIRUS RECEPTOR

i) Identification

All serotypes of group B coxsackieviruses have been shown to compete with each other for binding to host cell receptors (Crowell 1966; Lonberg-Holm 1976). However, CVBs did not compete with polioviruses, group A coxsackieviruses, or human rhinoviruses (Crowell 1966; Lonberg-Holm 1976). In contrast, evidence for a shared receptor between CVBs and adenovirus type C fiber protein was indicated by competition binding assays (Lonberg-Holm 1976). Experiments with proteolytic enzymes differentiated between receptors for CVBs and polioviruses (Zajac 1965a; Zajac 1965b). Chymotrypsin inactivates CVB receptors, while trypsin inactivates the poliovirus receptor. Because CVB receptors are also inactivated by glycosidases, but not lipases, evidence points to a basic glycoprotein structure (Zajac 1965a; Krah 1985). Detergents and solvents are required to dissociate the receptor from the membrane, indicating that it is an integral membrane protein (Crowell 1978; Krah 1982). Receptor binds virus at the optimal temperature of 37°C, and this binding occurs more rapidly for CVB serotypes 1, 3, and 5 (Crowell 1976). It was demonstrated
that there are at least $1.8 \times 10^4$ CVB3 receptor binding sites on the surface of each HeLa cell (Hsu 1988).

Purification of the CVB receptor was first accomplished by isolating a virus-receptor complex (VRC) from CVB3 infected HeLa cells (Mapoles 1985) and YAC-1 cells (Hsu 1989). The purified complex contained viral capsid proteins as well as an additional cellular protein with a molecular mass of about 50 kDa, termed Rp-a. A monoclonal antibody, RmcB, was prepared against this HeLa cell receptor and was found to protect against infection with all group B coxsackieviruses (Hsu 1988). CVB3 infection of YAC-1 cells was inhibited by a rabbit antiserum to Rp-a that was isolated from HeLa cells (Hsu 1989). Polyclonal antibody against mouse brain receptors (MBR), that was isolated from newborn BALB/c mice, was used to identify four receptor proteins with molecular masses of 46, 44, 36, and 33 kDa in mouse neural tissue (Xu 1995). Antisera to these proteins also protected against CVB3 infection. Anti-MBR did not protect HeLa cells from CVB3 infection and did not recognize any extracted HeLa cell receptor proteins. In contrast, a rabbit polyclonal anti-HeLa antiserum and RmcB do not inhibit infection of cultured murine neurons or astrocytes (Xu 1995). These findings highlight the antigenic differences between human and mouse receptors.

The receptor for group B coxsackieviruses that was the focus of the above investigations has been recently cloned, characterized and named the coxsackie-adenovirus receptor (CAR). Following isolation from HeLa cells using RmcB (Bergelson 1997a), or $^{35}$S-labeled CVB3 (Carson 1997) the human CAR (hCAR) receptor was digested into protein fragments and sequenced. Sequences from tryptic peptides matched a sequence tag cDNA from an infant brain library in the dbEST database, which in turn, was used to identify a 2.4 kb cDNA from a HeLa cell library. Murine CAR (mCAR) was identified by screening a transformed mouse kidney cell (TMCK-1) cDNA library constructed in a λ phage system with the antiserum to the 46kDa murine receptor protein (Xu 1995; Tomko 1997). Two 46kDa mCAR isoforms
were isolated by Bergelson et al. (1998) using a C57BL/6 mouse liver cDNA library screened with an EST cDNA found to encode a peptide sequence homologous to the C terminus of hCAR. One mCAR isoform (mCAR2) was identical to the clone isolated by Tomko et al. (1997). Transfection of CAR cDNA into CVB resistant Chinese hamster ovary cells (CHO) or the mouse fibroblast cell line NIH-3T3 conferred susceptibility to infection by all strains of CVB3 (Bergelson 1997a; Tomko 1997; Bergelson 1998; Martino 2000). CAR serves as the receptor for laboratory reference strains and clinical isolates of all six CVB serotypes. Echoviruses 1 and 9 and CAV9 do not bind to CAR, but it was shown to be the functional receptor for swine vesicular disease virus (SVDV), an animal enterovirus antigenically and phylogenetically related to the CVBs (Bergelson 1997a; Martino 2000).

ii) CAR structure and function

The gene for hCAR is located on human chromosome 21 at 21q11.2 and consists of seven exons (Bowles 1999). The CAR coding sequence does not have significant overlap with genes of known function. Sequence analysis of hCAR cDNA predicts a 365-amino acid surface protein with homology to the immunoglobulin superfamily. The CAR gene codes for a short leader sequence, a 222-amino acid extracellular domain containing two immunoglobulin-like domains, a membrane-spanning helical domain, and a 107-amino acid intracellular domain. The extracellular domain has two potential N-linked glycosylation sites, and the intracellular domain has one potential tyrosine phosphorylation site. The mCAR clones have a predicted overall amino acid identity of >90% to the extracellular domain of the human protein and up to 95% identical within the cytoplasmic domain (Bergelson 1998). The two mCAR isoforms were identical in the extracellular and transmembrane domains, but differed in the cytoplasmic domains. In clone mCAR2, the C-terminal 26 amino acids of mCAR1 were replaced by 13 different amino acids (Bergelson 1998). CAR proteins identified
in other species have highly homologous sequences and share the same general structural regions (Fechner 1999). Two isoforms with divergent cytoplasmic tails have now been isolated from the human and rat as well (Fechner 1999).

The specific interaction site between CVBs and CAR has not been determined, however mutagenesis studies have shown that cytoplasmic and transmembrane domains are not essential for coxsackievirus or adenovirus infection (Wang 1999). The extracellular domain of CAR is sufficient to mediate CVB binding and productive infection.

The role of CAR in cell function is unknown, however it has been speculated that given its relationship to other Ig superfamily members it functions in adhesion and intercellular communication (Ito 2000). MCAR expression promoted cellular aggregation, which was specifically inhibited by anti-mCAR (Honda 2000). In addition, the highly conserved cytoplasmic domain and the potential tyrosine phosphorylation sites suggest possible key interactions with other intracellular proteins.

iii) The role of CAR in group B coxsackievirus infection

The organ distribution of CAR expression is consistent with the known spectrum of clinical disease with CVB infection. Human CAR RNA expression is highest in heart, brain, pancreas and testes, with lower expression in kidney, lung and liver hCAR (Tomko 1997; Bergelson 1998; Fechner 1999). In Northern blots of mouse tissue using various cDNA probes, cDNA hybridized most strongly to RNA from the liver, and less strongly to heart, brain, lung and kidney (Tomko 1997; Bergelson 1998; Fechner 1999). In the pancreas CVB infection has been shown to cause massive acinar necrosis, while sparing the Islets of Langerhans (Vuorinen 1989; Gómez 1991). Correlating with this differential susceptibility, CAR is expressed at high levels in pancreatic acinar cells, but is barely detectable in the Islets (Mena 2000).
Developmental expression of CAR may be responsible for changing age-dependent susceptibility to CVB infection and also age-related patterns of organ tropism. In this context, mCAR is abundant in the hearts of newborn rats, but barely detectable in the hearts of adult rats (Ito 2000) and decreasing levels of CAR expression have been demonstrated in mouse brain with increasing age (Honda 2000).

iv) The role of CAR in adenoviral infection and gene therapy

Coxsackieviruses group B and adenovirus serotypes 2 and 5 are known to compete for a cell surface receptor (Philipson 1968; Defer 1990). Radiolabelled adenovirus 2 and isolated adenovirus 2 fibers bind specifically to CHO-CAR cells (Bergelson 1997a). CAR-CHO cells were found to be permissive to an adenovirus 5 vector with subsequent expression of the vector's reporter gene (Bergelson 1997a). CAR functions as a high-affinity cellular receptor for adenovirus serotypes from subgroups A, C, D, E, and F (Roelvink 1998). Additional receptors are required to complete the infection process, as CAR is considered to function solely as an attachment molecule. Recent mutational analysis revealed that the CAR binding site of adenovirus consists of residues from the AB loop, the B β sheet, and the DE loop, sites on the side of the fiber knob of adenovirus serotype 5 (Bewley 1999). Crystallization and structural analysis concur that the fiber AB loop is critical in the interaction of adenovirus and CAR (Roelvink 1999). These viral binding sites are exposed surface loops of the receptor. Based on crystal structure analysis domain 1 of CAR is thought to form a homodimer in its interaction with the adenovirus fiber (van Raaij 2000). Kinetic studies have suggested that three CAR molecules bind simultaneously to one fiber head protein (Lortat-Jacob 2000).

Adenoviruses are classified into six distinct subgroups A to F, with at least 49 serotypes (Horwitz 1996). Adenoviruses often cause respiratory and intestinal
infections, but have also been shown to infect the heart (Berkovich 1968; Henson 1971; Gardiner 1973; Lozinski 1994; Horwitz 1996; Grumbach 1999). Perhaps the common clinical presentation and cardiotropic nature of both CVBs and adenoviruses is associated with a mechanism related to their common receptor. In addition, the identification of CAR as an adenoviral receptor has significant implications for adenoviral gene therapy. Successful treatment can be hampered by low CAR expression, such as in hematopoietic cells and adult skeletal muscle tissue, or conversely by receptor expression in a broad range of cell types. Increasing CAR expression in alveolar macrophages resulted in an increase in adenovirus-mediated gene transfer (Kaner 1999; McDonald 1999). Ablation of CAR binding sites on adenoviral vectors can allow redirection of gene therapy to alternative receptor proteins, perhaps with a more selective tissue distribution (Roelvink 1999).

3. DECAY ACCELERATING FACTOR: THE GROUP B COXSACKIEVIRUS CORECEPTOR

i) Identification

An additional CVB receptor was suggested following the isolation of a host range variant selected for growth by human rhabdomyosarcoma (RD) cells (Reagan 1984). This second CVB receptor is different from Rp-a (now named CAR) that is recognized by monoclonal antibody RmcB (Hsu 1990). Another monoclonal antibody (RmcA) recognizes a 60 to 70 kDa cell surface protein on HeLa cells and RD cells (Crowell 1986; Hsu 1990; Bergelson 1997b). Following the observations that RmcA protected cells from echovirus 6 infection (Bergelson 1994; Ward 1994), it was hypothesized that the protein it recognized was DAF (Bergelson 1995; Shafren 1995). RmcA and MAb854, an antibody that inhibits replication of echovirus 7, protected
susceptible cells from infection by CVB serotypes 1, 3 and 5, as well as echovirus 6 (Bergelson 1995; Shafren 1995).

ii) DAF structure and function

DAF is a 70 kDa glycosyl-phosphatidylinositol-anchored protein on cell surfaces that protects from complement-mediated lysis by preventing either the formation, or the association of C3 convertases. DAF controls both classical and alternative pathway C3 convertases by dissociating enzymatically active C2a and Bb, or C4b and 2a from the binding sites (Fujita 1987). DAF expression is widespread, and found on most cells which are potentially exposed to serum complement (Nicholson-Weller 1994). DAF molecules have been identified in rabbits (Sugita 1987), guinea pigs (Nicholson-Weller 1981) and mice (Spicer 1995; Song 1996).

The molecule has five extracellular domains: four contiguous short consensus repeat [SCR] domains of approximately 60 amino acids each, followed by a serine/threonine-rich, heavily glycosylated C-terminal domain (Caras 1987; Nakano 1992). The carboxy-terminus is covalently attached to a glycoposphoinositol (GPI) segment which anchors the molecule to the outer lipid bilayer of cell membranes (Lublin 1991). Although all domains are important in the maintenance of the protein's three dimensional structure, SCR3 is the active site in complement regulation (Coyne 1992). This third SCR of DAF is also believed to be important to DAF's association with Src-family protein tyrosine kinase signal transduction pathways (Davis 1988; Stefanova 1991; Shenoy-scaria 1992; Shafren 1995).

iii) Role in group B coxsackieviral infection

DAF functions only as an attachment site in CVB infection, and CAR is required for production of progeny virus (Bergelson 1995; Shafren 1995; Pasch 1999). While prototype strains of CVB 1, 3 and 5 bind to DAF expressed on rodent cells, CVB2, 4
and 6 did not. Variable DAF-binding phenotypes have been identified in clinical isolates of CVB 1, 3 and 5 (Bergelson 1997b). Interestingly, strains of CVB3 cardiovirulent in mice bind to DAF with a high affinity, as compared to the less cardiotropic viruses (Martino 1998). The CVB-receptor binding sites have been mapped by systematic investigation using mutational analysis and domain-specific monoclonal antibodies to DAF (Bergelson 1995; Shafren 1995). Blocking of the interaction between virus and SCR 2 and SCR 3 of DAF with monoclonal antibodies prevented CVB binding to susceptible cells. Rather than the canyon region, which may be the site of CAR-virus attachment, it has been proposed that DAF binding may occur in the area of a surface depression at the twofold axes of the CVB capsid (Lindberg 1992).

4. PUTATIVE GROUP B COXSACKIEVIRAL RECEPTORS

As another receptor candidate, a 100 kDa virus binding protein was detected in various CVB-permissive human and monkey cell lines but was not detected in nonpermissive cell lines. Amino acid sequence analysis of tryptic fragments indicated the protein is related to nucleolin (de Verugo 1995).

Coxsackievirus B1 infection is enhanced by integrin αvβ6 in a colon cancer cell line. The integrin may function as a coreceptor molecule, particularly when upregulated as part of tissue response to injury (Agrez 1997). Interestingly, an RGD motif of the adenovirus penton base interacts with the integrins αvβ3 and αvβ5 (Wickham 1993).
5. ECHOVIRUS RECEPTORS

Echoviruses are now known to be common etiologic agents of febrile illnesses, rash and viral meningitis (Melnick 1996a). A common cellular receptor for echoviruses is postulated to be a multicomponent complex (Ward 1998). Virus competition assays and inhibition of viral replication by a monoclonal antibody to a 44 kDa surface protein suggest that many echoviruses share cellular receptors (Mbida 1991; Mbida 1992a; Mbida 1992b). The ligand for the monoclonal antibody raised against the putative 44kDa component of an echoviral receptor complex has been identified as β2-microglobulin, which associates with class I HLA heavy chains, and participates in antigen presentation (Ward 1998). The precise role of β2-microglobulin and possibly class I HLA await further investigation.

Several specific echoviral receptors have been reported. Using monoclonal antibodies that block susceptible cells from infection with echovirus 1, the α and β subunits of the integrin very late antigen (VLA-2, α2β1, CD49b/CD29), a cell attachment receptor for laminin and collagen, was recognized as a receptor for echovirus 1 (Bergelson 1992). Further investigation revealed that echovirus 8 was the only other virus of this group to use VLA-2 as its receptor (Bergelson 1993b). The α2 subunit likely determines virus specificity, as the other β1 integrins do not mediate echovirus 1 infection (Bergelson 1992; Bergelson 1993b). The binding sites for echovirus 1 and collagen are within the first domain of the α2 subunit (King 1997). Integrins transduce environmental signals from the cell surface to cell signal transduction pathways, and consistent with this is the observation that echovirus 1 can trigger the stress-activated p38 mitogen activated kinase (MAPK) pathway (Huttunen 1997; Huttunen 1998).

Another integrin, αvβ1 is used as a receptor by echovirus 22 (Pulli 1997). Monoclonal antibodies to both subunits inhibit echoviral binding to susceptible cells.
Of interest is that an arginine-glycine-aspartic acid (RGD) tripeptide motif is present in the VP1 capsid protein of this echovirus, and it is the RGD binding motif of αvβ1 that is used for binding to extracellular matrix components (Hyypia 1992). Virus binding is in fact inhibited by RGD-containing oligopeptides.

Decay accelerating factor (DAF) is a receptor for numerous echoviruses, including serotypes 6, 11, 12, 21 (Bergelson 1994), serotypes 7, 13, 21, 29, 33 (Ward 1994), and serotypes 3, 6, 11-13, 19, 21, 24, 25, 29, 30, 33 (Powell 1998). Echovirus 7 specifically requires short consensus repeats (SCRs) 2,3 and 4 virus binding and infectivity (Clarkson 1995; Powell 1997).

6. RECEPTORS FOR GROUP A COXSACKIEVIRUSES

Group A coxsackieviral receptors have received little attention, relative to other members of the Enterovirus genus, however three receptors to different group A coxsackieviruses (CAV) have been reported to date: αvβ3, ICAM-1, and DAF.

Integrin αvβ3 (the vitronectin receptor) is a cellular attachment molecule for CAV9, binding to RGD motifs located in the viral capsid (Roivainen 1994). The tripeptide is part of a 17 amino acid insertion at the C terminus of the VP1 protein, not present in other CAVs (Chang 1989; Chang 1992). CAV9 binding to host cells is inhibited by RGD-containing oligopeptides (Roivainen 1991). CAV9 is also capable of infecting cells in a non-RGD dependent manner (Roivainen 1996).

Competition binding assays demonstrated that CAV21 and human rhinovirus 14 (HRV14) share a common cell surface receptor (Lonberg-Holm 1976). Blocking monoclonal antibodies raised against lysates of HRV14 susceptible cells inhibit infection by CAV13, CAV18, CAV21 and HRV14 itself (Colonno 1986), and were found to recognize ICAM-1 (Greve 1989; Tomassini 1989; Staunton 1990). Transfection of nonpermissive cell lines with the gene encoding ICAM-1 renders them susceptible to
CAV21 infection (Shafren 1997a). ICAM-1 is a member of the immunoglobulin superfamily, with 5 extracellular Ig-like domains with up to eight potential N-glycosylation sites, a transmembrane anchor and a short cytoplasmic tail (Simmons 1988; Staunton 1988; Staunton 1990). The extent of protein glycosylation is dependent on the specific cell type in which it is found (Dustin 1986). ICAM-1 is a cell surface ligand for the adhesion molecule lymphocyte function antigen (LFA-1; CD11a) and Mac-1 (CD11b) (Makgoba 1988; Diamond 1991). Using ICAM mutants (Register 1991) and soluble receptor (Greve 1991) domain 1 was determined to be the binding site for CAV21 on ICAM-1 (Shafren 1997b).

CAV21 also binds to DAF, but requires ICAM-1 for viral entry and productive infection (Shafren 1997b). DAF and ICAM-1 appear to be closely associated on the cell surface, thus DAF may function as an attachment receptor for subsequent binding to ICAM-1, the high-affinity receptor. In contrast to other ligands and viruses, CAV21 binds to SCR1 of DAF, which shows the inherent variations in the function of these receptors.
E. INTRACELLULAR SIGNAL TRANSDUCTION IN IMMUNE AND CARDIAC CELLS

Protein phosphorylation is central to signal transduction networks within the cell (Krontiris 1995). Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulate intracellular signalling events following T cell activation. Phosphorylation induces a conformational change in the substrate protein or enzyme altering activity or function and creating docking sites to recruit other proteins for further interaction (Krontiris 1995; Sugden 1995). Numerous biological processes are regulated by protein kinases (Sugden 1995). Most protein kinases transfer a phosphate group from ATP to the side chain of the amino acid tyrosine or the side chains of serine and threonine (Sugden 1995). The primary amino acid sequence adjacent to the potential phosphorylation site determines the specificity of protein kinase groups for their substrates (Kemp 1990). Phosphatases, whose function is to dephosphorylate, are important for regulation and maintenance of signalling pathways. Adapter, anchoring and scaffold proteins form a framework to compartmentalize enzymes and substrates, contributing to regulation of signal transduction (Pawson 1997b; Schillace 1999)

Protein tyrosine kinases may be receptors themselves, or are non-receptor PTKs, which interact with molecules at the cell surface. Src-family, Syk (ZAP-70 and Syk) and Janus family (JAK1, JAK2, JAK3 and Tyk2) families of tyrosine kinases are the focus of much investigation. The serine/threonine kinases include mitogen activated protein kinases (MAPKs), cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinases (PKG), and the phospholipid-dependent protein kinase C (PKC) family (Krontiris 1995; Sugden 1995)

Crosstalk between signalling pathways has become one of the most intensively studied aspects of signal transduction. Different receptor systems interact in complex
ways, creating a network by which cells receive information from their microenvironment and then have the means to react. The impact of virus infection on these signalling events was of interest in this dissertation. In particular, exploration of signal transduction pathways in the T lymphocyte and the myocyte in response to viral infection may impact on susceptibility to group B coxsackieviral (CVB) infection.

1. SRC FAMILY TYROSINE KINASES

Src was first identified as the normal cellular homologue of v-src, the transforming gene of Rous sarcoma virus, an avian retrovirus responsible for tumor formation in infected animals (Rous 1911; Stehelin 1976). Src is the prototype for a family of at least ten tyrosine kinases, which are structurally related and involved in transducing signals from cell surface receptors (Pawson 1997a). Included in this family are Src, Fyn, Lyn, Lck, Yes, Fgr, Hck, Blk, Yrk and Rak (Pawson 1997a). Maximal expression of Src-family kinases has been observed in specific cell types and tissues, but low level of expression of Src-family members may occur in many different cell types and could have significant functional relevance (Corey 1999).

Src-family non-receptor tyrosine kinases are defined by a common structure, with five distinct regions identified (Brown 1996) (Figure 5). The amino-terminal seven amino acids contains a myristoylation site, which links the protein with the plasma membrane and has a role in biological activity (Resh 1994). As well a palmitoylation site may be present and participate in concentrating Src family kinases in caveolae and setting up potential interactions with glycophosphatidylinositol-linked surface proteins (Corey 1999). Downstream is the unique domain, which is highly variable among the kinases and contributes to kinase specificity and targeting. Similar Src Homology (SH) or SH3 and SH2 domains are shared between Src family kinases and a variety of other cytoplasmic signalling proteins (Pawson 1992). In the kinase domain,
Figure 5: Src family protein tyrosine kinases. (A) Typical structure of a Src family protein tyrosine kinase (PTK). (B) Regulation Src family protein tyrosine kinases. C-src kinase (Csk) phosphorylates the tyrosine of the negative regulatory site of the Src, driving the kinase into the "closed" inactive state. Dephosphorylation by CD45 opens the kinase and allows activation by phosphorylation of the kinase domain tyrosine. Modified from Weiss, 1999.
highly conserved residues are essential for the binding of ATP and kinase activation (Hunter 1985; Parsons 1989). The major autophosphorylation site is located in the activation loop of the kinase domain (Schwartzberg 1998). A conserved tyrosine residue (tyr 527 in Src) in the carboxy-terminal tail of the enzyme is the key regulatory site of the kinase (Brown 1996). Phosphorylation of this site, mediated by c-src kinase (Csk) (Okada 1989; Sabe 1992), represses kinase activity of Src, while mutation or dephosphorylation of Y527 constitutively activates the kinase, enabling it to transform cells (Courtneidge 1985; Cartwright 1987; Kmiecik 1987; Pawson 1997a). Dephosphorylation of the C-terminal residue by, for example, CD45 protein tyrosine phosphatase, can lead to Src activation by allowing the enzyme to assume an active conformation (Corey 1999). (Figure 5). When phosphorylated, this terminal tyrosine interacts with the Src's own SH2 domain, resulting in inactivation of the enzyme (Parsons 1989). Mutation of the SH2, and the SH3 domains also, leads to the activation of the Src kinase, giving both domains a major role in the regulation of Src kinase function.

The kinase activity of Src is essential for triggering pathways downstream of growth factor receptors, such as for platelet derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1) (Ralson 1985; Kypta 1990), as well as signalling from certain G-protein coupled receptors and in responses to oxidative and ultraviolet (UV) stress (Devary 1992; Mukhopadhyay 1995; Luttrell 1996; Schwartzberg 1998). Src family kinases initiate signalling cascades, ultimately affecting cell migration, adhesion and shape, cell-cycle progression, secretion and differentiation (Corey 1999). The second level of signalling molecules includes Ras, PI-3-K, and FAK (Corey 1999). Src activates Ras via SH2-domain-containing (SHCs), Grb2 and SOS adaptor proteins, and phosphatidylinositol 3'-hydroxyl kinase (PI3K) via the adaptor molecule Cbl (Datta 1997; Corey 1999). Other nonreceptor protein tyrosine kinases are activated or associate directly with Src kinases, including ZAP70, Syk, Tec family kinases and
Focal Adhesion Kinase (FAK). FAK, a substrate for Src following integrin receptor interaction (Schaller 1994; Schlaepfer 1996; Schaller 1999), becomes a docking site for Src and PI3K, and Ras pathway activation follows (Erwin 1992; Schlaepfer 1994; Thomas 1995; Chen 1996; Schlaepfer 1997; Corey 1999).

2. LCK and FYN: THE T CELL TYROSINE KINASES

P56\text{Lck} (Lck) is a cytoplasmic membrane-associated member of the Src family of nonreceptor protein tyrosine kinases which interacts with CD4 and CD8 coreceptors (Voronova 1984). Structurally, Lck is comparable to other members of the Src kinase family. The N-terminus of Lck is palmitoylated at 2 sites in addition to being myristoylated. Two promoters are responsible for transcription of Lck mRNA: the proximal type I promoter and the distal type II promoter, which produce type I and type II transcripts, respectively (Leung 1989; Takadera 1989; Reynolds 1990). The transcripts differ only in their 5' untranslated regions. In mature thymocytes and T cells Lck transcription is almost exclusively from the type II promoter (Abraham 1991a; Abraham 1991b). Although it is known to be maximally expressed in T lymphocytes (Voronova 1984), recently, Lck has been reported in the heart and isolated myocytes of adult rabbits and found to be activated with ischemic preconditioning, an established cardioprotective technique (Ping 1999). This observation challenges the belief that Lck expression is restricted to T cells, B cells and NK cells (Bolen 1992). Lck, from promoter I, is also expressed in colon carcinoma cells under the influence of an alternate pathway of transcriptional activation (McCracken 1997).

P59\text{Fyn} (Fyn), also a member of the Src family, interacts with the TCR\text{\(\zeta\)} chain and the CD3\text{\(\gamma,\delta,\varepsilon\)} chains through its N-terminal region (Samelson 1992). Two isoforms have been identified, one expressed in brain and other cell types, with the second primarily expressed in lymphoid cells (Cooke 1989).
Lck and Fyn influence multiple T lymphocyte functions. Lck has been implicated in functions as diverse as thymic development (Molina 1992), T cell stimulation (Glaichenhaus 1991; Molina 1992; Straus 1992; Xu 1993), lymphokine production (Abraham 1991c; Luo 1992), T cell mediated cytotoxicity (Karnitz 1992), endocytosis (Pelchen-Matthews 1991; Cefai 1992) and in apoptosis (Julius 1993). Lck has a critical role in thymic maturation as demonstrated by the block in T cell maturation after gene-targeted knockout of the tyrosine kinase (Molina 1992). Lck is also important in cytotoxic and antibody-mediated responses to viral infection (Molina 1993). Fyn is involved in regulation of T cell function including T cell stimulation and IL-2 production, however a redundancy may exist with the ability of Lck to compensate. Knockout mice lacking Fyn are partially defective in TCR mediated signalling (Appleby 1992; Stein 1992).

3. MITOGEN ACTIVATED PROTEIN KINASES

Mitogen-activated protein kinases (MAPKs) are important mediators of signal transduction from the cell surface to the nucleus (Sturgill 1988; Rossomando 1989). They provide a link between diverse extracellular stimuli and cellular events responsible for proliferation and differentiation, including the cell cycle, generation of phospholipid messengers, transcription, and translation. Three separate groups of mammalian MAPKs have been characterized. Cascades consist of a three-tiered arrangement of protein serine/threonine kinases, conserved from yeast to humans, in which the MAPK is activated by a MEK, which is activated by a MEKK. Extracellular-signal regulated kinases (ERK), p38 MAPK and c-jun NH2-terminal kinase (JNK) pathways are illustrated in figure 6. Other ERK-related proteins have been identified, ERK3 (Boulton 1991), ERK 5, or big MAPK (BMK), and ERK 7. Currently, little is known about these ERKs, however, ERK5 may be involved in the regulation of cell
Figure 6: Mitogen-activated protein kinase (MAPK) signalling pathways. The three classic MAPK cascades are depicted in this illustration. These tyrosine/serine kinases transduce signals from the cell surface to the nucleus. Effects on gene transcription direct the cellular response to growth and stress messages. Modified from Gutkind, 1998.
proliferation, while ERK7 can phosphorylate c-Fos (Saxena 2000).

i) Extracellular-signal regulated kinases 1 and 2

 ERK1/2, 44 kDa and 42 kDa cytosolic proteins, were the first MAPK isoforms cloned and characterized (Boulton 1990; Boulton 1991). They were originally identified when activated by growth factor signalling through receptor tyrosine protein kinases (Sturgill 1988; Rossomando 1989; Sugden 1995). These serine/threonine (ser/thr) kinases are distributed widely, with distinct developmental and tissue distributions (Boulton 1991). MAPKs preferentially phosphorylate proteins on ser/thr residues within a Pro-X-(ser/thr)-Pro consensus sequence (Alvarez 1991; Gonzalez 1991).

 ERKs play a central role in the regulation of cell proliferation and differentiation in response to mitogens and a wide variety of growth factors and cytokines (Sturgill 1988; Ahn 1991; Boulton 1991; Ettehadieh 1992; Davis 1993; Seger 1995). Inhibition of ERKs impairs cell proliferation in response to a number of growth stimulating agents (Pages 1993), whereas constitutive activation of the MAPK pathway is itself sufficient for tumorigenesis (Schlessinger 1993; Dhanasekaran 1995). ERK1/2 activation also has a specific role in thymocyte development and CD4 and CD8 lineage commitment (Bommhardt 1999; Pages 1999). Upstream regulators of the ERK signal pathway are activated by receptor tyrosine kinases, G-protein coupled receptors (GPCRs), and non-receptor tyrosine kinases. Src family kinases (Fyn, Lyn Yes) (Doolittle 1983; Ptasznik 1995; Wan 1996) link βγ subunits of heterotrimeric G proteins to activation of Ras through phosphorylation of the adapter protein Shc and the recruitment of the Grb2 adapter then SOS, a guanine nucleotide exchange factor for Ras (Luttrell 1996; Gutkind 1998; Saxena 2000). Raf is recruited to the plasma membrane and activated by Ras, and downstream MEK1/2 is activated, which dual phosphorylates ERK1 and ERK2 (Cobb 1995). Prolonged activation of MAPK is mediated by PI3K and PKC,
independently of MEK activation (Grammer 1997). ERK1/2 homodimerize and translocate to the nucleus following activation to influence cell function.

Downstream targets of ERKs include a large number of substrates (Davis 1993), including transcription factors (c-myc, c-jun, NF-IL6, ATF-2, AP-1 and Elk-1) (Treisman 1994), cytoskeletal proteins (ie myelin basic protein and microtubule-associated protein 2), other protein kinases (the ribosomal protein S6 kinase, p90RSK) (Sturgill 1988), and other proteins involved in intracellular signalling (cytoplasmic phospholipase A2) (Lin 1993). A role for ERKs in cytokine production is implicated for IL-2 (Perkins 1993; Whitehurst 1996), IL-3, IL-4, IFN-γ and GMCSF (Egerton 1996) and lymphotoxin (Li 1999a), indicating a broad influence of this pathway on inflammatory mediators. Although JAK-STAT pathways are central to cytokine mediated signal transduction (Kishimoto 1994; Ihle 1995), the Ras-MAPK pathway can be activated downstream of a variety of growth factors and cytokine receptors and may play a regulatory role in cytokine stimulated gene expression (David 1995; Joneson 1997).

ii) Stress-activated MAPKs

c-jun NH2-terminal kinase (JNK), initially identified in 1990, shares a proline-directed substrate specificity and an activation requirement for dual tyrosine/threonine phosphorylation with the ERKs (Kyriakis 1990; Davis 1994). The most potent activators of JNK are UV radiation, environmental stress and the proinflammatory cytokines TNF-α and IL-1β (Derijard 1994). Upstream of JNK, Ras can cause partial activation of the JNK cascade, but primarily potentiates the effects of other stimuli (Derijard 1994). The transcription factor c-jun, a component of the AP-1 transcription factor was the first identified substrate of JNK (Pulverer 1991). ATF-2 (Gupta 1995) and Elk-1 (Whitmarsh 1995) are also activated by JNK. Elk-1, with serum response factor controls serum response element directed transcription such as expression of immediate-early genes c-fos and Egr-1.
p38 MAPK is also a stress activated serine/threonine kinase (Han 1994), responding to lipopolysaccharide (endotoxin from gram negative bacteria) (Rouse 1994), hyperosmolar medium (Han 1994), TNF-α, interleukin-1β (Freshney 1994) and heat shock (Davis 1994; Rouse 1994). In addition to activation of the transcription factors c-jun and ATF-2, p38 activates MAPK kinase-2 (Rouse 1994) which phosphorylates and regulates the heat shock protein Hsp25/HSP27.

4. JANUS (JAK) FAMILY TYROSINE KINASES

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway mediates diverse effects downstream of cytokine interactions with their receptors. There are four members of the JAK family: JAK1, JAK2, JAK3 and Tyk2, and seven STATs (1 to 4, 5a and 6, 7). JAKs are differentially stimulated in response to different cytokines, and interact with various STATs (Ihle 1995; Leonard 1998) (Table 5). Many cytokines and growth factors activate JAK1, JAK2 and Tyk2, but JAK3 is only activated by ligands of receptors with a γc subunit. Associations between particular cytokine receptors, JAKs and STATs confer specificity to the system. All JAKs have a characteristic JH1 C-terminal kinase domain adjacent to a JH2 pseudokinase domain (Ihle 1995). STATs are composed of an SH2 domain, a conserved tyrosine residue, a DNA binding domain, and an N-terminal STAT dimerization region.

JAKs associate with designated box 1 and box 2 motifs in the membrane-proximal region of cytokine receptors. Genetic knockout of individual JAKs and STATs has demonstrated their requirement in normal hematopoiesis and immune function (Ward 2000). Following binding of the ligand to a cytokine receptor, tyrosine residues of JAKs are autophosphorylated and thus activated. Activated JAKs
<table>
<thead>
<tr>
<th>JAK/STAT type</th>
<th>knockout phenotype</th>
<th>cytokines affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK 1</td>
<td>impaired lymphoid development, defective response to class 2 cytokines and those using γc or gp130 receptor subunits</td>
<td>IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-15, LIF, all IFNs</td>
</tr>
<tr>
<td>JAK 2</td>
<td>no definitive erythropoiesis</td>
<td>EPO, TPO, IL-3, IL-5, GM-CSF, IFN-γ</td>
</tr>
<tr>
<td>JAK 3</td>
<td>defective lymphoid development and myelopoiesis</td>
<td>IL-4, IL-7, IL-9, IL-15</td>
</tr>
<tr>
<td>STAT 1</td>
<td>IFN responses absent</td>
<td>IFNs</td>
</tr>
<tr>
<td>STAT 2</td>
<td>type 1 IFN responses absent</td>
<td>IFN-α/β</td>
</tr>
<tr>
<td>STAT 3</td>
<td>embryonically lethal</td>
<td></td>
</tr>
<tr>
<td>STAT 4</td>
<td>IL-12 responses absent</td>
<td>IL-12</td>
</tr>
<tr>
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<td>proliferation signalling affected</td>
<td>IL-2, IL-3, IL-7, GM-CSF, G-CSF</td>
</tr>
<tr>
<td>STAT 6</td>
<td>IL-4 responses absent</td>
<td>IL-4</td>
</tr>
</tbody>
</table>

IL = interleukin; IFN = interferon; EPO = erythropoietin; TPO = thrombopoietin; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; Modified from Ward et al, 2000.
phosphorylate the receptor, enabling interaction with STATs. JAKs phosphorylate a STAT C-terminal tyrosine residue, triggering the formation of stable STAT homodimers and heterodimers. Dimers translocate into the nucleus and interact with specific gene promoters to direct gene transcription (Ihle 1995; Ward 2000).

Multiple mechanisms of downregulation of the pathway have been identified, and are important in maintaining a balanced growth or immune response (reviewed in Ward et al, 2000). Receptor-mediated endocytosis and subsequent degradation in endosomes, endogenous dominant-negative STAT variants, and tyrosine phosphatases, such as SHP1 have been implicated in control of the pathway. As well, suppressor of cytokine signalling (SOCS) family proteins directly interact with particular JAKs, inhibiting kinase activity.

Recently, CD45 PTP has been shown to act as a Janus kinase (JAK) phosphatase, with the potential to alter signalling pathways downstream of cytokine interactions with their cell surface receptors (Irie-Sasaki 2001). Following observations that CD45 inhibits the IL-3-activated JAK2-STAT3/5 signalling pathway in bone-marrow-derived mast cell lines, further investigation established the PTP as a JAK phosphatase, able to negatively regulate cytokine receptor signalling in several haematopoietic cell types (Irie-Sasaki 2001).

5. PROTEIN KINASES AND T CELL SIGNAL TRANSDUCTION

T cells differentiate and proliferate following presentation and recognition of processed antigen in an MHC-restricted manner (Crabtree 1989). A complex cascade of protein phosphorylation is crucial for signal transduction mediated by the TCR, as shown in figure 7. T cell stimulation by antigen or anti-TCR antibodies leads to a rapid rise of intracellular tyrosine protein phosphorylation (Hsi 1989), although the TCR itself lacks intrinsic protein tyrosine kinase activity. Lck associates with the cytoplasmic
Figure 7: T cell signal transduction pathways. Multiple signalling pathways are activated downstream of the T cell receptor (TCR). Ultimately the genes encoding IL-2 and other proteins, are transcribed, stimulating T cell activation, differentiation and proliferation. Modified from Elder, 1998.
domains of the TCR coreceptors CD4 and CD8, whereas Fyn complexes with TCRζ/CD3 (Sleckman 1988; Shaw 1989; Turner 1990; Samelson 1992; Straus 1992; Penninger 1993b; Rudd 1994). Mutants of the Jurkat T cell line, JCaM T cells, are defective in signal transduction following TCR stimulation due to the absence of Lck tyrosine kinase function (Goldsmith 1987; Straus 1992; Rouer 1999). Lck is required for the initial events in the TCR signalling process (Goldsmith 1987; Straus 1992).

Crosslinking of the TCR activates Lck, which phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of the CD3 zeta chain inducing recruitment of ZAP-70, a member of the Syk-family of tyrosine kinases (Chan 1994; Iwashima 1994; Isakov 1995; van Oers 1996). Lck phosphorylates ZAP70, and downstream signalling pathways are activated (Williams 1998). Initiation of T cell signalling requires Lck and ZAP-70 to function sequentially (Iwashima 1994). Downstream PLCγ, PI-3K, PKC and SHC adaptor proteins are recruited and activated (Kolanus 1993; Ravichandran 1993; Rudd 1994). SHC proteins activate Ras by Grb-2 and SOS (Rozakis-Adcock 1993) The extracellular signal-regulated kinases (ERKs) are activated rapidly via adaptor proteins and Ras following TCR engagement and Lck activation (Franklin 1994; Izquierdo 1995; Saxena 2000). Lck associates with and activates the ERK1/2 pathway via the SH3 domain of the Src kinase (Ettehadieh 1992; August 1996). But, while SH3 is required for ERK activation, it is not necessary for ZAP-70 and phosphatidylinositol pathway activation (Denny 1999). In T lymphocytes ligand interaction with the IL-2 receptor also stimulates ERK phosphorylation (Satoh 1991; Turner 1991; Watts 1993). PKC mediated events also activate ERKs in T cells, however are not coupled to TCR activation (Izquierdo 1993; Izquierdo 1994). JNK and p38 MAPK are also stimulated in activated T lymphocytes (Su 1994; Crawley 1997). The regulation of JNK is a point of interaction for TCR activation and CD28 costimulatory signals (Su 1994; Cantrell 1996).
CD45, a transmembrane protein tyrosine phosphatase (PTP) expressed in all haematopoietic cells, is important in the regulation of immune cell responses (Penninger 1993b; Chan 1994). The extracellular region is variable among isoforms, while the cytoplasmic region is conserved and composed of two PTP domains and a C-terminal tail (Townbridge 1994). The first intracytoplasmic domain of CD45 cytoplasmic region contains the primary tyrosine phosphatase activity (Tonks 1988), which targets the Src-family protein tyrosine kinases (PTKs) Lck and Fyn (Ostergaard 1989; Sieh 1993). This PTP is critical to Lck signalling, and upregulates the activity of Src kinases by dephosphorylating the negative regulatory kinase site at the C terminus of Lck and Fyn (Mustelin 1989; Ostergaard 1989). The tyrosine kinase Csk phosphorylates the same site, thus inhibiting T cell activation (Chow 1993b).

Inositol lipid metabolism is regulated following T cell activation (Sieh 1994; Weiss 1994; Cantrell 1996). Phospholipase Cγ1 (PLCγ1) regulates inositol phospholipid hydrolysis, generates inositol polyphosphates and diacylglycerols, enabling TCR stimulation to lead to regulation of intracellular calcium levels and recruit the PKC family, respectively (Berridge 1993). PI3K requires both TCR and CD28 costimulatory signals for optimal activation (Ward 1993).

Interleukin-2 production is characteristic of T cell activation. The coordinate activities of several elements of T cell signalling pathways are required for IL-2 gene expression (Cantrell 1996). Lck and MAPK association during S phase of the cell cycle plays a role in regulating IL-2 mediated DNA synthesis and lymphocyte proliferation (Taieb 1995; Li 1999b). The involvement of ERK may be via Elk1 regulation of Fos, which associates with AP-1 and nuclear factor of activated T cells (NFAT) (Perkins 1993; Cantrell 1996). The calcium phosphatase calcineurin is involved in IL-2 gene expression via activation of NFAT, which translocate to the nucleus in TCR-activated cells, combining with AP-1 to form a functional transcription complex (Clipstone 1992; Rao 1994).
Interestingly, Lck can mediate signal transduction independently of TCR engagement, by the IL-2Rβ chain and glycoprophatidylinositol (GPI)-linked surface molecules such as decay accelerating factor (DAF), CD59, CD48, CD24, CD14 and murine Thy-1 and LY-6 (Hatakeyama 1991; Stefanova 1991; Shenoy-scaria 1992). Antibody crosslinking of GPI-anchored DAF, in combination with phorbol esters, induces T cell proliferation (Davis 1988; Shenoy-scaria 1992). DAF-mediated signal transduction involves Lck and Fyn (Shenoy-scaria 1992; Shenoy-scaria 1993). DAF triggered Lck phosphorylation induces Ras activation, and depends on CD3-TCR complex expression, however IL-2 production requires the presence of the TCRζ chain alone (Tosello 1998)

6. PROTEIN KINASES AND THE HEART

Cardiac hypertrophy is a compensatory response to increased wall stress. Hypertrophy is a major risk factor for the development of congestive heart failure and leading to death. The role of intracellular signalling pathways in the evolution of cardiac hypertrophy is currently being addressed. Cardiac myocyte hypertrophy can be induced by mechanical stretch (Komuro 1990; Komuro 1993) and several ligands of G protein coupled receptors (GPCR) including norepinephrine (Simpson 1985), endothelin-1 (Ito 1991; Kovacic 1996) and angiotensin II (Baker 1990; Yamazaki 1998). In cardiac myocytes the ERK1/2 cascade, which is activated by these same factors, is important for the induction of hypertrophy (Sadoshima 1993; Yamazaki 1995). Cardiac hypertrophy can also be induced by IL-6 and the related cardiotropin-1 (CT-1) (Pennica 1995). IL-6-related cytokines share the gp130 subunit as the signal transducing component of their receptor complex (Yamauchi-Takahara 2000). The binding of ligand to receptor induces the dimerization of gp130, leading to JAK activation and gp130 tyrosine phosphorylation (Murakami 1993), with subsequent
activation of signal transducer and activator of transcription (STAT), Ras-MAPK, and PI3K (Hirano 1997).

Evidence has also accumulated for a cardioprotective role for IL-6 family cytokines. Leukemia inhibitory factor (LIF) protects cardiac myocytes from apoptotic cell death via PI3K/AKT pathway activation of Bcl-xL and Bad phosphorylation (Oh 1998; Yamauchi-Takahara 2000). Conditional knockout of cardiac ventricular gp130 produced rapid onset dilated cardiomyopathy and induction of myocyte apoptosis in mice exposed to acute pressure overload of the heart (Hirota 1999), illustrating the importance of a gp130-dependent myocyte survival pathway. The role of JAK/STAT, MAPK and PI3K pathways in gp130-mediated cardiac hypertrophy and myocyte protection may be relevant to the role of IL-6 in myocarditis (Kanda 1996). Ischemic preconditioning, which is cardioprotective, is dependent on intracellular signalling pathways, including kinases such as protein kinase C (PKC). Downstream, activation of Lck and Src in the myocardium are PKC-dependent (Superti-Furga 1995; Ping 1999).

7. PROTEIN KINASES AND VIRAL INFECTION

Several viruses have evolved diverse mechanisms to stimulate signal transduction pathways which have a role in viral oncogenic transformation of cells, promotion of viral replication, and regulation of host inflammatory responses to infection (Table 6).

Signal transduction pathways are involved in human immunodeficiency virus (HIV) infection at several points in the replicative process, and have been extensively studied. A link between Src kinases and HIV-1 was first indicated by the activation of HIV-1 long terminal repeat (LTR) and gene expression by the v-src oncoprotein (Dehbi 1994). Lck has been shown to have a role in the transduction of signals following HIV-
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<th>virus</th>
<th>pathways</th>
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<tr>
<td>adenovirus</td>
<td>1. Virus internalization by αV integrins requires activation of PI-3K, but</td>
<td>Li et al, 1998</td>
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<td>αV integrin-mediated cell motility depends on activation of the ERK1/2</td>
<td>Bruder and Kovesdi, 1997;</td>
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<td>pathway</td>
<td>Whalen et al, 1997</td>
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<td>2. ERK1/2 phosphorylate the adenovirus E1A protein, and induce IL-8</td>
<td>See and Shi, 1998</td>
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<td>production with infection</td>
<td>Perez and White, 1998</td>
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<td>3. Adenovirus infection, and specifically E1B/19K protein activate</td>
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<td>JNK/SAPK depends on activates SAPK, protects cells from apoptosis</td>
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<td>4. E1B 19K, the adenovirus Bcl-2 homologue inhibits Fas-mediated</td>
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<td>apoptosis</td>
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<td>cytomegalovirus</td>
<td>1. Viral gene expression is dependent on host transcription factors</td>
<td>Rodems and Spector, 1998</td>
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<td>CREB, AP-1, SRE and Elk-1.</td>
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<td>2. Inhibits ERK specific phosphatases, leading to sustained activation</td>
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<td>Epstein-Barr Virus</td>
<td>1. EBV activates ERK1/2 and JNK/SAPK pathways</td>
<td>Fenton and Sinclair, 1999</td>
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<td>2. ERK activation interrupts viral latency</td>
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<td>3. p38 MAPK participates in EBV-encoded latent membrane protein 1</td>
<td>Eliopoulos et al, 1999</td>
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<td>(LMP1)-mediated IL-6 and IL-8 production</td>
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<td>hepatitis B virus</td>
<td>1. HBx, a hepatitis regulatory protein, activates protein kinases</td>
<td>Benn and Schneider, 1994</td>
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<td>including Ras, ERKs, JNK/SAPK, PI-3-K, IKK, PKC, JAK/STAT, and</td>
<td>Benn et al, 1996,</td>
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<td>several transcription factors including NF-κB, AP-1, CREB, TBP</td>
<td>Henkler et al, 1998,</td>
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<td>2. Src activation by HBx stimulates viral polymerase activity and viral</td>
<td>Klein et al, 1999,</td>
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<td></td>
<td>replication</td>
<td>Diao et al, 2001</td>
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<td>Virus Type</td>
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<td>Herpes simplex virus type 1</td>
<td>The virion transactivator protein VP16 stimulates p38 and JNK MAPK pathways and activates AP-1</td>
<td>Zachos et al, 1999</td>
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<td>HTLV-1</td>
<td>The HTLV-1 oncprotein TAX protein interacts with cellular transcription factors such as CREB/ATF, CREB binding protein and NFKB</td>
<td>Kuo et al, 2000, Yao and Wigdahl, 2000, Kibler and Jeang, 2001</td>
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<td>Herpesvirus saimiri</td>
<td>The p56Lck (Lck) binding domain of the virus activates Lck leading to T cell transformation and oncogenesis</td>
<td>Lund et al, 1999</td>
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<td>murine hepatitis virus 3</td>
<td>Virus induces activation of ERK1/2 and p38 MAPK in macrophages, promoting the procoagulant activity associated with fulminant viral hepatitis</td>
<td>McGilvray et al, 1996</td>
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<td>simian virus 40 large T antigen</td>
<td>Virus activation of signalling pathways protects cells from apoptosis</td>
<td>Rouquet et al, 1995</td>
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1 binding to CD4 receptor. The nef gene is important in the development of AIDS (Kestler 1991) and contributes to viral replication (Skowronski 1993; Miller 1994; Greenway 1995). Nef interacts directly with Lck and MAPK, decreasing kinase activity, potentially altering the ability of HIV-infected T cells to mount a defense against viral infection (Greenway 1996; Greenway 1999). A role for Lck in HIV-mediated signal transduction is consistent with its binding to CD4, the major HIV receptor (Manna 2000). Crosslinking of CD4 with HIV-1 envelope glycoprotein gp120 or monoclonal antibodies specific for the gp120 binding site on CD4 triggers Lck activation (Juszczak 1991; Hivroz 1993; Goldman 1994; Phipps 1996; Briand 1997). As well, HIV-1 binding to CD4 receptors activates the MEK/ERK signalling pathway (Popik 1998). Downstream of ERK1/2, nuclear expression factors AP-1, NFκB and C/EBP are activated and gene expression contributing to the inflammatory process is stimulated (Popik 1998). Lck is required for activation of NF-κB, AP-1, JNK, and ERK1/2 by the HIV regulatory protein Tat (Manna 2000). ERK1/2 can influence the infectivity of HIV-1 virions themselves (Yang 1999b). Evidence supports a role for ERK1/2 in the establishment of a functional reverse transcription complex within the host cell (Jacques 1998).

8. SIGNAL TRANSDUCTION AND PICORNAVIRUSES

Little is known about the effects of picornaviruses on cell signalling pathways. Reports on the effects of viruses, including coxsackievirus, echovirus and encephalomyocarditis virus (EMCV), on signal transduction have focused on later stages of infection, at the time of virus-induced host protein synthesis shutoff (Huber 1997a; Huttunen 1998; Huber 1999; Iordanov 2000).

In response to EMCV infection the mediators of interferon activation, RNase L and double stranded RNA (dsRNA)-activated protein kinase (PKR) are activated.
(Stark 1998). The antiviral action of RNase L, and PKR is mediated by inhibition of cellular protein translation, degradation of mRNAs, and elimination of infected cells by apoptosis (Iordanov 2000). Activation of the p38 MAPK pathway with EMCV infection triggers IL-6 production, which may mediate inflammatory responses via cytokine production (Iordanov 2000).

Echovirus 1 attachment is mediated by α2β1 integrin, but echovirus 7 uses DAF as its cell surface receptor (Bergelson 1994; Ward 1994). In both infections ERK1/2 activation was shown to be induced by 5 h pi and p38 MAPK activation by 10 h pi, with associated induction of immediate-early genes c-jun, junB and c-fos 10h pi (Huttunen 1997; Huttunen 1998). The time of ERK1/2 activation is consistent with this signal transduction being mediated by viral replication or induction of secondary responses, such as cytokine elaboration, rather than virus-receptor binding (Huttunen 1997).

CVB3 infection induces tyrosine phosphorylation of 48kDa, 70kDa and 200kDa host proteins in HeLa cells from 3 to 5 hours post infection (Huber 1997a). As well, herbimycin A, an inhibitor of tyrosine kinases, resulted in the reduction of virus-induced tyrosine phosphorylation and virus yield (Huber 1997a). In response to CVB3 infection Sam 68, a cellular target of Src kinases which is known to interact with poliovirus, was shown to associate with RasGAP (Huber 1999). Subsequently, RasGAP was cleaved at 6 hours post infection, possibly activating the Ras/MAPK pathway (Huber 1999). Persistent MAPK activation was also observed at this late stage of infection, possibly playing a role in CVB3-induced cytotoxicity or apoptosis (Huber 1999). Apoptosis of picornavirus infected cells has been indicated by observations of nuclear condensation and DNA fragmentation following poliovirus (Tolskaya 1995) and Theiler's murine encephalitis virus infection (Jelachich 1996; Tsunoda 1997). Caspase 3 activation and cleavage of substrates follows typical cytopathic effect seen with CVB3 infection, perhaps facilitating clearance of infected cells or the release of progeny virus (Carthy 1998).
HYPOTHESES

Group B coxsackieviruses are responsible for a wide range of disease phenotypes, including febrile illnesses, respiratory infections, meningitis, hepatitis, pancreatitis, and myocarditis. Although the nature of the infecting virus is relevant, different hosts infected with the same CVB3 strain may develop completely different clinical presentations. Host susceptibility to CVB3 still remains an enigma. The marked differences in host response to viral infection within a population are likely multifactorial. However, based on the current understanding of CVB3 myocarditis, specific hypotheses were developed and subsequently tested in the studies that comprise this thesis.

HYPOTHESIS #1

The T lymphocyte response to CVB3 infection promotes severe myocarditis in murine models of this disease. The specific elements of the T cell response that promote myocardial inflammation and destruction have not yet been identified. Therefore, it is hypothesized that individual αβ+ TCR T cell populations differentially determine the severity of CVB3 myocarditis by activating distinctive cellular and cytokine responses. This hypothesis was addressed in studies outlined in Chapter 3.

HYPOTHESIS #2

The Src-family protein tyrosine kinase p56Δck (Lck), essential to T lymphocyte activation, is a key determinant of susceptibility to CVB3 myocarditis. Lck is an integral component of intracellular signal transduction pathways. It is hypothesized that Lck is required for CVB3 activation of the extracellular signal-regulated kinase (ERK1/2).
signalling cascade, and subsequently, activation of the ERK cascade promotes CVB3 infection and myocarditis in mice. This hypothesis was addressed in studies outlined in Chapter 4.

HYPOTHESIS # 3

The initial interface between virus and host occurs at the cell membrane with the interaction between CVBs and their functional receptor coxsackie-adenovirus receptor (CAR). Following the establishment of a functional model of murine susceptibility to CVB3 infection, two hypotheses were addressed. Firstly, the hypothesis that the tissue distribution of CAR, and receptor isoform expression are determinants of differential host susceptibility to CVB myocarditis was explored. Secondly, the hypothesis that multiple isoforms of CAR exist and influence the phenotype of CVB3 disease was tested. These hypotheses were addressed in studies outlined in Chapter 5.

In the course of studies designed to test these hypotheses, evidence is presented which illustrate that the T cell response to CVB3 infection, viral activation of cellular signal transduction pathways, and the interaction between the virus and its receptors all contribute to susceptibility to coxsackieviral myocarditis.
CHAPTER 3

SUSCEPTIBILITY TO MYOCARDITIS IS DEPENDENT ON THE RESPONSE OF αβ T LYMPHOCYTES TO GROUP B COXSAKIE VIRAL INFECTION

1. PURPOSE

To investigate the role of specific T lymphocytes in the pathogenesis of group B coxsackieviral myocarditis, and to identify effector mechanisms important to the T cell response.

Based on the publication -


All experiments presented were performed or supervised by the candidate. The assistance of F Dawood, W-H Wen and K Aitken is much appreciated.
2. ABSTRACT

Viral myocarditis is an important cause of heart failure and dilated cardiomyopathy. T lymphocytes are implicated in myocardial damage in murine models of coxsackievirus B3 (CVB3) myocarditis. Knockout mice lacking CD4 (CD4<sup>-/-</sup>), CD8 (CD8<sup>-/-</sup>), both coreceptors (CD4<sup>-/-</sup>-CD8<sup>-/-</sup>), or the T cell receptor β chain (TCRβ<sup>-/-</sup>) were used to address the contribution of T cell subpopulations to host susceptibility to CVB3 myocarditis. Severity of disease was magnified in CD8<sup>-/-</sup> mice, but attenuated in CD4<sup>-/-</sup> mice, consistent with a pathogenic role for CD4+ lymphocytes. Elimination of both CD4 and CD8 molecules from T lymphocytes by genetic knockout better protected mice from myocarditis, demonstrating that both CD4+ and CD8+ T cells contribute to host susceptibility. Likewise, in TCRβ<sup>-/-</sup> mice, with prolonged survival and minimal myocardial disease was observed following CVB3 infection. Elevated interferon-γ and decreased tumor necrosis factor-α expression were associated with attenuated myocardial damage in CD4<sup>-/-</sup>-CD8<sup>-/-</sup> mice. These findings show that the presence of TCRα,β<sup>+</sup> T cells enhances host susceptibility to myocarditis. The severity of myocardial damage and associated mortality are dependent upon the predominant T cell type available to respond to CVB3 infection. One mechanism by which CD4+ and CD8+ T cell subsets influence the pathogenesis of myocarditis may involve specific cytokine expression patterns.
3. INTRODUCTION

Viral myocarditis is an important cause of heart failure and dilated cardiomyopathy (Liu 1996a; Opavsky 1998). The spectrum of disease spans from a fulminant course including arrhythmias or sudden death to mild early symptoms, later progressing to chronic heart failure (Liu 1996a; Opavsky 1998). Group B coxsackieviruses (CVB) have been associated commonly with viral myocarditis (Opavsky 1998). Current evidence from studies on CVB3 myocarditis in the mouse model suggests that the acute phase of disease involves both the development of direct viral-mediated myocyte dysfunction and tissue injury mediated by the immune response (Liu 1996a). While some elements of the immune response attenuate viral replication and protect the myocardium, others contribute to the development of myocardial inflammation and necrosis (Liu 1996a). In SCID mice, which lack both T and B cell functions, CVB3 infection induces severe disease (Chow 1992). In contrast, depletion of T lymphocytes through thymectomy, irradiation and bone marrow reconstitution; or, by injection of antithymocyte serum attenuates myocarditis (Woodruff 1974). Monoclonal antibody treatment directed against T cell populations can modify the severity of myocarditis in CVB3-infected mice, however depletion is not complete by this process (Lodge 1987; Kishimoto 1989). A proportion of each T cell subset remains available to participate in the immune response following antibody administration (Lodge 1987; Kishimoto 1989).

The majority of circulating T lymphocytes express α and β chains of the T cell receptor (TCR) with either CD4 or CD8 coreceptor molecules, which augment TCR signalling pathways (Mak 1994). Following viral infection, the host immune system responds via activation of TCRαβ+CD4+ helper T cells and TCRαβ+CD8+ cytotoxic/suppressor T cells in a major histocompatibility (MHC) II- or MHC I-restricted
manner, respectively (Mak 1994). In addition, TCRαβ+CD4−CD8− double negative (DN) T cells can also participate in the immune response (Suda 1993).

Proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, and the antiviral interferons (IFNs), participate in the immune response to CVB3 myocarditis (Seko 1997; Freeman 1998). Interferons are protective, while TNF-α is destructive in CVB3 myocarditis in animal models. Multiple cell types can be the source of these cytokines. Natural killer (NK) cells and macrophages, cellular elements of the innate immune system, respond to viral infection early, with the characteristic production of IFN-γ and TNF-α, respectively. CD4+ and CD8+ T cells, the key elements of the specific immune response, elaborate IFN-γ and TNF-α as well (Abbas 1996).

Models using transgenic knockout technology provide a highly specific system to examine the contributions of specific subsets of T lymphocytes to pathology of virus infection (Penninger 1996). In the study described below, mice with targeted gene disruptions of CD4+, CD8+, both CD4+ and CD8+ or TCRβ genes were used to examine the role of αβ T lymphocytes in the pathogenesis of viral myocarditis. Cytokine expression patterns were subsequently analyzed as a mechanism by which T cells may influence the pathogenesis of CVB3 myocarditis.
4. METHODS

i) Virus
The cardiovirulent strain of CVB3 was adapted by Woodruff and passaged in the laboratory of Dr. Charles Gauntt (Martino 1998). Virus stock was prepared by passage through HeLa (ATTC-CCL2) cell cultures, then titres were determined by plaque assay and stored at -70°C. Aliquots from the same stock were used for all animals.

ii) Mice
Transgenic knockout mice, created by disruption of the CD4, CD8 (Ly-2α) or TCRβ chain gene in embryonic stem cells through homologous recombination, have been described previously (Fung-Leung 1991; Rahemtulla 1991; Mombaerts 1992). Mice heterozygous for CD4 and CD8 genes were interbred to produce homozygotes for the CD4-/- or CD8-/- genotype. CD4-/-CD8+/+ mice were subsequently bred with CD4+/+CD8-/- mice to obtain CD4+/+CD8+/+ offspring (Schilham 1993). After mating of double heterozygotes, CD4-/-CD8+/+ offspring were produced (Schilham 1993). Mice of the CD4-/-CD8+/+ or CD4-/-CD8+/+ genotype will be referred to as CD4-/- throughout the text. Mice with genotypes CD4+/-CD8-/- or CD4+/-CD8-- will be designated CD8-/-.

No differences were found between the two genotypes within each group. CD4+/+CD8+/+ (control) mice, with both CD4 and CD8 molecules intact, served as controls. All animals were backcrossed into an A/J strain (H2/kk) to generate mice (5th generation) with uniform CVB3 susceptibility. An H2/kk haplotype was confirmed for all models using flow cytometry. Mice were housed and handled in an aseptic manner, including use of microisolator cages, sterilized mouse chow and drinking water, and use of continuous laminar airflow. Care of animals was in accordance with the policies of The Toronto Hospital and the protocol was approved by the hospital Animal Care Committee.
iii) Experimental Protocol

CD4-/- (n=47), CD8-/- (n=35), CD4-/-CD8-/- (n=56), TCRβ-/- (n=16) and control (n=64) mice, aged 4 to 6 weeks, were inoculated intraperitoneally (ip) with $10^5$ plaque forming units (pfu) of CVB3. Animals were observed for spontaneous mortality and a subgroup was randomly assigned to sacrifice on 4, 7, 10, 14 or 28 days post-infection. The animals who were selected at random for sacrifice were censored from the mortality data.

iv) Histopathology

Transverse midsections of hearts were fixed in 4% paraformaldehyde and processed for hematoxylin-eosin staining. Histopathological grading of cellular infiltrate and necrosis of the myocardium was on a scale of 0 to 4: 0 absence of infiltration or necrosis; 1 limited focal areas of infiltration or necrosis; 2 mild to moderate infiltration or necrosis; 3 moderate infiltration or necrosis; and, 4 extensive areas of infiltration or necrosis involving the entire examined heart tissue (Dong 1992). Paraffin-embedded pancreases and livers were examined qualitatively for evidence of inflammation and necrosis following CVB3 infection.

v) Viral titres

After aseptic removal, hearts, pancreases, livers and spleens were stored individually in RPMI medium (Gibco BRL, Burlington, Ontario) at -70°C for titre determination. Organ samples were homogenized in 5 ml of RPMI medium. Following 3 freeze-thaw cycles and centrifugation at 3000 rpm for 15 mins, virus titres were determined in duplicate by standard plaque formation assay.
vi) Neutralizing antibody titres
Neutralizing antibody titres were measured by inhibition of viral cytopathic effect (CPE). Sera were inactivated at 56°C for 30 mins. Serial dilutions, in 2-fold increments in RPMI plus 10% FCS (Gibco BRL), were incubated for 1 hour at 37°C with 100 pfu CVB3-CG. Sera were adsorbed onto HeLa cell monolayers in 96 well plates for 1 hour at RT, then replaced with RPMI plus 10% FCS and incubated for 48h at 37°C. The highest dilution of sera that inhibited CPE, determined following staining with 1% crystal violet in 10% formalin, was found to be the titre of neutralizing antibody against CVB3. The positive control was commercially produced anti-coxsackievirus antibody (Chemicon, Temecula, CA), and the negative control was uninfected mouse serum.

vii) Immunohistochemistry
Sections of paraffin-embedded heart tissue were deparaffinized and rehydrated, then endogenous peroxidase activity was blocked in 3% hydrogen peroxide in methanol for 10 mins. Sections were permeablized with 0.125% trypsin (Zymed Laboratories, San Francisco, CA) at 37°C for 10 mins and washed in PBS with 0.05% Tween 20 (PBST). After blocking with 10% normal goat serum (Zymed Laboratories) in PBS for 30 mins, samples were incubated overnight at 4°C with a rabbit polyclonal anti-CD3 antibody (Zymed Laboratories) to detect T cells or a monoclonal rat anti-mac-3 antibody (1:100; PharMingen, San Diego, CA) to detect macrophages, then washed with PBST. Sections were incubated for 20 mins with biotinylated secondary antibody (goat anti-rabbit; 1:250; Gibco BRL or goat anti-rat; 1:100; Jackson ImmunoResearch Laboratories, WestGrove, PA), then rinsed with PBST. Streptavidin-horseradish peroxidase conjugate (SA-HRP; 1:500; Jackson ImmunoResearch Laboratories) was then applied for 10 mins. After washing, sections were developed for 5 mins with aminoethyl carbazole (AEC; red staining; Zymed Laboratories).
Sections of OCT-embedded frozen myocardium were fixed in acetone at 4°C for 3 mins, then incubated in 3% hydrogen peroxide in methanol for 30 mins. After washing with PBS samples were blocked with 10% goat serum (Sigma Chemical Co, St. Louis, MO) and 3% BSA (Bioshop, Burlington, Ontario) in PBS for 1 h at RT. Samples were then incubated in rabbit anti-mouse asialo GM1 polyclonal antibody (Cedarlane, Hornby, Ontario) for 1 h at RT to detect natural killer (NK) cell infiltration. After washing in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:500), followed by SA-HRP (1:500) and AEC detection, as described above.

Primary antibody was replaced with normal rat serum or rabbit serum, as negative controls. As well, sections were processed in the absence of primary antibody or control serum. Sections of normal mouse spleen and thymus were used as positive controls. All samples were counterstained with hematoxylin.

To quantify differences in infiltrating cell populations at day 10, positive cells and total infiltrating cells were counted at high power in 5 randomly selected myocardial foci for each heart examined, then expressed as percent CD3 (for T lymphocytes) or mac-3 (for macrophages) positive cells. Alternatively, at day 4 the total number of asialo GM positive cells in each section was expressed as the number of positive cells per high power field (hpf, X250), with greater than 10 hpf counted per section.

viii) RNA isolation and RT-PCR
Hearts were snap-frozen in liquid nitrogen at the time of sacrifice. Total RNA was isolated using Trizol Reagent (Gibco BRL) as directed. First-strand cDNA synthesis was performed by incubation of 1 μg RNA with 200 ng/ml random hexamers (Gibco BRL) at RT for 10 mins, followed by the addition of 200 units Superscript II (Gibco BRL), 10mmol/L DTT, 0.5mmol/L of each dNTP (Pharmacia Biotech Inc., Baie d'Urfe,
Quebec) and first strand buffer (Gibco BRL). The 20 μl reaction was incubated at 42° for 45 mins, then reverse transcriptase was denatured at 70° for 5 mins.

Cytokine PCR primer sets for IFN-γ, TNF-α, IL-6, IL-4, IL-10 and β-actin were synthesized based on published sequences (Platzer 1995). Each 25 μl PCR reaction contained 0.5 mmol/L of each primer pair, 100 μmol/L of each dNTP (Pharmacia), 0.3 U Taq polymerase (Pharmacia) in reaction buffer (Pharmacia). Reactions were subjected to thermal cycling (Perkin-Elmer, Cetus) as follows: 94° X1 min; then, 95° X2 min, 60° X2 min, and 72° X2 min for 35 cycles; then, 95° X1 min, 60° X1 min, and 72° X7 min. PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis. Bands were recorded under ultraviolet light with a digital camera (GelDoc 1000, Bio-rad Laboratories). Band intensities representing cytokine PCR products were compared in a semi-quantitative fashion with Molecular analyst software (version 2.1.2, Bio-rad Laboratories) after expression as a ratio to β-actin for the same cDNA sample. Splenic cDNA and the plasmid pMCQ (courtesy of Dr. Cornelia Platzer), which contains gene fragments of all cytokines and β-actin, served as positive controls (Platzer 1995).

ix) Statistical analysis
Differences in survival were evaluated by Chi squared analysis. Results were subjected to analysis of variance with post hoc testing using Neuman-Keul comparisons (SuperAnova, Abacus Concepts, Inc.). Results were considered statistically significant when the probability of a type 1 error was less than 0.05.
5. RESULTS

i) Knockout of $\alpha\beta$ T cell subsets alters survival after CVB3 infection
To examine the role of TCR$\alpha\beta^+$ T cells in susceptibility to CVB3 disease, animal survival was followed for 28 days following virus inoculation. Survival was 46% in the control group (Figure 8). In CD4$^{-/-}$ mice a similar survival rate of 42% was observed, but in CD8$^{-/-}$ mice survival tended to be lower than control, at 30%. Outcome was significantly improved in both CD4$^{-/-}$CD8$^{-/-}$ and TCR$\beta^-$ mice, with increased survival rates of 72% and 82%, respectively. Susceptibility to CVB3 infection was decreased substantially by knockout of both CD4$^+$ and CD8$^+$ T cells, or alternatively, by knockout of all $\alpha\beta$ T cells.

ii) TCR$\alpha\beta^+$ T cells regulate cellular infiltration and necrosis in the heart
Following CVB3 infection, cardiac pathology was consistent with observed mortalities. Widespread myocardial infiltration was seen in controls on days 7 and 14, and remained unaltered by elimination of CD8$^+$ T cells (Figures 9 and 10). Less infiltration was seen in CD4$^{-/-}$ mice versus CD8$^{-/-}$ and control groups on day 14. Infiltration was mild in CD4$^{-/-}$CD8$^{-/-}$ and TCR$\beta^-$ mice. By day 14 myocardial necrosis tended to be most severe in control and CD8$^{-/-}$ mice in accord with the extent of infiltration observed in these groups (Figures 9 and 10). In CD4$^{-/-}$ and CD4$^{-/-}$CD8$^{-/-}$ mice necrosis was less extensive at day 14. Limited myocyte necrosis was observed at day 7 in TCR$\beta^-$ mice. The severity of myocardial infiltration and tissue damage was most dramatically attenuated in CD4$^{-/-}$CD8$^{-/-}$ and TCR$\beta^-$ strains. In CD4$^{-/-}$ mice myocarditis was less severe. In the CD8$^{-/-}$ group, with the CD4$^+$ T cell subset intact, myocardial infiltration and necrosis was severe and comparable with control.

No differences were found in the extent of pancreatic or liver disease among knockout groups. In the pancreas moderate to severe acinar destruction was observed.
Figure 8. Survival of immune knockout mice following CVB3 infection. CD4\(^{-/-}\) (n = 47), CD8\(^{-/-}\) (n = 35), CD4\(^{-/-}\)CD8\(^{-/-}\) (n = 56), TCR\(\beta^{-/-}\) (n = 16) and control mice (n = 64) were followed for 28 days after inoculation of 10\(^5\) pfu CVB3. Percent survival was highest in CD4\(^{-/-}\)CD8\(^{-/-}\) and TCR\(\beta^{-/-}\) mice. *p<0.01 vs CD4\(^{-/-}\) and control, and p<0.001 vs CD8\(^{-/-}\).
Figure 9. Histopathology of CVB3 myocarditis in knockout models. On day 7 post-inoculation with $10^5$ pfu CVB3 large confluent areas of myocardial necrosis and cellular infiltration were seen in control mice (A). Multiple scattered myocarditic foci were apparent in CD4$^-$ mice (B) and CD8$^-$ mice (C). In CD4$^-$CD8$^-$ mice (D) and TCRβ$^-$ mice (E) myocardial involvement was less extensive. Hematoxylin-eosin. X50.
Figure 10. Myocardial infiltration and necrosis in knockout models following CVB3 infection. At days 7, 14 and 28 following inoculation with $10^5$ pfu CVB3, the degree of infiltration (A) and necrosis (B) were scored (mean score ± SEM) from 0 to 4. Cardiac tissue was not available (n/a) for histological scoring on day 14 post CVB3 infection in TCRB$^-/-$ mice. n=3 to 7 per group; *p<0.05 vs control and CD4$^-/-$; and p<0.01 vs CD8$^-/-$; †p<0.05 vs control and p<0.01 vs CD8$^-/-$; ‡p<0.05 vs control; and, §p<0.05 vs CD8$^-/-$ and p<0.01 vs control.
in all groups, with islet cells remaining intact. Although mild to moderate cellular vacuolation and cytolysis of hepatocytes was evident in all groups, no frank necrosis was seen. Minimal inflammatory infiltrate was found in both organs, in striking comparison to the heart.

iii) Knockout of T cell subsets influences the participation of immune cells in CVB3 myocarditis

Not only were differences in the extent of myocardial infiltration observed (Figure 10); but, differences in the contribution of T cells and macrophages to the infiltrate were identified as well. On day 10 post-infection the proportion of T cells present in myocarditic foci of CD4+/-, CD8+/- and CD4+/-CD8+/- mice was decreased versus controls (Table 7 and Figure 11). Infiltration of macrophages was also less extensive in CD4+/- and CD8+/- mice as compared to control mice (Table 7 and Figure 11). The proportion of macrophages infiltrating the myocardium was lowest in CD4+/-CD8+/- mice. The extent of macrophage infiltration appears to depend on the T cell population available to respond to CVB3 infection. Macrophages may contribute to pathology or play a protective role, depending on the other elements involved in specific T cell knockout mice.

Infiltration of the myocardium by NK cells was evident at day 4 after infection in our T cell knockout models. NK cell infiltration tended to be more prominent in the myocardium of CD4+/- (2.5 ± 0.8 cells/hpf) and CD4+/-CD8+/- mice (3.3 ± 1.7 cells/hpf). Only 0.7 ± 0.5 cells/hpf and 0.4 ± 0.2 cells/hpf were found in the myocardium of CD8+/- and control mice, respectively.

The neutralizing antibody response, representative of B cell activation, was attenuated in some CD4+/- and CD4+/-CD8+/- mice (Figure 12). However, a rise in titre was observed in the majority of these mice, suggesting that in the absence of CD4+ T cells neutralizing antibody can still be produced.
Table 7: Infiltrating cell populations in myocarditic foci on day 10 following CVB3 infection

<table>
<thead>
<tr>
<th>genotype</th>
<th>T lymphocytes</th>
<th>macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>33.6 ± 5.2</td>
<td>40.6 ± 8.4</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>6.0 ± 1.2*</td>
<td>18.8 ± 4.0†</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>10.6 ± 3.8*</td>
<td>20.6 ± 5.9‡</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-/-&lt;/sup&gt;CD8&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>4.5 ± 1.5*</td>
<td>6.1 ± 3.6*</td>
</tr>
</tbody>
</table>

Sections of hearts 10 days after CVB3 infection were immunostained for T lymphocytes (CD3) and macrophages (mac-3). Data are reported as mean % CD3<sup>+</sup> or mac-3<sup>+</sup> cells ± SEM per group (n=5 to 9). *p < 0.0005 and †p < 0.05 within each column.
Macrophages were identified. Hematoxylin counterstain. X250.

H. Macrophages were identified. Few focal in hearts of CD4−/− (F) and CD8−/− (G) mice. In CD4−/CD8− hearts (H), few were seen in control mice (E), less macrophage infiltration was found in myocardial foci, in hearts of CD4−/CD8−-mice (D), T cells were rarely identified. Despite clear necrotic lesions, extensive macrophage infiltration was injection. In hearts of CD4−/− (E), CD8−/− (G), and CD4−/−CD8− (F) mice fewer T cells were seen in myocardial foci. In hearts of CD4−/− mice (A) and CD8−/− mice (B) T cells were rarey seen in myocardial foci. In hearts of CD4−/−CD8− mice (C) T cells were rarey seen in myocardial foci.

Figure 1.1. Immunohistochemical staining for CD3+ T cells and macrophages in hearts of CVB3-injected mouse models.
Figure 12. Neutralizing antibody titers following CVB3 infection of knockout models. Sera were collected from day 7 to day 10 post-infection, inactivated and serially diluted 1:2 to determine the highest dilution able to completely inhibit CPE by CVB3. • represent individual mice. Titers are expressed as reciprocal dilutions of serum. Serum from uninfected mice did not inhibit viral replication.
iv) **Myocardial cytokine expression varies among CD4 and CD8 knockout strains.**

To examine the hypothesis that the host's T cell repertoire affects the cytokine response in myocarditis, cardiac cytokine gene expression was evaluated in the knockout mice. On day 4, when cellular infiltration was minimal, cytokine expression differed among knockout groups (Figure 13). In CD4⁻/⁻ and CD4⁻/⁻CD8⁻/⁻ mice, myocardial IFN-γ gene expression was increased. This difference tended to persist until day 10. An association between day 4 IFN-γ expression and NK cell infiltration is demonstrated by regression analysis ($r=0.58; p=0.08$). Day 4 TNF-α expression was lowest in the hearts of CD4⁻/⁻CD8⁻/⁻ mice. Also in CD4⁻/⁻CD8⁻/⁻ mice, IL-6 expression in the heart was significantly higher than in CD8⁻/⁻ and CD4⁺/⁺CD8⁺/⁺ mice at day 4. At day 10, no differences in myocardial IL-6 and TNF-α expression were evident. There were no differences in IL-10 expression in the heart among knockout models at either timepoint. Expression of IL-4 was undetectable in the myocardium of any mice.

v) **Viral titres do not correlate with severity of myocarditis and mortality in CD4 and CD8 knockout mice.**

To test whether the differences in cardiac disease were due to differences in viral replication, CVB3 titres in the myocardium were analyzed. No significant differences among genotypes were observed, and no direct relationship between viral titres in the heart and outcome was apparent (Figure 14). The similarity in viral titres observed in the heart was apparent in other organs as well. No significant differences in splenic and hepatic viral titres were found among groups (Table 8). Pancreatic CVB3 titres were significantly lower in CD4⁻/⁻CD8⁻/⁻ mice versus control, although this was not reflected in differences in pancreatic tissue damage. Virus was cleared from spleens, livers and pancreases in the majority of animals in all groups by day 7 (data not shown).
Figure 13. Semiquantitative cytokine expression in the myocardium of CVB3 infected knockout models at day 4 and day 10 post-inoculation. A: interferon-γ (IFN); B: tumor necrosis factor-α (TNF); C: interleukin-6 (IL-6). Following RT-PCR, band intensities were determined by image analysis and data expressed as mean cytokine:β-actin ratio ± SEM with n=3 per group. *p<0.05 vs CD8−/− and **p<0.05 vs control and CD8−/−; †p<0.05 vs control and CD4−/−; ‖p<0.05 vs control and CD8−/−.
Figure 14. Cardiac viral titers following CVB3 infection of knockout models. Following CVB3 infection (10^5 pfu), the viral content of hearts was determined on days 4 and 7 post-infection. Cardiac tissue was not available (n/a) for analysis on day 4 in TCRβ-/- mice. Data are expressed as mean ± SEM with n=3 to 5 per group. No significant differences in viral titers were found between knockout groups.
Table 8: Organ viral titers day 4 following CVB3 infection of immune knockout models

<table>
<thead>
<tr>
<th>genotype</th>
<th>pancreas (log$^{10}$pfu/g tissue)</th>
<th>liver (log$^{10}$pfu/g tissue)</th>
<th>spleen (log$^{10}$pfu/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>5.99 ± 0.32</td>
<td>4.54 ± 1.38</td>
<td>5.21 ± 1.76</td>
</tr>
<tr>
<td>CD4$^{-/-}$</td>
<td>4.97 ± 0.35</td>
<td>4.57 ± 0.79</td>
<td>6.84 ± 0.21</td>
</tr>
<tr>
<td>CD8$^{-/-}$</td>
<td>4.95 ± 0.71</td>
<td>3.38 ± 0.23</td>
<td>5.46 ± 0.62</td>
</tr>
<tr>
<td>CD4$^{-/-}$CD8$^{-/-}$</td>
<td>4.66 ± 0.18*</td>
<td>5.76 ± 0.39</td>
<td>6.12 ± 0.85</td>
</tr>
</tbody>
</table>

Organ viral titers 4 days after CVB3 inoculation. Data are expressed as mean log$^{10}$ pfu/g tissue ± SEM. n=3 to 5, except CD8$^{-/-}$ liver and pancreas where only 2 samples were available. *p<0.05 vs control
6. DISCUSSION

T lymphocytes are essential participants in the host immune response to viral infection. While they can inhibit viral replication, T cell effector mechanisms can also promote host tissue damage via excess activation of cytokines and cellular cytotoxic responses (Liu 1996a). Gene-targeted removal of T cell subsets alters the course of viral myocarditis, illustrating two key factors in the pathogenesis of myocarditis following viral infection. First, host susceptibility to myocarditis is dependent upon activation of both CD4+ and CD8+ αβ T cells. Second, the predominant T cell type available to respond to CVB3 infection determines the severity and course of myocarditis after CVB3 infection. Viral replication in the heart does not correlate with myocardial damage in αβ T cell deficient mice, underscoring the pivotal role of the T lymphocyte in the development of CVB3 myocarditis.

Evidence from CD4-/- and CD8-/- knockout models demonstrates an important pathogenic role of CD4+ T cells in viral myocarditis. Knockout of CD4+ T cells alone provided protection from the myocardial infiltration and necrosis seen in control mice, but did not impact on survival. This is in contrast to anti-CD4 monoclonal antibody treatment experiments, which had no effect on myocarditis following CVB3 infection in A/J mice (Lodge 1987). The incomplete depletion of CD4+ T cells by monoclonal antibody administration likely explains the differences observed. In CD4-/- mice, total T cell numbers are normal and the CD8+ cytotoxic T cell response is intact (Rahemtulla 1991). Approximately 10% of peripheral T cells are TCRαβ+CD4-CD8- (TCRαβ+DN), which can provide MHC II-restricted help for CD8+ T cells and B cells (Rahemtulla 1991; Penninger 1993a). Therefore, activation of the intact CD8+ T cell population in CD4-/- mice may proceed in response to CVB3 infection, albeit at a less intense level, resulting in less extensive myocardial damage. Survival was lowest in the CD8-/- mice, while the severity of myocardial disease was comparable to that observed in control mice. Animal survival is likely inversely related to the extent of myocardial infiltration.
and necrosis, such that more mice in the CD8^{-/-} group had severe myocardial damage that is incompatible with life. Histopathological examination of pancreases and livers did not reveal any differences in inflammation or tissue necrosis among knockout groups, indicating that factors other than fulminant heart dysfunction did not lead to fatal outcome. This is consistent with the cardiovirulent nature of this strain of CVB3 (Chow 1991; Martino 1998). CD8^{-/-} mice are unable to produce any detectable CTL responses against viral antigens, but do have normal total T cell numbers, and a normal CD4^{+} T cell population which can mediate expected levels of T cell help and immunoglobulin class switching (Fung-Leung 1991). In conjunction with the less extensive myocarditis observed in CD4^{-/-} mice, the severe disease in the CD8^{-/-} model supports a deleterious role for CD4^{+} lymphocytes in myocarditis. Also, as suggested following monoclonal antibody-depletion of CD8^{+} T cells in A/J mice, removal of suppressive CD8^{+} T cells may play a role in the development of cardiac disease (Kishimoto 1989).

It was found that CD4^{-/-}CD8^{-/-} mice had increased survival and decreased myocardial damage after CVB3 infection, without any impairment of viral clearance, indicating that both CD4^{+} and CD8^{+} T cells participate in cardiac tissue damage. Although CD4^{+} and CD8^{+} T cells do not develop in CD4^{-/-}CD8^{-/-} mice, other functional T cell subsets are present. The majority of T cells (10% of normal numbers), are TCR\alpha\beta^{+} DN, with TCR\gamma\delta^{+} T cells increased to 3% of the total leukocyte number (Schilham 1993). The CD3^{+} cells identified in the myocardial infiltrate of CD4^{-/-}CD8^{-/-} mice were most likely TCR\alpha\beta^{+}DN or TCR\gamma\delta^{+} T lymphocytes. It has been shown that MHC II-restricted TCR\alpha\beta^{+}DN T cells provide T cell help in CD4^{-/-} mice in response to vesicular stomatitis virus (Pfeffer 1994). Human TCR\alpha\beta^{+}DN T cell clones can lyse target cells in a non-MHC-restricted manner (Porcelli 1992). Cytokine regulation of TCR\alpha\beta^{+}DN T cell effector functions supports a functional diversity and physiologic relevance of this T cell population (Nishizawa 1997). TCR\gamma\delta^{+} T cells can mediate both
a cytotoxic response (Hoq 1997; Welsh 1997) and B cell activation with neutralizing antibody production (Maloy 1998) in viral infection, and can influence effector cells via cytokine production (Kaufmann 1996). Thus, in CD4⁻/⁻CD8⁻/⁻ mice TCRαβ⁺DN and TCRγδ⁺ T cells may participate in the host response to CVB3 infection with cytolytic capacity and cytokine expression.

Susceptibility to myocarditis was decreased in TCRβ⁻/⁻ mice, with the outcome comparable to that observed in CD4⁻/⁻CD8⁻/⁻ mice. TCRβ⁻/⁻ mice completely lack TCRαβ⁺ T lymphocytes, however, development of TCRγδ⁺ T cells is not impaired (Mombaerts 1992). The total thymocyte number in TCRβ⁻/⁻ mice is 8% of controls, with peripheral T cells all γδTCR positive (Mombaerts 1992). In the absence of TCRαβ⁺ T cells, TCRγδ⁺ T cells may participate in viral clearance in conjunction with NK cells, macrophages and B cells. We have shown that removal of all TCRαβ⁺ T cells is not required to optimally protect the host from myocarditis. Genetic knockout of CD4⁺ and CD8⁺ T cell subsets is sufficient. These findings allow for the advancement of the concept that the exuberant response of this T cell repertoire confers host susceptibility.

The elimination of specific T cell subsets has the potential to alter other elements of the host response to CVB3 infection, including antibody production, cytokine expression and NK cell response. The detection of neutralizing antibody in all knockout groups indicates that in the absence of CD4⁺ T cells, other lymphocytes, perhaps TCRαβ⁺DN and TCRγδ⁺ T cells, can participate in B cell activation and antibody production in CVB3-infected mice. Neutralizing antibody is likely not an important protective factor in these models with an A/J background, as titre does not correlate with outcome.

Cytokines are active participants in the induction of the immune response to viral infection, and can influence both viral replication and immune-mediated tissue damage (Curfs 1997). Cardiac expression of IFN-γ, IL-6 and TNF-α may be significant factors in determining susceptibility to disease. The pattern of cytokine expression is
dependent upon the specific T cell subset available to respond to CVB3 infection. The findings of increased IFN-γ and IL-6 expression, and decreased TNF-α expression in association with resistance to CVB3 myocarditis in CD4−/−CD8−/− mice are consistent with published reports of the beneficial and detrimental effects of these cytokines (Heim 1992; Lane 1992).

Interferon-γ may protect CD4−/− and CD4−/−CD8−/− groups from myocarditis via its role in activation of NK cells and macrophages, or by recruiting or on activating the available T cell subsets in these models (Curfs 1997). Activation of NK cells occurs as early as day 3 post CVB3 infection (Godeny 1986), and is compatible with the myocardial infiltration of NK cells observed on day 4. The increased NK cell infiltration of the myocardium in CD4−/− and CD4−/−CD8−/− mice in association with elevated IFN-γ expression may reflect a shift in the balance of the immune response, in favour of protective immune elements such as IFN-γ and NK cells. Similar day 4 cardiac viral titres among groups does not support widespread inhibition of viral replication by IFN-γ, however, perhaps myocyte to myocyte spread of virus is limited by local IFN-γ production, thus limiting the size and number of myocarditic foci.

In an encephalomyocarditis virus (EMCV) model, IL-6 protects infected mice from myocarditis, decreasing mortality from 70 to 40% in association with a decrease in virus and an increase in neutralizing antibody levels (Kanda 1996). In CD4−/−CD8−/− mice lower cardiac TNF-α expression, together with elevated IFN-γ and IL-6, may contribute to decreased mortality by preserving heart function despite high viral titres. It has been shown previously that TNF-α administration to CVB3 resistant mice worsens myocarditis (Lane 1992), while anti-TNF-α antibody treatment prior to EMCV infection decreases the severity of myocarditis (Yamada 1994). In addition, the negative inotropic effects associated with TNF-α have been well documented (Finkel 1992; Freeman 1998).
Cytokine gene expression in the myocardium on day 4 may reflect responses of cardiac myocytes, fibroblasts, endothelial cells, and dendritic cells. Alternatively, early isolated infiltrating cells may play a role. Myocardial cytokine expression may be influenced directly by the virus, or in response to differences in splenic cytokine production, determined by T cell subsets in the knockout models.

In T cells CD4 and CD8 coreceptors participate in the activation of p56\textsuperscript{Lck} (Lck), the Src-family protein tyrosine kinase essential to the TCR signalling process (Goldsmith 1987; Straus 1992). In turn, CD45 protein tyrosine phosphatase (PTP) upregulates Lck activity by dephosphorylating the negative regulatory site of the Src kinase (Mustelin 1989; Ostergaard 1989). Recently, CD45 has been shown to act as a JAK phosphatase as well, with the potential to alter signalling pathways downstream of cytokine interactions with their cell surface receptors (Irie-Sasaki 2001). In CD45\textsuperscript{-/-} T cell lines JAK1 is hyperphosphorylated following treatment with the antiviral cytokine interferon-\(\alpha\) (IFN-\(\alpha\)). Gene-targeted knockout of Lck or CD45 protein tyrosine phosphatase (PTP) have been shown to protect mice from CVB3-related mortality and myocarditis (Liu 2000; Irie-Sasaki 2001). Data exploring the role of CD45 in the antiviral cytokine response to CVB3 infection is presented in the appendix to this thesis. Enhanced inhibition of CVB3 replication by IFN-\(\alpha\) in T cells lacking CD45 PTP demonstrated a mechanism by which the CD45\textsuperscript{-/-} mice may be protected from viral myocarditis.

It has been suggested that the variable responses of murine strains to immunotherapy are the result of differences in the host's genetic repertoire (Lodge 1987; Martino 1995; Huber 1997b). This may explain observations, in different murine backgrounds, that CD4 knockout mice develop severe myocarditis, while \(\beta\)2 microglobulin knockout mice have minimal myocardial disease following CVB3 infection (Henke 1995). This is in contrast to the milder myocarditis observed in the CD4\textsuperscript{-/-} mice shown here. Extrapolation of the present study suggests that it is in fact the
complement of specific T cell subpopulations in each host which determines susceptibility to myocarditis in mice and man. The relevance of T cell subset responses may have important diagnostic, prognostic and therapeutic implications in the management of the disease.

In summary, the present study used a strategy of gene targeting to elucidate the role of the T cell response in the pathogenesis of myocarditis after CVB3 infection. The severity of myocarditis is dependent upon the responding T cell subset. Both CD4+ and CD8+ T lymphocytes can contribute to myocarditis and mortality following CVB3 infection. Distinct patterns of cytokine expression, associated with specific T cell populations, may alter the intensity of the inflammatory response and severity of myocarditis. It appears that the balance of protective and destructive immune elements can be altered by the elimination of one or more T cell subsets. The role of the T cell in myocarditis is complex, and the contribution of elements in addition to a specific αβ T cell effect cannot be underestimated. Thus, elimination of the total αβ T cell population may not always be advantageous to the host. Further investigation of the relationship between the T cell and cytokines in the host response to CVB3 infection may identify other important factors of host susceptibility, and lead to better understanding of the disease and more precise targeting of therapy.

Elements of the T cell response which influence susceptibility to CVB3 myocarditis have been presented in this chapter. The strategy of genetic elimination of T lymphocyte populations has revealed multiple elements of the immune response that are able to influence the pathogenesis of the disease, including αβ T cells, distinct cytokine expression patterns correlated with specific T cell subsets, Lck and the role CD45 PTP can play in regulating the cellular response to cytokines via control of JAK activation. To pursue the mechanisms by which intracellular signal transduction can influence host susceptibility to CVB3 myocarditis, the next chapter will present
evidence for the involvement of Src-family kinase and mitogen-activated protein kinase signal transduction pathways in the pathogenesis of CVB3 disease.
CHAPTER 4

INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS AS DETERMINANTS OF HOST SUSCEPTIBILITY TO GROUP B COXSACKIEVIRUS INFECTION

1. PURPOSE

To discover the mechanism by which p56Lck protein tyrosine kinase determines host susceptibility to group B coxsackieviral (CVB3) myocarditis, and evaluate the role of downstream mitogen-activated protein kinase signal transduction pathways in the disease process.

Based on the publications:

Opavsky MA, Martino T, Rabinovitch M, Penninger J, Richardson C, Petric M, Trinidad C, Liu P. The extracellular signal-regulated kinase signalling cascade is a key determinant of susceptibility to coxsackievirus B3 infection and myocarditis in mice. To be submitted.


All experimental data presented in this chapter done by the candidate. The candidate conceived, performed or supervised 100% of the experiments in the first paper and contributed 10% of the second paper. The assistance of Fayez Dawood and Wen-Hu Wen with animal infection is much appreciated. Thanks to Cathy Trinidad and the summer students Janice Chen and Lisa Butcher for their help with the protein analysis.
2. ABSTRACT

Group B coxsackieviral (CVB) infection can cause significant morbidity and mortality in susceptible hosts, leading to meningitis, pancreatitis, myocarditis and dilated cardiomyopathy. The T cell response to CVB3 infection contributes to severe myocarditis. Gene-targeted knockout of both CD4+ and CD8+ T cells, all T cell receptor (TCR) β+ T cells or p56lck (Lck) protein tyrosine kinase protects mice from myocarditis following CVB3 infection. While Lck can influence host susceptibility to CVB3 myocarditis, its mechanism of action remains unknown. In Lck knockout mice viral replication is attenuated, and disease is prevented. Downstream of Lck, the extracellular signal-regulated kinases (ERKs) are known to be activated following TCR engagement and are important mediators of gene transcription and cell function. The ERK1/2 cascade may be a key mediator of host susceptibility to CVB3 infection and disease. The role of the ERK1/2 signalling cascade in the pathogenesis of CVB3 myocarditis is explored here using a Jurkat T cell culture model, and a mouse model of susceptibility to viral infection. At the time of initial virus-host interaction the ERK1/2 cascade is rapidly activated in an Lck-dependent manner. Inhibition of this growth-activated mitogen-activated protein kinase (MAPK) pathway significantly reduced CVB3 replication in Jurkat cells and cardiac myocytes. A relationship between CVB3 infection and ERK1/2 activation was confirmed in mice. ERK1/2 phosphorylation was correlated with severity of myocarditis and cardiac viral load following CVB3 infection. In the hearts of A/J mice, which are susceptible to severe CVB3 myocarditis, exuberant activation of the ERK1/2 cascade was observed, while in contrast, in the hearts of C57BL/6 mice, which are resistant to myocarditis, ERK1/2 activation was weaker. These data demonstrate that the ERK1/2 signalling pathway is triggered by CVB3 infection in immune and myocardial cells, perhaps playing a role in host susceptibility to viral myocarditis.
3. INTRODUCTION

Enteroviruses are common infectious agents which are responsible for a broad spectrum of diseases, including febrile illnesses, respiratory infections, meningitis, hepatitis, pancreatitis, and myocarditis (Liu 1996a; Opavsky 1998). Group B coxsackieviruses (CVBs), members of the Enterovirus genus, are the etiologic agent most often associated with acute viral myocarditis and chronic dilated cardiomyopathy (DCM) (Liu 1996a; Opavsky 1998). Investigations using murine models have demonstrated that both the T lymphocyte response to viral infection and direct CVB-mediated injury have an important role in the development of myocarditis (Woodruff 1974; Chow 1992; Liu 1996a; Opavsky 1998). A strategy using transgenic knockout mice to explore the deleterious effect of T lymphocytes in CVB3 myocarditis has been presented in Chapter 3. Gene-targeted knockout of both CD4+ and CD8+ T cells or all T cell receptor (TCR) β+ T cells results in minimal heart pathology following CVB3 infection (Opavsky 1999). Genetic elimination of CD45, a tyrosine and JAK phosphatase expressed in haematopoetic cell lines, also protects mice from CVB3 infection (Irie-Sasaki 2001). CD45 PTP is required for p56lck (Lck) activation following TCR engagement (Ostergaard 1989; Thomas 1999).

Mice infected with CVB3 which lack Lck, required for normal T cell function, did not develop disease, and manifest impaired viral replication (Liu 2000). Lck is a cytoplasmic membrane-associated member of the sarcoma (Src) family of nonreceptor protein tyrosine kinases, which is expressed primarily in T and B lymphocytes (Voronova 1984). T cell stimulation by antigen or anti-TCR antibodies leads to a rapid rise of intracellular tyrosine protein phosphorylation (Hsi 1989). This signal transduction and activation of T lymphocytes is dependent upon Lck, which associates with the cytoplasmic domains of the TCR coreceptors CD4 and CD8 (Straus 1992; Penninger 1993b). Crosslinking of the TCR activates Lck, which phosphorylates the CD3 zeta chain inducing recruitment of ZAP-70, a member of the Syk-family of tyrosine
kinases (Chan 1994; Isakov 1995). Lck phosphorylates ZAP70, and downstream signalling pathways are activated. These include mitogen-activated protein kinases (MAPKs) which are important mediators of signal transduction from the cell surface to the nucleus. In addition, the extracellular signal-regulated kinases (ERKs) are activated rapidly through threonine and tyrosine phosphorylation following TCR engagement and Lck activation via adaptor proteins and Ras (Franklin 1994; Su 1996). The distribution of these serine/threonine kinases is broad, but specific for distinct stages of development and tissue type (Boulton 1991). ERKs respond to diverse stimuli of cell proliferation and differentiation and alter cell function by the activation of gene transcription factors (Sturgill 1988; Ahn 1991; Boulton 1991; Ettehadieh 1992; Davis 1993; Seger 1995).

In this study, the hypothesis that the ERK1/2 signalling cascade is a key mediator of host susceptibility to CVB3 infection and disease is explored. In T cell lines CVB3 infection specifically and rapidly activates the ERK1/2 pathway in an Lck-dependent manner. CVB3 replication in T cell lines and myocytes depends upon activation of Src-family kinases and the ERK1/2 signalling pathway. In A/J mice, severe myocarditis and high viral load in the myocardium were associated with activation of the ERK1/2 signalling pathway early in the course of infection. The attenuation of ERK1/2 signalling in the hearts of C57BL/6 mice was correlated with less severe myocardial disease. The ERK1/2 signal cascade is an important determinant of host susceptibility to CVB3 infection and ensuing disease.
4. MATERIALS AND METHODS

i) Antibodies and Reagents

Phosphoprotein-specific monoclonal antibodies (MAb) detecting dual phosphorylated p44/42 MAPK and phospho-p38 MAPK, p44/42 MAPK and p38 MAPK Abs to detect total MAPK, horseradish peroxidase (HRP)-conjugated goat anti-rabbit (GAR) secondary Ab, and a nonradioactive p38 MAPK assay kit were purchased from New England Biolabs (Beverly, MA). The anti-CVB3 VP1 MAb, HRP-conjugated goat anti-mouse (GAM) secondary Ab and the ARK staining system were obtained from Dako (Carpinteri, CA). The anti-CAR MAb RmcB and soluble CAR (sCAR) were kind gifts provided by Dr. Jeffrey Bergelson. Anti-DAF MAb 914 (clone BRIC 216) and FITC-conjugated anti-DAF 1614F were obtained from Serotec (Oxford, England). The anti-Lck Mab was obtained from Transduction Labs (Lexington, KY). Phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus cereus was from Molecular Probes (Eugene, OR). MEK1 inhibitor PD98059, which is a highly selective inhibitor of MEK1 activation and the MAPK cascade was from New England Biolabs. All media, fetal calf serum (FCS) and additives were obtained from Gibco-BRL (Burlington, Ontario) unless otherwise noted.

ii) Cell culture

Jurkat T cells (E6-1), JCaM T cells and TCRβ negative (TCRβ−/−) T cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). Cells were grown at 37°C with 5%CO₂ in RPMI medium supplemented with 10mM HEPES, 10 mM sodium pyruvate, 0.5% penicillin and streptomycin (P/S) and 10% FCS. Jurkat/Lck cells and Jurkat/vector cells required additional supplementation with hygromycin B (Boehringer Mannheim, Laval, Quebec) and geneticin. Prior to
stimulation experiments, T cells were starved in RPMI supplemented with 0.5% FCS for 18 hours.

Neonatal mouse cardiac myocyte cultures were prepared as previously described (Simpson 1985; Parker 1990), with modifications. Briefly, hearts were removed from A/J or C57BL/6 mice (Harlan/Sprague-Dawley) within 72h of birth. Following trimming, ventricles were mechanically minced and then subjected to stepwise enzymatic digestion with 0.15% trypsin. Isolated cells were washed with media (DMEM/HAM F12 [1:1] pH 7.4 with 0.5% P/S) plus 10% FCS. Nonmyocytes were depleted by a 1 hour preplating on 10cm² petri dishes (Primaria). The myocytes, which remain in suspension were collected, washed and resuspended in media plus 10% FCS, with 0.1mM 5-bromo-2-deoxyuridine (BRDU; Sigma) to inhibit growth of nonmyocytes in the presence of serum, and plated in 24 well laminin-coated tissue culture plates (Biocoat) at a density of 1 X 10⁵ cells/well. Myocytes were routinely present at approximately 90%. After 24 hours adherent myocytes were washed and resuspended in serum-free media (DMEM/HAM F12 [1:1] pH 7.4 with 0.5% P/S) plus additives, including 5 μg/ml transferrin, 1nM lithium chloride, 1nM selenium oxide, and 25 μg/ml ascorbic acid (all from Sigma), and 1 μg/ml insulin. Myocytes were incubated a further 24 hours before use.

iii) Viruses

The cardiovirulent CVB3 strain CVB3-CG (CVB3 throughout this paper) was adapted by Woodruff and Woodruff (Woodruff 1974) and passaged in the laboratory of Dr. Charles Gauntt (Martino 1998). CVB3-VR30 (Nancy) was obtained from the ATCC. Viruses were prepared by passage once through HeLa cells. Stocks were frozen and thawed three times, clarified by centrifugation and stored at -70°C after titres were determined by plaque assay on HeLa cells. Radiolabelled CVB3 was prepared in HeLa cell monolayers as described previously (Martino 1998). To prepare purified
virus, clarified CVB3-CG and CVB3-VR30 stocks were separately overlaid on 15 to 45% (wt/wt) sucrose gradients in collection buffer (0.01M NaCl, 0.01M Tris-HCl, 0.05M MgCl₂). Gradients were then centrifuged at 24,000 rpm at 25°C for 3 h in an SW28 rotor (Beckman, Mississauga, Ontario, Canada). Prior to application to the gradients virus stocks were spiked with 1000 cpm of [³⁵S]-methionine labeled CVB3. Following centrifugation, fractions with peak radioactivity and virus were pooled and dialyzed in 10K Slide-A-Lyzer Cassettes (Pierce, Rockford, IL) against RPMI medium. Virus titres were determined by plaque assay and aliquots were stored at -70°C.

iv) Flow cytometry and immunocytochemistry

Cells (2 X 10⁶) were washed in serum-free RPMI and labeled with FITC-conjugated anti-DAF for 30 mins at 37°C. Alternatively, cells were incubated with anti-CAR RmcB MAb for 1 h at RT, washed and incubated with FITC-conjugated goat-anti-mouse secondary antibody for 30 mins at 37°C. After washing in PBS, cells were analyzed on a COULTER(R) EPICS(R) Acquisition Flow Cytometer. When indicated cells were pretreated with 1 U PIPLC at 37°C for 30 mins before labeling with anti-DAF MAb.

Twenty-four hours following infection of Jurkat cells with 1 pfu/cell CVB3, cells were washed with PBS and applied to microscope slides by cytopspin, then stained with anti-CVB3 VP1 Mab using the Dako ARK system as instructed.

v) CVB3 binding and replication

Binding assays of radiolabelled CVB3 were performed as described previously (Martino 1998). After washing in RPMI, cells (5 X 10⁶ cells/test) were incubated with about 30,000 cpm [³⁵S]-CVB3 for 1 hour at RT. Supernatant and 2 subsequent washes were collected and pooled (free virus), separately from the cell pellet (membrane-bound virus). The [³⁵S] cpm were monitored by scintillation spectroscopy.
Percent virus binding was calculated by: [cpm membrane-bound virus/(cpm membrane-bound + cpm free virus)].

CVB3 yield in T cell lines was determined by incubating 1 X 10^6 cells CVB3 in serum-free RPMI for 1 h at RT. Cells were then washed to remove unbound virus, resuspended in RPMI-10% FCS and immediately frozen at -70°C or incubated at 37°C for 24 h or 48 h. In blocking experiments, cells were incubated with anti-CAR Rmcb (hybridoma, 1:2), anti-DAF MAb 914 (40 µg/ml) or PIPLC (1 U/ml) for 1 h at 37°C, then washed with serum-free RPMI twice prior to virus inoculation.

To examine the functional role of the ERK1/2 cascade in CVB3 infection, T cells or myocyte monolayers were incubated with the specific MEK1/MAPK inhibitor PD98059 for 1 hour before infection and during adsorption with CVB3. Cells were washed and resuspended in the presence or absence of PD98059, according to treatment protocol. At the end of the incubation period cells were frozen at -70°C and thawed 3 times, cellular debris was removed by centrifugation, and virus was quantitated by plaque assay on HeLa cell monolayers.

vi) CVB3 treatment of Jurkat cells

Following incubation in RPMI-0.5% FCS for 18 h, cell aliquots (2 X 10^6 or 1 X 10^7 cells/ml) were washed in and resuspended in 200 µl serum-free RPMI, and preincubated at RT for 10 mins. CVB3 in serum-free RPMI or control RPMI were added to aliquots for the indicated times. Cells were washed in PBS containing 400 µM sodium orthovanadate, 5 mM EDTA, and 10 mM sodium fluoride. The cells were solubilized on ice for 15 mins in lysis buffer containing 0.5% Triton X-100, 50 mM Tris, pH 7.6, 300 mM NaCl, 1 mM sodium orthovanadate, 5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF. The lysates were spun at 12,000 X g for 10 mins at 4°C. The total protein concentrations of the supernatants were determined with the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, Ontario).
vii) Western blot analysis and kinase assay

Whole cell lysates (50 μg of protein) were mixed with 2X Laemmli sample buffer, boiled and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-16% gradient gel; Novex, San Diego, CA), transferred to PVDF membranes, blocked for 1 h at RT with 5% powdered skim milk in TBS with 0.05% Tween (TBST) and reacted with anti-phospho-ERK1/2 (1:2000) at 4°C overnight, and then incubated for 1 h at RT with HRP-conjugated GAM Ab (1:1000). Blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Baie d'Urfe, Quebec). After membranes were incubated in stripping buffer (10mM β-mercaptoethanol, 2% [w/v] SDS, 62.5 mM Tris, pH 6.7) for 30 mins at 50°C they were washed in TBST, blocked, and incubated with anti-ERK1/2 (1:1000) at 4°C overnight. Following incubation of membranes for 30 mins at RT with HRP-conjugated GAR antibody (1:4000), the presence of specific proteins was detected with ECL. Band intensities were measured using densitometry (NIH Image Version 1.60) and ratios of phosphorylated to total ERKs were compared among different virus treatments. Alternatively, membranes were probed with anti-phospho-p38 MAPK (1:2000) and then anti-total p38 MAPK (1:2000) using HRP-conjugated GAR secondary Ab as described above.

To detect activation of the p38 MAPK pathway, a nonradioactive p38 MAPK assay kit was used according to manufacturer's protocol. Briefly, 100 μg protein from the cell lysate was incubated with immobilized phospho-p38 MAPK MAb overnight at 4°C. After washing twice in lysis buffer and twice in kinase buffer the pellet was resuspended in kinase buffer plus ATP (200μM) and ATF-2 fusion protein (2 μg) as substrate, and incubated at 30°C for 30 mins. After termination of reactions, samples were subjected to SDS-PAGE (8-16%), transferred to PVDF, blocked with 5% skim milk in TBST, then incubated with phospho-ATF-2 antibody (1:1000) overnight at 4°C. After incubation with HRP-conjugated goat anti-rabbit secondary antibody (1:4000) at
RT for 30 mins, ECL was used to detect and quantitate p38 MAPK. Reactivity of specific samples was compared using densitometry.

viii) Lck RT-PCR and protein detection

Hearts extracted from A/J and C57BL/6 mice were processed as described previously for isolation of RNA and synthesis cDNA (Opavsky 1999). The PCR primers MLck II forward (5'-AGCATCATGTGAATAGGCCAGAAGGCTCCC-3') and MLck EX2 reverse (5'-ATAGGTGACCAGTGGGTCCCGCACTTCAGA-3') were used to amplify Lck using the Expand High fidelity PCR system (Boehringer Mannheim). The conditions were as follows: 96°C for 3 min; 30 cycles of 55°C for 2 min, 72°C for 2 min, 94°C for 1 min; and 1 cycle of 50°C for 2 min and 72°C for 7 min. Lck protein was extracted and analyzed by Western blot as described above, using anti-Lck monoclonal antibody. Equal protein loading was confirmed by staining with coomassie brilliant blue.

ix) CVB3 infection in mice

A/J and C57BL/6 mice (Harlan-Sprague Dawley), with differential susceptibility to CVB3 myocarditis (Chow 1991), were inoculated intraperitoneally with $10^5$ plaque forming units (pfu) of CVB3 at age 5 to 6 weeks. Animals were sacrificed on day 0 (uninfected controls), 2, 4 and 7 days after infection. Transverse sections of hearts were examined histopathologically for evidence of inflammation and necrosis. For detection of infectious CVB3, homogenates of cardiac or splenic tissue were analyzed for virus as described previously (Opavsky 1999). Organs were snap frozen in liquid nitrogen for subsequent homogenization and suspension in lysis buffer, then processed as described above.
5. RESULTS

i) CVB3 infection in Jurkat cells

Two receptors for CVBs have been identified. The coxsackievirus and adenovirus receptor (CAR), a transmembrane glycoprotein with 2 extracellular immunoglobulin-like domains is essential for virus internalization and productive infection (Bergelson 1997a). Decay accelerating factor (DAF; CD55), a complement inhibiting cell surface molecule collaborates with CAR to enhance infection by serving as an attachment protein (Bergelson 1995; Shafren 1995; Martino 1998). Both receptors are widely distributed among a number of tissues and cell types.

T lymphocytes and T cell lines are known to be permissive to CVB infection (Vuorinen 1994; Vuorinen 1996). Productive infection in about one third of Jurkat cells was demonstrated by immunocytochemistry 24 hours following virus inoculation (Figure 15). Both CAR and DAF are expressed in Jurkat cells and JCaM T cells (no functional Lck) as determined by flow cytometry (Liu 2000)(data not shown). Depletion of DAF from T cells by PIPLC pretreatment was confirmed by flow cytometry. Binding of radiolabelled $[^{35}S]$-CVB3 was doubled in JCaM cells, as compared to Jurkat cells (data not shown). However, CVB3 replication in Jurkat cells was significantly higher in comparison to Lck negative JCaM cells (Figure 16a). CVB3 yields were impaired in cells lacking Lck, despite increased viral binding in the JCaM cells, and inoculation with a higher moi than reported previously (Liu 2000). This implies that events subsequent to virus-receptor binding play a significant role in susceptibility to CVB3 infection.

Viral receptors, are the initial point of interaction between virus and cell, but may also influence cell signalling pathways. Exposure to the monoclonal antibody against CAR significantly inhibited CVB3 replication in Jurkat cells (Figure 16b). In contrast, anti-DAF MAb 914, specific for SCR3, considered to be a site of CVB3 binding
Figure 15: Jurkat cells are permissive to CVB3 infection. Twenty-four hours following infection with CVB3 at an moi of 1 pfu/cell, cells were cytopspun onto microscope slides and fixed in acetone. Infected cells were identified using anti-CVB3 VP1 Mab (brown colour) as shown in panel A. Infected cells, incubated with IgA control did not stain brown (panel B).
Figure 16: CVB3 infection of Jurkat and JCaM cells. (a) Jurkat and JCaM cells (1 X 10^6) were infected with CVB3 at an moi of 1 pfu/cell and progeny virus was quantitated at 24 and 48 h post-infection by plaque assay. Concentrations of progeny virus are expressed as mean log pfu/1 X 10^6 cells (± SEM, n=3 per group) and represent three separate experiments. Viral titres were 50% higher at 24 h post-infection in Jurkat cells as compared to JCaM cells. *p< 0.001 at 24 h, and **p< 0.005 at 48 h (Student T test). (b) Following incubation of Jurkat cells (1 X 10^6) with anti-CAR RmcB (hybridoma supernatant 1:2), anti-DAF (40 μg/ml), phosphatidylinositol-specific phospholipase C (PI-PLC; 1U/1 X 10^6 cells) or serum-free media (nil) for 1 h at 37°C, cells were infected with CVB3 at an moi of 1 pfu/cell. At 24 h post-inoculation, treatment with anti-CAR resulted in reduced titres, while anti-DAF MAb and PIPLC had no effect. These titres are represented as mean log pfu/1 X 10^6 cells (± SEM) for triplicate cultures. * P< 0.001 for anti-CAR vs nil for each cell type. **p< 0.01 for anti-DAF vs control (Student T test). The basal growth rate of Jurkat and JCaM cells were found to be similar for a period of 4 days.
(Bergelson 1995; Shafren 1995; Martino 1998), and PIPLC removal of GPI-anchored DAF did not reduce viral yields in infected Jurkat cells. The viral-CAR interaction was also important to progeny production in JCaM cells (data not shown). The absence of DAF has little effect on the efficiency of viral production in these T cells. Thus, taken together, these findings support the concept that CAR is the functional receptor in CVB3 infection of Jurkat and JCaM cells.

ii) CVB3 infection activates the ERK1/2 pathway.

To determine the effect of CVB3 infection on the ERK1/2 signalling cascade whole cell lysates were collected from CVB3-treated and control Jurkat cells. Cardiovirulent CVB3-CG (CVB3) induced rapid ERK1/2 phosphorylation at 5 mins post-treatment, which declined over 30 to 60 min (Figure 17a). Similar results were observed with CVB3 purified by sucrose-gradient ultracentrifugation. In dose-response experiments an moi of 25 pfu/cell was the threshold dose with an moi of 50 pfu/cell consistently resulting in at least a 2- to 5-fold increase in ERK1/2 phosphorylation. A minimal number or percentage of viral receptors may need to be bound by virus or cross-linked by the multivalent CVB3 in order to trigger subsequent signalling events. Activation of the ERK 1/2 cascade was also observed with CVB3-VR30 (Nancy) (Figure 17b).

iii) p56Lck is essential for ERK1/2 activation in Jurkat cells

To explore the role of Lck in CVB3 activation of the ERK signalling pathway the intensity of ERK1/2 phosphorylation between infected Jurkat cells and JCaM cells was compared. The dramatic activation of ERK1/2 after exposure to CVB3 observed in Jurkat cells is essentially absent in the JCaM cells which have no functional Lck (Figure 18a). To determine if there may have been a delayed effect in JCaM cells, incubation was continued for as long as 60 mins before processing. This difference in
Figure 17: CVB3 infection activates the ERK1/2 pathway in Jurkat cells. At specific times after exposure to CVB3, whole cell lysates were resolved by SDS-PAGE (50 µg protein/lane), transferred to PVDF membrane and probed with antibodies specific for phosphorylated (P-ERK1, P-ERK2) and total (ERK1, ERK2) ERKs. (a) Jurkat T cells (1 X 10⁷) were lysed preexposure (pre) or following incubation with CVB3 at an moi of 50 pfu/cell (+) or media alone (-) at RT for the indicated times. One result representative of five experiments is shown. (b) Five or 10 mins after exposure at RT to 50 pfu/cell of CVB3-CG (CG) or CVB3-VR30 (Nancy; N), or media alone (-), cells (1 X 10⁷) were washed, lysed and subjected to immunoblot with ERK1/2-specific antibodies as above. Both CVB3 strains induced ERK1/2 phosphorylation as indicated by P-ERK1/2:total ERK1/2 ratio. One result is representative of three experiments is shown.
Figure 18: Early activation of the ERK1/2 pathway by CVB3 infection is dependent on p56lck (Lck). (a) Jurkat cells or JCaM cells (1 X 10^7) were incubated with CVB3 at an moi of 50 pfu/cell (+) or media alone (-) at RT for 5 or 10 mins followed by lysis. Whole cell lysates were resolved by SDS-PAGE (50 μg/lane), transferred to PVDF membrane and probed with antibodies specific for phosphorylated (P-ERK1, P-ERK2) and total (ERK1, ERK2) ERKs. One result representative of three experiments is shown. (b) The ERK1/2 pathway is activated by coxsackievirus B3 (CVB3) in JCaM cells with restored Lck (JCaM/Lck). Jurkat, JCaM, JCaM/vector and JCaM/lck cells (1 X 10^7) were incubated with CVB3 at an moi of 50 pfu/cell (+) or media alone (-) at RT for 5 mins followed by lysis and resolution by SDS-PAGE (50 μg/lane), transferred to PVDF membrane and probed with antibodies specific for phosphorylated (P-ERK1, P-ERK2) and total (ERK1, ERK2) ERKs. One result representative of two experiments is shown. (c) TCR interaction with Lck is not necessary for CVB3 triggered ERK1/2 cascade activation. TCRβ negative Jurkat cells (1 X 10^7/test) were lysed following incubation for 5 or 10 mins with CVB3 at an moi of 50 pfu/cell (+) or media alone (-) followed by lysis and resolution by SDS-PAGE (50 μg/lane), transferred to PVDF membrane and probed with antibodies specific for phosphorylated (P-ERK1, P-ERK2) and total (ERK1, ERK2) ERKs. One result representative of two experiments is shown.
phosphorylation of ERK1/2 was not due to lack of MAPK expression as equivalent levels of total ERK1/2 are present in both cell lines. Treatment with the phorbol ester PMA rapidly activates the ERK1/2 cascade in JCaM cells, indicating that the cell line does have an intact response (data not shown).

To confirm that Lck has an important role in signal transduction, JCaM cells expressing Lck constitutively after transfection (JCaM/Lck) or controls transfected with vector alone (JCaM/vector) were exposed to CVB3. Phosphorylation of ERK1/2 stimulated by CVB3 was observed in both Jurkat and JCaM/Lck cells, in contrast to JCaM and JCaM/vector cells, which exhibited no phosphorylation of ERK1/2 (Figure 18b). Constitutive phosphorylation of ERK1/2 was slightly higher in transformed JCaM/Lck cells than other cell lines in this study. A functional TCR, upstream of Lck, is not required for CVB3 triggered activation of the MAPK cascade as stimulation of ERK1/2 phosphorylation in TCRβ−/− Jurkat cells occurs on infection with CVB3 (Figure 18c). These findings indicate that ERK1/2 activation triggered early in infection with CVB3 is dependent on the presence of Lck.

Activation of the stress-activated p38 MAPK and SAPK/JNK pathways were examined as well by p38 kinase assay (Figure 19) and phospho-specific antibodies (data not shown). Exposure to CVB3 resulted in no consistent change in p38 MAPK or SAPK/JNK activity in Jurkat or JCaM cells. From this it can be concluded that the effect of exposure to CVB3 on early signalling events in these T cell lines appears to be specific for the growth-activated MAPK pathway.

iv) CVB3 replication in T cells is dependent on ERK1/2 activation

The dramatic activation of ERKs observed with exposure of Jurkat cells to CVB3 led to the examination of the role of this signalling pathway in CVB3 infection of T cells. The specific MEK1/ERK inhibitor PD98059, which blocks the ERK cascade by preventing activation of MEK1 upstream of ERK1/2, significantly reduced virus yield in
Figure 19: The p38 MAPK pathway is not activated by CVB3 infection in T cell lines. Jurkat or JCaM cells (1 X 10⁷) were incubated with CVB3 at an moi of 50 pfu/cell (+) or media alone (-) at RT for the indicated times before lysis. Phosphorylated p38 MAPK was immunoprecipitated and kinase activity was determined using ATF-2 as the substrate for phosphorylation. The intensity of phosphorylated ATF-2 (P-ATF) was measured by densitometry for each of three individual experiments (one of which is shown here) and the mean ± SEM for each group is expressed as the fold change from pretreatment (0). Differences between untreated and CVB3-treated groups were not significant.
Jurkat cells (Figure 20a). Inhibition of ERK1/2 activation also decreased CVB3 yield in JCaM cells (Figure 20b), despite the low baseline ERK1/2 activation, as shown above (Figure 18a). Different pre-infection cell culture conditions could influence the degree of baseline ERK1/2 phosphorylation. Alternatively, additional upstream activators of the ERK1/2 cascade, other than Lck, may be important to optimal viral replication after the initial virus-receptor interaction. It was confirmed that the increase in ERK1/2 phosphorylation following initial virus exposure of Jurkat cells is inhibited by PD98059 (data not shown). From the initial virus-receptor interaction, the ERK pathway may be involved in many aspects of host cell function important to productive CVB3 infection. To investigate the importance of ERK1/2 cascade activation in viral attachment, as the first step in infection of cells, we measured binding of radiolabelled CVB3 to T cells in the presence and absence of PD98059. MEK1 inhibition did not reduce binding of radiolabelled CVB3 to either cell line (Figure 20c).

v) CVB3 replication is dependent on Src family kinase and ERK1/2 activation in cardiac myocytes

To explore a role for the ERK1/2 signalling cascade in CVB3 infection of the heart parallel to that observed in the T cell model, neonatal mouse cardiac myocyte cultures were infected at an moi of 5 pfu/cell in the presence of PP1, a specific Src-family kinase (Lck, Lyn, Src, and Hck) inhibitor. CVB3 replication was suppressed in the presence of 50µM PP1 (Figure 21a). The roles of individual Src-family kinases in CVB3 infection of the cardiac myocyte are yet to be elucidated. However, findings thus far suggest that Src-family tyrosine kinases in myocytes may have a comparable role to Lck in the T cell. Furthermore, ERK1/2 activation is important to CVB3 replication in cardiac myocytes, as in the T cell model. Neonatal mouse cardiac myocytes were infected at an moi of 5 pfu/cell. Viral replication was inhibited by up to 77% with 25 µM of PD98059 and up to 96% when myocytes were infected in the presence of 50 µM
Figure 20: CVB3 replication in T cells is reduced by blocking the ERK1/2 signal transduction pathway. Following incubation of (a) Jurkat and (b) JCaM cells (1 X 10^6) in the presence of the specific MEK1/MAPK inhibitor PD98059 (50μM in DMSO) or control DMSO alone for 1 h at 37°C, cells were infected with CVB3 at an moi of 1 pfu/cell. Following virus adsorption for 1 hour, cells were washed, resuspended in RPMI-10% FCS (plus DMSO or PD98059) and immediately frozen (1 h) or incubated at 37°C for 24 h post infection (pi), then frozen. Viral titers were determined by plaque formation assay. Virus titres are expressed as mean log pfu/1 X 10^6 cells (± SEM, n=3 per group) and represent three separate experiments. *p<0.005 at 24 h for untreated vs PD98059 for each cell type (Student T test). (c) Inhibition of the ERK1/2 signalling cascade by PD98059 did not impair viral binding. Jurkat and JCaM cells (5 X 10^6) were incubated with [35S]-CVB3 for 1 h in the presence (PD98059) or absence (DMSO). Binding of virus is expressed as mean percentages (± SEM) for triplicate cultures. Viral binding to JCaM cells was twice as high as to Jurkat cells (*p=0.0001; Student's T test).
Figure 21: CVB3 replication in neonatal mouse cardiac myocyte cultures is dependent on protein tyrosine kinase activation. (a) Cardiac myocyte cultures from neonatal mice were incubated with DMSO (0), or 10 or 50 μM of the Src-kinase inhibitor PP1 for 1 h at 37°C, then infected with CVB3 at an moi of 5 pfu /cell. Following virus adsorption for 1 hour, myocytes were washed, resuspended in media (plus DMSO or PP1) and incubation was continued for 48 h. Cell lysates were analyzed for infectious virus by plaque assays. Virus titres are expressed as mean pfu/1 X 10^5 cells (± SEM, n=3 per group). *P< 0.05 for DMSO vs 50 μM PP1 (Student T test). (b) Cardiac myocyte cultures from neonatal mice were incubated with DMSO (0), or 10 or 50 μM PD98059 (PD) for 1 h at 37°C, then infected with CVB3 at an moi of 5 pfu /cell. Following virus adsorption for 1 hour, myocytes were washed, resuspended in media (plus DMSO or PD) and incubation was continued for 48 h. Cell lysates were analyzed for infectious virus by plaque assays. Virus titres are expressed as mean pfu/1 X 10^5 cells (± SEM, n=3 per group). *P< 0.05 for DMSO vs 25 μM PD and **P< 0.01 for DMSO vs 50 μM PD. (Student's T test). These findings are representative of three experiments, using both A/J or C57BL/6 neonatal mouse cardiac myocytes.
PD98059 (Figure 21b). Observations were consistent for cardiac myocytes from both A/J and C57BL/6 mice. These findings suggest that in neonatal cardiac myocytes Src kinases and ERK1/2 activation are important to productive CVB3 infection.

vi) Activation of the ERK1/2 cascade is correlated with susceptibility to CVB3 myocarditis

Following the observations that CVB3 stimulates the ERK1/2 cascade in T cell lines, and that ERK1/2 activation is required for optimal viral infection of T cells and cardiac myocytes, the contribution of this signalling pathway in the development of myocarditis was explored in mice. A/J and C57BL/6 mice were infected with CVB3 to investigate whether ERK1/2 phosphorylation is involved in the pathogenesis of CVB3 myocarditis. CVB3 infection with 1 X 10^5 pfu cardiovirulent CVB3 led to acute and severe myocarditis in A/J mice (Figure 22a), while minimal myocardial inflammation and necrosis was observed in C57BL/6 mice (Figure 22b). High CVB3 titres were isolated from hearts and spleens by day 2 after CVB3 inoculation. Cardiac viral titres were comparable between the two strains at day 2 following inoculation. However, by day 4 viral titres were significantly higher in the hearts of A/J mice compared to C57BL/6 mice (Figure 22c). In contrast, viral titres in the spleen were similar between the two strains of mice (data not shown).

ERK1/2 phosphorylation in the myocardium of CVB3 infected A/J mice increased in parallel with high viral titres and severity of myocarditis. In susceptible A/J mice cardiac ERK1/2 phosphorylation was greater than in hearts of uninfected mice by day 2 and increased to 3-fold above control levels by day 4 pi (Figure 23a&b). ERK1/2 phosphorylation in the hearts of CVB3-infected C57BL/6 mice was less intense 2 days after infection, and similar to uninfected mice by day 4. The differential effect between mouse strains was not observed in the spleen. In infected spleens of both strains, a 1.5 fold increase in ERK1/2 phosphorylation day 2 pi was observed, and by day 4 levels of
Figure 22: A/J mice are more susceptible to CVB3 myocarditis. Mice (n=25 per group) were inoculated with $1 \times 10^5$ pfu CVB3 and observed for 7 days. At 4 and 7 days mice were autopsied and tissues examined for evidence of myocarditis. (a) Inflammatory cell infiltration and myocyte necrosis are apparent in the A/J heart. (b) Minimal disease is evident in hearts of C57BL/6 mice at the same time point. At day 4 pi, myocyte cytolysis is evident in A/J mice, but not in C57BL/6 mice, prior to any identifiable mononuclear infiltrate (not shown). (c) Cardiac viral titers were determined by plaque forming assay of clarified tissue homogenates (n=3 per group). On day 4 pi CVB3 titers were significantly greater in A/J mouse hearts, consistent with the more aggressive heart disease evident on histopathology. *$P<0.05$ for day 4 viral titers in A/J vs C57BL/6 hearts (Student's T test). Hematoxylin & eosin, original magnification X250.
Figure 23: ERK1/2 phosphorylation was increased in the hearts of CVB3 infected mice. (a) Levels of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2) in the heart are shown for 2 A/J and C57BL/6 mice for uninfected controls (0) and day 2 and 4 post-CVB3 infection (pi). Relative to total ERK1/2 expression, ERK1/2 phosphorylation (P-ERK1/2) was significantly and persistently increased in the A/J myocardium. ERK1/2 activation was not as high and normalized by day 4 in the hearts of C57BL/6 mice. (b) Two days following CVB3 infection myocardial ERK1/2 activation increased by 2.5 fold in A/J mice, and 1.5 fold in C57BL/6 mice. By 4 days myocardial ERK1/2 activation increased by 3-fold in A/J mice, but had normalized to uninfected levels in C57BL/6 mice. Changes in the ratio of phosphorylated:total ERK1/2 were quantified using densitometry and values are expressed as mean fold change (± SEM). *P< 0.005 for A/J uninfected vs day 2 pi, and **P< 0.001 for A/J uninfected vs day 4 pi, and ***P< 0.05 for C57BL/6 uninfected vs day 4 pi (ANOVA; n=3 per group, except n=2 for A/J day 4, and n=4 for C57BL6 day 2).
phosphorylated ERK were similar to control. Thus, ERK1/2 activation in the hearts of infected mice correlated with severity of CVB3 myocarditis.

The pattern of activation of the stress-activated p38 MAPK pathway in the heart following CVB3 infection was found to be different than that observed for the ERK1/2 cascade. Phosphorylated p38 MAPK in hearts of infected C57BL/6 mice was twice as high on day 4 compared to uninfected controls in the hearts of myocarditis-resistant mice. (Figure 24a&b). This observation correlated with lower cardiac CVB3 titre. In contrast, in susceptible A/J mice p38 MAPK activation in the heart tended to decline by day 4, although CVB3 titres were higher than in myocarditis-resistant C57BL/6 mice. No differences in activation of the JNK pathway were found (data not shown). The reciprocal pattern observed for the ERK1/2 and p38 MAPK signalling pathways merits further study.

vii) Myocardial Lck expression

Several Src-family kinases are expressed in the heart, including Lck. In the adult rabbit heart and isolated myocytes Lck is found to be activated with ischemic preconditioning, an established cardioprotective technique (Ping 1999). This observation challenges the belief that Lck expression is restricted to T cells, B cells and NK cells (Bolen 1992). Lck is also expressed in colon carcinoma cells under the influence of an alternate pathway of transcriptional activation (McCracken 1997). Using RT-PCR Lck RNA was detected in the hearts of A/J and C57BL/6 mice (Figure 25a). As well, Lck protein was found in the myocardium using a monoclonal antibody generated to the first 191 amino acids at the N-terminal end of the kinase (Figure 25b). Perhaps variable myocardial Lck expression influences host susceptibility to CVB3 myocarditis, as demonstrated by the Lck knockout mouse model (Liu 2000).
Figure 24: p38 MAPK phosphorylation was increased in the hearts of myocarditis-resistant C57BL/6 mice. (a) Levels of phosphorylated p38 MAPK (P-p38 MAPK) and total p38 MAPK (p38 MAPK) in the heart are shown for 2 A/J and C57BL/6 mice for uninfected controls (0) and day 2 and 4 post-CVB3 infection (pi). Relative to total p38 MAPK expression, p38 MAPK phosphorylation (P-ERK1/2) was significantly increased in the C57BL/6 myocardium by 4 days following CVB3 infection. p38 MAPK activation tended to be suppressed by day 4 in the hearts of A/J mice. (b) Four days following CVB3 infection myocardial p38 MAPK activation increased by 2 fold in C57BL/6 mice. Changes in the ratio of phosphorylated:total p38 MAPK were quantified using densitometry and values are expressed as mean fold change (± SEM). *p< 0.05 for C57BL/6 uninfected vs day 4 pi (ANOVA; n=3 per group, except n=2 for A/J day 4, and n=4 for C57BL/6 day 2).
Figure 25: \textit{p56}^{Lck} (Lck) expression in the hearts and spleens of A/J and C57BL/6 mice. (a) Lck RT-PCR of the myocardium identified a 198 bp PCR product representing Lck in the hearts of A/J and C57BL/6 (C57) mice (results not quantitative). A/J spleen was the positive control, and the water control (not shown) was negative. Findings are representative of duplicate experiments (b) Lck was identifiable in mouse myocardial tissue by immunoblot using a specific anti-mouse Lck monoclonal antibody. Uniform gel loading was documented by coomasie staining. Findings are representative of 3 (A/J) or 4 (C57BL/6) hearts analyzed.
6. DISCUSSION

Identifying the host factors involved in susceptibility to viral myocarditis will allow for the identification of potential pathogenic pathways in viral heart disease and lead to new areas for therapeutic intervention. Evidence supporting a role for the activation of the ERK1/2 signalling cascade associated with susceptibility to CVB3 myocarditis has been shown in this study. Viral replication in T cell lines, myocytes and in the myocardium is correlated with activation of Src-family kinases and the ERK1/2 signal transduction pathway. Viral activation of the ERK1/2 cascade, which links the cell surface to cellular events involved in growth and differentiation, could impact on host cell functions essential to viral replication and dissemination. In addition, the importance of Lck in the pathogenicity of CVB3 may depend on downstream ERK1/2 activation. The ERK1/2 cascade could direct host cell functions necessary for optimal viral replication, or alternatively, be responsible for triggering the fulminant T cell response often seen with CVB3 myocarditis. Early activation of ERK1/2, while it is not essential for virus binding, may be important to facilitate cytoskeletal reorganization involved in virus internalization (Cole 1995). CVB3 replication, although significantly attenuated, does proceed in the absence of Lck and with ERK1/2 inhibition, indicating that other host or viral factors contribute to viral infection.

Several viruses have been shown to stimulate intracellular protein kinase phosphorylation. The hepatitis regulatory protein HBx can influence the functions of several protein kinases, including protein kinase C, JAK/STAT, PI3K, and SAPK/JNK (Diao 2001a; Diao 2001b). Src activation by HBx stimulates replication of Hepatitis B virus, an enveloped dsDNA virus, in cell culture (Klein 1999). The role of Lck in human immunodeficiency virus (HIV) mediated signal transduction is consistent with the role of CD4 as the major HIV receptor (Manna 2000). Activation of Lck is required for downstream stimulation of ERK1/2, JNK, NF-κB and AP-1 by HIV tat protein (Manna 2000). Murine hepatitis virus 3, an enveloped positive strand RNA virus of the
Coronavirus family, induces activation of ERK1/2 and p38 MAPK in macrophages, promoting procoagulant activity found with fulminant viral hepatitis (McGilvray 1998). The important role of signal transduction in the natural history of these diverse viruses suggests that post-receptor events are important for the establishment of viral disease.

Little is known about the effects of picornaviruses on cell signalling pathways. Reports on signal transduction have focused on virus-induced host protein synthesis shutoff with viruses such as coxsackievirus, echovirus and encephalomyocarditis virus (Huber 1997a; Huttunen 1998; Huber 1999; Iordanov 2000). CVB3 replication is known to be inhibited by herbimycin A, a protein tyrosine kinase inhibitor (Huber 1997a). As well, tyrosine phosphorylation of 48kDa, 70kDa and 200kDa HeLa cell proteins has been observed from 3 to 5 hours post infection with CVB3 (Huber 1997a). In response to CVB3 infection Sam 68, a cellular target of Src kinases which is known to interact with poliovirus, associates with RasGAP. Subsequently, RasGAP is cleaved at 6 hours post infection, possibly activating the Ras/MAPK pathway (Huber 1999). It was hypothesized that the persistent MAPK activation also observed at this late stage of infection, may be consistent with a role for the pathway in CVB3-induced cytotoxicity or apoptosis. However, in this study it was observed that inhibition of ERK1/2 attenuates CVB3 replication, thus, the late-stage MAPK activation may in fact promote viral replication, possibly by phosphorylating viral polymerases or inhibiting host anti-viral defense mechanisms.

The CVB receptor, DAF, a glycosyl-phosphatidylinositol (GPI) -anchored protein which associates with Lck (Nicholson-Weller 1994), is not essential to viral replication in Jurkat T cells, as shown here. However, monoclonal antibodies to short consensus repeat 3 (SCR3) of DAF were able to stimulate activation of EL-4 mouse lymphoma cell lines transfected with GPI-anchored DAF (Shenoy-scaria 1992). A CVB-binding site in the vicinity of this same domain has been identified (Bergelson 1995; Shafren 1995; Martino 1998). Thus, crosslinking of DAF by CVB3 may trigger activation of the
ERK1/2 signalling cascade following CVB3 attachment. Wang and Bergelson (Wang 1999) have shown that the extracellular portion of CAR is necessary for CVB3 binding and infection. The cytoplasmic domain of CAR does contain tyrosine residues, suggesting it may trigger intracellular signalling pathways. Signalling events following the binding of CVB3 to CAR may in fact mimic a pathway activated by the as yet unidentified natural ligand(s) of the receptor. Alternatively, signal transduction may result from CVB3 interaction with another cell surface binding site capable of initiating intracellular signal transduction.

Consistent with the initial observations made in cell culture in both T cells and cardiac myocytes, activation of the ERK1/2 cascade was found to be correlated with the severity of CVB3 infection and myocarditis in mice. Viral infection can activate the ERK1/2 cascade, which can subsequently influence host cell function and viral replication in the heart. The exuberant T cell response following CVB3 infection, which is prevented in T cell knockout mouse models, may result from CVB3 activated T cell signal transduction pathways. The discovery of ERK1/2 activation in the hearts of virus infected mice highlights a juncture downstream of viral receptors that could be targeted in the therapy of severe CVB disease. The investigation of the role of CVB receptors in the pathogenesis of viral myocarditis is important to the understanding of susceptibility to CVB3 infection, and one of the mechanisms by which virus can activate intracellular signalling pathways.
CHAPTER 5

THE COXSACKIE-ADENOVIRUS RECEPTOR IS ASSOCIATED WITH DIFFERENTIAL SUSCEPTIBILITY TO CVB3 INFECTION AND MYOCARDITIS

1. PURPOSE

To investigate the expression of mouse coxsackie-adenovirus receptor (mCAR) in myocarditis susceptible and resistant mice, and identify novel mCAR mutations as determinants of the course of CVB3 infection.

Based on the publication:


All experiments conceived, performed or supervised by the candidate. The technical assistance of Cathy Trinidad is much appreciated, as is the assistance of Fayez Dawood and Wen-Hu Wen with the animal experiments. Thanks to Barbara Kellam for help with sequence analysis.
2. ABSTRACT

Group B coxsackieviruses (CVBs) are responsible for a wide spectrum of disease phenotypes, ranging from mild febrile illness to fulminant multi-system disease affecting the heart, the brain, the pancreas and the liver. The T cell response and intracellular signal transduction pathways have been identified as host determinants of CVB3 infection. However, the expression of specific viral receptors, such as the coxsackie-adenovirus receptor (CAR), may be the primary determinant of host susceptibility and organ tropism. To explore CAR as a determinant of myocarditis susceptibility, the pathogenesis of CVB3 infection was investigated in A/J and C57BL/6 mice. The organ distribution of murine CAR (mCAR) RNA and protein was compared between strains. In addition, mCAR1 and mCAR2 were cloned and sequenced systematically to screen for mutations derived from cardiomyocytes and various murine tissues. Overall survival was similar between mouse strains, but on histopathological examination, myocarditis was dramatically more severe in A/J mice. In contrast, pancreatitis was worse in C57BL/6 mice. CAR protein expression was highest in the hearts of the myocarditis-susceptible A/J mice. This is in association with increased CVB3 replication in A/J hearts. Interestingly, levels of CAR protein and CVB3 titres were similar in pancreatic tissues of both strains. Sequencing of DNA clones from A/J myocytes and Vero cells led to the identification of a truncated CAR isoform, highly homologous between murine and monkey cells. The transmembrane and cytoplasmic regions of full length mCAR1 are replaced by a 24 amino acid segment at the C-terminal end of the protein. In addition, a recurring amino acid substitution in the first immunoglobulin loop (D1) of the extracellular portion of mCAR2 was identified. The ability of truncated CARs to mediate virus attachment was demonstrated by CVB3 binding assays. But, interestingly, productive infection was not observed. In conclusion, increased mCAR expression, and differential expression of receptor isoforms may play a role in susceptibility to myocarditis. The diversity of CAR
isoforms in CVB3 infection may be significant. Mutations of the extracellular domain of CAR, or absent cytoplasmic domains may impact on the pathogenesis of CVB3 infection, in influencing both signal transduction and viral replication.
3. INTRODUCTION

Viral myocarditis is an important cause of acute heart failure and dilated cardiomyopathy (DCM). Group B coxsackieviruses (CVBs) have been identified as one of the major etiologic agents of this disease. It has been estimated that 70% of the population is exposed to cardiotropic viruses at some point in their life. These individuals do not however, all develop viral heart disease (Smith 1970; Grist 1974; Woodruff 1980; McManus 1986). In patients with documented CVB infection the heart was shown to be involved in about 5% of cases (Woodruff 1980). Why the heart becomes infected in specific cases is unknown. CVB3 infection in mice has provided a working model to investigate the pathogenesis of these viruses. Among strains of mice, significant differences in the severity of CVB3 myocarditis have been identified (Gauntt 1984; Wolfgram 1986; Buie 1987; Lodge 1987; Chow 1991; Klingel 1993), suggesting that specific host factors determine susceptibility to CVB myocarditis. T cell knockout models have illustrated that the T lymphocyte influences the response of the host to coxsackievirus infection by regulating cytokine responses to infection (Opavsky 1999; Irie-Sasaki 2001). Multiple components of signal transduction pathways, including Lck, CD45, ERK1/2, p38 MAPK and JAK1, have now been identified as key factors in the pathogenesis of CVB3 infection (Opavsky 1999; Irie-Sasaki 2001)(Chapter 4).

All viruses initiate infection by binding to a specific receptor on the cell surface, thus, the virus-receptor interaction may represent a first determinant of host susceptibility and tissue tropism (Ren 1992). The response of T cells and intracellular signal transduction pathways to CVB3 infection could occur subsequent to virus interaction with its receptor(s). The diversity of disease caused by CVBs and variations in host susceptibility may reflect the presence of specific receptors in various tissues. Coxsackie-adenovirus receptor (CAR) has been shown to be the functional cellular
receptor for CVBs (Bergelson 1997a; Tomko 1997; Bergelson 1998) and mediates binding to the fiber binding protein of adenoviruses serotypes from subgroups A, C, D, E, and F (Freimuth 2000; Lortat-Jacob 2000; Tomko 2000). Murine CAR (mCAR) has a >90% identity with human CAR (hCAR), with the protein being well-conserved across species (Bergelson 1997a; Tomko 1997; Bergelson 1998; Fechner 1999). The CAR gene codes for a transmembrane protein composed of an extracellular region containing two immunoglobulin-like domains, a transmembrane helical domain, and an intracellular domain. The extracellular domain has two potential N-linked glycosylation sites, and the intracellular domain has one potential tyrosine phosphorylation site (Bergelson 1997a; Tomko 1997; Bergelson 1998). Two isoforms have been identified in the human, mouse and rat, differing at the C-terminal end of the protein (Bergelson 1997a; Bergelson 1998; Fechner 1999). While the extracellular portion of the receptor is required for productive CVB infection, the specific interaction site(s) between CAR and CVBs has not yet been determined.

The investigation of the pathogenesis of CVB3 myocarditis in mouse models is important to the identification susceptibility factors for the development of severe CVB disease, including myocarditis. In the following study, a functional model of murine susceptibility using two mouse strains which have a high differential susceptibility to myocarditis (Chow 1991) was characterized. Subsequently, the hypothesis that the tissue distribution of CAR, and receptor isoform expression are determinants of host susceptibility to CVB infection was explored. Increased mCAR expression was found in the hearts of A/J mice, correlated with more severe myocarditis and higher cardiac viral load as compared to C57BL/6 mice. In addition, a novel truncated isoform of mCAR, initially cloned from A/J neonatal cardiac myocytes, may influence susceptibility to CVB3 infection.
4. MATERIALS AND METHODS

i) Cell culture
Polyoma T antigen transformed Chinese hamster ovary cells (CHOP) (gift of Dr. Jim Dennis) are not susceptible to group B coxsackievirus infection. Cells were grown in α-MEM supplemented with 0.5% penicillin and streptomycin (P/S) and 10% fetal calf serum (FCS). HeLa cells (CCL-2) and Vero cells (CCL-81), an African green monkey kidney cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and grown in RPMI 1640 medium supplemented with 0.5% P/S and 10% FCS. All media and supplements were from Gibco BRL (Burlington, Ontario). Neonatal mouse myocytes were isolated and cultured, as described previously (Chapter 4).

ii) Virus
The cardiovirulent CVB3 strain CVB3-CG (hereafter referred to as CVB3) was adapted by Woodruff and Woodruff (Woodruff 1980) and passaged in the laboratory of Dr. Charles Gauntt (Martino 1998). Virus stock, prepared following one passage through HeLa cells, was maintained as described previously (Opavsky 1999).

iii) CVB3 infection of A/J and C57BL/6 mice
To characterize an in vivo model of host susceptibility to CVB3 infection, A/J (H2\(^{k/k}\)) and C57BL/6 (H2\(^{b/b}\)) mice age 5 weeks (n=25 per group), obtained from Harlan/Sprague-Dawley, were infected with 1 X 10^4 or 1 X 10^5 pfu of a CVB3 and monitored for survival. A subgroup of mice infected with the higher dose of virus was randomly assigned to euthanization on days 2, 4, or 7 post-infection (pi). Mice infected with the lower dose were followed to day 10 pi. Uninfected controls were sacrificed at
day 0. Mouse organs were collected for histopathology, viral titres and isolation of RNA and protein as described previously (Opavsky 1999).

iv) RNase protection assay for CAR

To prepare an RNA probe for the mCAR RNase protection assay (RPA), first-strand cDNA synthesis was performed using 2 μg RNA with the Superscript II (GIBCO-BRL) system as described previously (Opavsky 1999). For detection of murine CAR RNA, a cDNA fragment 484 bp in length was amplified by PCR using the Expand high fidelity PCR system (Boehringer Mannheim) with the sense primer A1 (5'-GATTTCACCAGTGGTTTGAG-3') and antisense primer A2 (5'-GCCATTCAAACGTAGTGGA-3'). Samples were analyzed for mCAR in a thermal cycler (Perkin-Elmer) using the following conditions: 94°C for 3 min; 5 cycles of 44°C for 30 sec, 72°C for 60 sec, 94°C for 15 sec; 30 cycles of 50°C for 30 sec, 72°C for 60 sec, 94°C for 15 sec; and 1 cycle of 50°C for 2 min and 72°C for 7 min. Using Lig'N'Scribe (Ambion, Austin, Texas) a T7 RNA polymerase promoter was added to the 3' end of the PCR fragment using A1 and the supplied T7 adaptor primer, according to manufacturer's protocol. From this template MAXIscript (Ambion), an in vitro transcription kit, was used to transcribe a nonisotopic biotin-16-UTP (Sigma, St. Louis, MO)-labeled antisense mCAR probe complementary to the portion of mCAR RNA encoding an area spanning both extracellular immunoglobulin loops common to both published mCAR isoforms (nucleotide 43 to 527). A reaction mixture containing 1 μg DNA template, 2 μl 10X transcription buffer, 1 μl each ATP, CTP, and GTP, 0.6 μl UTP, 0.4 μl biotin-16-UTP (10mM; Sigma) and 2 μl RNA polymerase was incubated at 37°C for 1 hour, then DNase treated to remove template DNA (Ambion). The mCAR probe and a β-actin probe transcribed from pTRI-actin-Mouse vector (Ambion) were gel-purified on a 6% TBE-urea polyacrylamide gel (Novex), eluted and used for CAR RNA detection by ribonuclease protection assay (RPA).
Fifteen μg of mouse organ RNA, the mCAR RNA probe and the mouse β-actin probe were co-precipitated, denatured and hybridized for 18 hours at 42°C. As directed, following RNase digestion for 15 mins, the RNase was inactivated, and mouse RNA hybridized to the mCAR and β-actin probes was precipitated, resuspended in gel loading buffer and analyzed by electrophoresis on a 6% TBE-urea polyacrylamide gel. Biotinylated RNA probes were detected after electrophoretic transfer to a positively charged nylon membrane using a chemiluminescent detection system (BrightStar Biodetect Kit, Ambion). Band intensities were measured with image densitometry.

v) Reverse transcriptase-polymerase chain reaction

Two μl of each cDNA reaction was amplified using high fidelity Taq DNA and Pwo DNA polymerases (Expand; Boehringer Mannheim) according to the recommended protocol. To determine mouse tissue expression of mCAR1 and mCAR2 RNA, RT-PCR was performed using the conditions described above. The common forward primer A8 (5'-AGCCGGAACGATCGCG-3') was matched with reverse primers A5 (5'-CTATACTAGACCCGTCCT-3') for mCAR1 or A6 (5'-TTATACCACTGTAATGCCATC-3') for mCAR2, which target the divergent cytoplasmic tails of mCAR. Samples were analyzed for mCAR using the following conditions: 94°C for 3 min; 5 cycles of 44°C for 30 sec, 72°C for 60 sec, 94°C for 15 sec; 30 cycles of 50°C for 30 sec, 72°C for 60 sec, 94°C for 15 sec; and 1 cycle of 50°C for 2 min and 72°C for 7 min. To confirm the integrity of the cDNA, previously published (Platzer 1995) β-actin PCR primers were used with similar conditions for mCAR, except only 25 cycles of annealing, elongation and denaturation were used. After resolution on a 1.4% agarose gel, images were acquired with a digital camera (GelDoc 1000, Bio-Rad Laboratories) and band intensities were measured with densitometry (NIH Image Version 1.60) as described previously (Opavsky 1999). To compare relative expression of the two mCAR isoforms,
the band intensities for each sample were compared as a ratio of mCAR1:mCAR2, and tripli cate samples averaged. The change from a unit of one, where both isoforms are equal was then determined for each mean.

For PCR amplification of mCAR cDNA for cloning purposes, a common mCAR forward primer (5'-CTGATATATAAGCTTATAGGCGCGCCTACTGTGCTTCTCGTCTCT-3'), a mCAR1 reverse primer (5'-AGTGCCGTTCTTAGACTATACTATAGACCCCGTCCT TGCTCTG-3'), and a mCAR2 reverse primer (5'-AGTGATGTTCTTAGATCGGTTGGA GGTTGGGACACGTCTAG-3') were used. The 5' primer consisted of a HindIII site (underlined) and the first 28 nucleotides of the mCAR open reading frame. The 3' primers consisted of an XbaI site (underlined) and 27 nucleotides complementary to the terminal region of mCAR1 or mCAR2. Cloning primers TM31 and TM32 amplify the entire coding region of hCAR (Martino 2000) and were used for RT-PCR of Vero cell RNA. For PCR of murine decay accelerating factor (mDAF) the primers forward mDAF 5'-CTTCTACCTGGGGCTATG-3' and reverse mDAF 5'-TATGTAAGTAGCCAATG AGTG-3' were designed based on the published sequence (Spicer 1995) to amplify the 1031 bp coding region.

To amplify mCAR and mDAF, RT-PCR was performed using the conditions described above for mCAR. PCR for Vero cell CAR (vCAR) was performed as follows: 94°C for 3 min; 30 cycles of 50°C for 30 sec, 72°C for 45 sec, 94°C for 30 sec; and 1 cycle of 50°C for 2 min and 72°C for 7 min.

vi) Cloning and sequencing of CVB receptors

PCR products were resolved on a 1.4% agarose gel. A band about 1100 bp in size, corresponding to the expected size of the coding region of CAR, or a band corresponding to the expected size of mDAF were gel-purified with Sephaglas Band Prep (Pharmacia), ligated into pCR2.1 cloning vector using the TA cloning kit (Invitrogen, Carlsbad, CA) and transformed into INVαF' One Shot cells (Invitrogen).
Two clear bands representing Vero cell CAR were separately excised, purified and cloned. Following purification with Qiagen chromatography (Qiagen Inc) two to four clones of mCAR1-pCR2.1 and mCAR2-pCR2.1 from each mouse organ, vCAR clones, and mDAF clones were bidirectionally sequenced with SequiTherm Long-Read Cycle Sequencing (Cedarlane Laboratories, Hornby, Ontario) by automated DNA sequencing (LI-COR and ABI Prism 377, Perkin-Elmer).

Following sequencing, two clones of mCAR1, [full-length mCAR1 and truncated mCAR1 (t-mCAR)] and two mCAR2 clones [mCAR2 and mCAR2(111)] were selected. In addition, one clone of vCAR, comparable in length to mCAR1, and a second shorter clone, t-vCAR, as well as the A/J neonatal cardiomyocyte mDAF clone were selected for subcloning. Clones were digested with XbaI and HindIII restriction endonucleases (Pharmacia) and subcloned into pcDNA1.1/Amp eukaryotic expression vectors (Invitrogen). Following purification sequence fidelity was confirmed by bidirectional sequencing.

vii) CAR expression in CHOP cells

CHOP cells were transiently transfected with 1 µg mCAR1-pcDNA1.1, mCAR2-pcDNA1.1, mCAR2(111)-pcDNA1.1, t-mCAR1-pcDNA1.1, vCAR-pcDNA1.1, or t-vCAR-pcDNA1.1, or controls, using 2 µl Lipofectamine reagent (GIBCO-BRL), according to manufacturer’s instructions, as described previously (Martino 2000). HCAR1-pcDNA1.1 (Martino 2000) served as a positive control, and negative controls included antisense CAR inserted into the expression vector (reverseCAR = rCAR), mDAF-pcDNA1.1, and lipofectamine alone. Briefly, 2 to 2.5 × 10⁵ cells/well, plated in 24 well culture dishes (Corning) were washed in antibiotic and serum-free media, then incubated with DNA-lipofectamine complexes for 5 hours at 37°C. Supernatant was then replaced with α-MEM+10%FCS and incubated further, for a total of 24 h. CAR expression was confirmed with western blot of extracted proteins and
immunocytochemistry. Cell culture supernatant was collected, protein precipitated with acetone, and analyzed by western blot.

viii) Analysis of mCAR protein expression
Transfected and control CHOP cell monolayers were washed with PBS containing 400 \(\mu\)M sodium orthovanadate, 5 mM EDTA, and 10 mM sodium fluoride. The cells were solubilized on ice for 15 mins in lysis buffer containing 0.5% Triton X-100, 50 mM Tris, pH 7.6, 300 mM NaCl, 1 mM sodium orthovanadate, 5 mM EDTA, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, and 1 mM PMSF. A/J and C57BL/6 mouse organs were homogenized and suspended in lysis buffer. The lysates were spun at 12,000 X \(g\) for 10 mins at 4°C. The total protein concentrations of the supernatants were determined with the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, Ontario).

Proteins (50\(\mu\)g) from mouse organs or transfected cell lines were analyzed by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (Novex). Following transfer to PVDF membranes (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec), blots were blocked for 1 h at RT with 5% powdered skim milk in TBS with 5% Tween (TBST) and incubated overnight at 4°C with a rabbit antiserum to mCAR (anti-mCAR;1:5000), kindly provided by Dr. Jeffrey Bergelson. After incubation for 1 h at RT with HRP-conjugated goat anti-rabbit (GAR; New England Biolabs) antibody (Ab), blots were detected using an enhanced chemiluminescence detection system (ECL, Amersham). Equal protein loading was confirmed by staining with coomassie brilliant blue. Band intensities were measured with image densitometry.

CAR expression was also monitored by immunocytochemistry. CHOP cells plated on 8-well slide chambers (Becton Dickinson Labware, Franklin Lakes, NJ) were transfected with mCAR1-pcDNA1.1, mCAR2-pcDNA1.1, vCAR-pcDNA1.1, t-vCAR-pcDNA1.1, or controls, incubated at 37°C for 24 hours, then fixed in PBS:acetone (1:1) for 5 mins, acetone at RT for 5 mins, then ice-cold acetone for 5 mins. After air drying
for 30 mins, monolayers were incubated with anti-mCAR (1:100) for 1 hour, washed and incubated with FITC-labeled GAR antibody (1:500; Jackson Labs, Bar Harbour, Maine) combined with Evans blue reagent for 30 mins, then examined with fluorescent microscopy.

ix) CVB3 binding and infection of CAR-CHOP transfectants

Binding of $^{35}$S-methionine-labeled CVB3 was performed as previously described (Martino 2000)(Chapter 4). Briefly, 24 hours following CAR transfection, cells were washed with serum-free medium and incubated with $^{35}$S-CVB3 for 3 h at RT. Bound and free virus were collected and analyzed by scintillation spectroscopy. CVB3 replication in transfected CHOP cells and controls was determined 24 hours after transfection of $2.5 \times 10^5$ cells per well of 24 well plates (Corning). Cells were inoculated with 1 pfu/cell, incubated at RT for a 1 hour period of adsorption, then unbound virus was removed and cells washed twice with PBS. Cells were resuspended in $\alpha$-MEM+10% FCS and then incubated for 24 hours at 37°C. Cultures were then frozen and thawed three times, and viral titre was determined by plaque assay on HeLa cell monolayers.
5. RESULTS

i) A model of host susceptibility to CVB infection

A mouse model of differential susceptibility to CVB3 infection was explored by examining survival, pathology and organ viral replication in infected A/J and C57BL/6 mice. Overall survival was similar in each strain at 8% in A/J and 13% in C57BL/6 in mice infected with $1 \times 10^5$ pfu CVB3 (Figure 26). The majority (ten) of C57BL/6 mice died at day 3, which was 24 to 48 h earlier in time than the deaths in the A/J mice. Interestingly, only the A/J mice looked clinically unwell, with ruffled coats and lethargy. Histopathology in the A/J mice revealed more severe myocarditis on day 7 pi with diffuse areas of myocyte vacuolation, cytolysis and early inflammatory infiltrate. In fact, even earlier at day 4 isolated myocyte damage was evident in A/J mice, before any cellular infiltration was evident. In contrast, on microscopic examination, C57BL/6 hearts appeared almost normal (Figure 27a & b).

The pancreatic pathology was reversed, with C57BL/6 mice being more affected. Massive infiltration and acinar destruction was apparent on day 4 and day 7 pi, with sparing of the Islets of Langerhans (Figure 27c & d). Pancreatitis was evident in A/J mice, however, the disease was less severe, with acinar structure still clear, and less inflammatory infiltrate. Hepatocyte cytolysis was evident earlier following infection in C57BL/6 mice, but little cellular infiltration was apparent in either strain (Figure 27e & f).

Mice infected with the lower ($1 \times 10^4$ pfu) dose of CVB3 also had similar survival rates, of 90% in the A/J mouse strain, and 85% in the C57BL/6 mouse strain. The pattern of organ tropism was conserved, independent of virus inoculum size. The degree of myocarditis was assessed by a histological score which incorporates severity of cellular infiltration and myocyte damage, as described previously (Opavsky 1999). The myocarditis scores for A/J mice ($3.4 \pm 0.2$) were consistent with
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153 (Fig. 26)

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Figure 27: The pattern of disease following coxsackievirus B3 (CVB3) infection was different between mouse strains. (a) Histopathology revealed that myocarditis was severe in the A/J mice, at day 7 post-infection (pi), with diffuse areas of myocyte vacuolation, cytolysis and early inflammatory infiltrate. (b) In contrast C57BL/6 hearts were almost normal in appearance. The pancreas was more affected in both mouse strains, (c) mild in A/J mice, and (d) severe in C57BL/6 mice more affected. Massive infiltration and acinar destruction is observed on day 7 pi with Islets of Langerhans spared. Hepatitis following CVB3 infection was observed in (e) A/J and (f) C57BL/6 mouse strains, as indicated by cytolysis, vacuolation, but little inflammation. Hematoxylin & eosin, original magnificationX250.
widespread, multifocal myocardial inflammation and necrosis. In contrast, the myocarditis score for C57BL/6 mice (0.8 ± 0.2) indicated significantly less severe disease (p<0.005).

CVB3 replication in the myocardium was consistent with the differences observed in survival and cardiac pathology. The more severe myocarditis observed in the A/J mice was associated with significantly higher cardiac virus titres by day 4 than in C57BL/6 mice. No differences in CVB3 titre were found in pancreas, livers or spleens on day 4 pi (Figure 28). Higher viral titres in the hearts of A/J mice may reflect the increased opportunity for virus-receptor interaction and lead to increased susceptibility to infection. The differences in severity of myocarditis and cardiac viral production observed 4 days following infection, prior to the development of inflammatory foci, indicates that certain susceptibility factors are important before targeting of the heart by the T cell response.

ii) CAR expression in organs of susceptible and resistant mice
To explore the basis for differential CVB3 replication in the hearts of A/J and C57BL/6 mice, tissue expression of the CVB receptor CAR was determined. Using an RNA probe designed to detect RNA encoding the extracellular region of CAR, which is conserved between isoforms, it was found that in both A/J and C57BL/6 mice mCAR RNA expression was highest in liver, heart and kidney (Figure 29). Lower expression was detected in the pancreas, spleen and brain. Due to organ differences in β-actin expression, pancreatic mCAR RNA is also abundant when expressed relative to tissue β-actin RNA. Generally, levels of expression were similar in myocarditis-susceptible A/J mice and pancreatitis-susceptible C57BL/6 mice. Semi-quantitative RT-PCR with primers designed to distinguish between the two full-length mCAR isoforms, was used to compare the relative tissue expression of the two mCAR isoforms (Figure 30). mCAR1 expression tended to be more abundant in the heart, kidney and brain of both
Figure 28: Organ viral load following coxsackievirus B3 (CVB3) infection. Significantly higher virus titres were found in the hearts of A/J mice as compared to C57BL/6 mice at day 4 following infection with 1 X 10^5 pfu CVB3. Viral titres are expressed as log pfu CVB3/g tissue ± SEM (n = 3 per group). *p ≤ 0.001 (Student's T test). No differences in CVB3 titres were found in pancreases, livers or spleens at the same time.
Figure 29: Coxsackie-adenovirus receptor (CAR) mRNA expression in mouse organs. (a) Expression of murine CAR (mCAR) and β-actin were determined by RNase protection assay on heart (H), liver (L), pancreas (P), kidney (K), spleen (S) and brain (B) from A/J and C57BL/6. (b) mCAR expression in two organs per strain were compared following image analysis. Levels of expression were not different between myocarditis-susceptible A/J mice and pancreatitis-susceptible C57BL/6 mice.
Figure 30: The relative tissue expression of mCAR1 and mCAR2 isoforms. RT-PCR was used to compare CAR RNA isoform expression in organs from A/J and C57BL/6 (C57) mice. Findings are presented as % change from an mCAR1:mCAR2 ratio = 1 (n = 3 to 4 per group). A pattern of more abundant mCAR1 expression was observed in cardiac and brain tissue of both strains, while mCAR2 RNA was more abundant in the spleen. *p <0.05 for the proportion of mCAR1 RNA in hearts and brains of C57BL/6 mice vs spleens (z test).
strains, while mCAR2 RNA was more abundant in the spleen. The different cytoplasmic tails of mCAR1 and mCAR2 may influence receptor function and interactions with other cellular proteins, however, the putative tyrosine phosphorylation site is present in both isoforms.

Differences in mCAR protein expression were evident in the hearts of myocarditis-susceptible and resistant mice. A doublet at 44 to 46 kDa was evident on western blot with anti-mCAR in certain mouse organs, most predominantly in A/J hearts and brains (Figure 31). A doublet has been described previously in both mCAR transfected cell lines, and murine tissue (Xu 1995; Bergelson 1998) and may represent glycosylation differences. In addition, the intensity of the 46 kDa band was 26% higher in the myocardium of A/J versus C57BL/6 mice. The abundance of mCAR in hearts of A/J mice, as compared to hearts of C57BL/6 mice, could be responsible for elevated CVB3 titres and the associated severe myocarditis found in this strain. The similar level of mCAR in pancreatic tissue suggests that the difference in susceptibility to pancreatitis between strains was due to factors other than the quantity of mCAR expressed.

To determine if CVB3 infection regulates viral receptor expression, mCAR RNA and protein levels were examined after viral infection. Myocardial mCAR RNA levels, measured relative to β-actin, tended to decrease by day 4 in both A/J and C57BL/6 mice. However, this may reflect increased β-actin RNA expression during infection (Figure 32a&b). Immunoblots demonstrated that the amount of mCAR protein in infected hearts was increased on day 2 in the hearts of A/J mice, but was lower by day 4 in both mouse strains (Figure 32c).

iii) Novel CAR isoforms isolated from the mouse and Vero cells

Susceptibility to coxsackieviral infection may depend on variations in receptor affinity for the virus. Systematic sequencing of mCAR1 and mCAR2 clones from A/J and
Figure 31: Coxsackie-adenovirus receptor (CAR) protein expression in mouse organs. (a) Differences in mCAR protein expression between A/J and C57BL/6 (C57) mice were identified by Western blot with polyclonal anti-mCAR antiserum. A doublet observed at 44 to 46 kDa (arrows) was evident and strongest in A/J hearts and brains. Findings are representative of three to four mice per strain. (b) MCAR protein expression was quantitated by image analysis and presented as band intensity (arbitrary units) ± SEM (n = 3 per group). *p ≤ 0.05 for heart vs liver, p ≤ 0.005 for heart vs pancreas and spleen, p ≤ 0.005 heart vs kidney. **p ≤ 0.05 liver vs pancreas. ***p ≤ 0.05 for brain vs liver, p ≤ 0.005 brain vs kidney, p ≤ 0.0005 brain vs pancreas and spleen. §p ≤ 0.005 for heart vs pancreas, p ≤ 0.05 heart vs spleen. §§p ≤ 0.01 brain vs heart, p ≤ 0.005 brain vs liver, p ≤ 0.0005 brain vs pancreas, kidney and spleen. Equal protein loading was confirmed by Coomassie brilliant blue staining.
Figure 32: The effect of coxsackieviral B3 (CVB3) infection on mCAR RNA and protein expression in the heart. (a) RNase protection assay with probes for mCAR and β-actin in uninfected (0) and days 2 and 4 post-infection (pi) in A/J and C57BL/6 mice. (b) Determination of CAR:β-actin ratio following measurement of band intensity by densitometry suggested that cardiac mCAR RNA expression decreases following CVB3 infection. (c) mCAR protein expression in the hearts (two per group) of A/J and C57BL/6 mice was determined by Western blot. The mCAR doublet at 46 and 44 kDa is apparent in infected A/J mice and tends to increase on day 2 pi. Four days following infection mCAR protein levels are lower. CAR-CHOP is the positive control. Equal protein loading was confirmed by Coomassie blue staining. Results are representative of 3 mice per group.
C57BL/6 mouse organs and neonatal cardiac myocytes revealed two striking changes in CAR. Sequence analysis of clones from A/J myocytes revealed a truncated mCAR1 clone (t-mCAR1) (Figure 33). Deletion of a 152 bp from nucleotide 702 to 853 (inclusive) resulted in a frameshift and a truncated protein. This clone encodes a protein identical to the common area of mCAR1 and mCAR2 until a point of divergence at amino acid 235, just outside to the described transmembrane domain. A 24 amino acid segment which completes t-mCAR1 is substituted for the hydrophobic transmembrane and intracellular domains of the receptor.

On screening of CVB3-susceptible cell lines for CAR RNA by RT-PCR, two amplicons were detected in the Vero cell line (Figure 34). Following cloning of the DNA from these 2 bands separately a truncated form of vCAR, t-vCAR, 99.2% homologous to t-mCAR from A/J myocytes was identified. Full length vCAR which is 98.7% homologous to mCAR1 was also sequenced (Figure 35). As well, two amino acid substitutions were found between the full length and truncated forms of vCAR.

In the mCAR2 isoform, a persistent change at 447 bp, was also identified. This translated into an amino acid substitution of glycine to arginine at amino acid 111 in D1, the first Ig loop of the extracellular domain. Following identification of this amino acid substitution in 2 of 4 A/J heart clones sequenced, screening of other organs revealed the mutation in 2 of 2 A/J pancreas clones, and 3 of 3 C57BL/6 kidney clones. This substitution was found in isolation, or in association with one or more other changes from the published mCAR2 sequence.

iv) The functional role of the novel CAR isoforms
To confirm the translation of t-mCAR RNA into protein, nonsusceptible cells transfected with CAR cDNA were analyzed. The expression of mCAR1, mCAR2, and the truncated isoforms t-mCAR and t-vCAR in CHOP cells was confirmed by western blot. Anti-CAR detects a protein at 46 kDa in CHOP cells transfected with full-length mCAR (Figure
Figure 33: Comparison of truncated and full length amino acid sequences. The truncated mCAR1 clone (t-mCAR1) isolated from A/J myocytes has a 152 bp deletion resulting in a frameshift and a truncated protein 257 aa in length. Red arrows indicate points of divergence from the aa sequence of mCAR1. * indicates the potential tyrosine phosphorylation site. The transmembrane region is underlined. At the site in mCAR2 indicated by the blue arrowhead (at amino acid 111) the glycine is substituted by an arginine in the mCAR2[111] clone. Sequences were compared with DNAsis (Mac v-2).
Figure 34: Coxsackie-adenovirus receptor PCR of Vero cells. Arrows indicate double PCR bands identified in Vero cell line (Vero). The adjacent lane represents a negative water control.
Figure 35: Comparison of truncated and full length amino acid sequences of Vero cell CAR (vCAR) with truncated mCAR1 (t-mCAR). Full length vCAR is 98.7% homologous to mCAR1 and the truncated form of vCAR (t-vCAR) is 99.2% homologous to t-mCAR. Green arrows indicate two amino acid differences between mCAR1 and vCARs. Blue arrowheads indicate substitutions between full length vCAR and t-vCAR. Red arrows indicate points of divergence in t-vCAR from the aa sequence of vCAR, and a tail substitution in vCAR vs mCAR1. * indicates the potential tyrosine phosphorylation site. The transmembrane region is underlined. Sequences were compared with DNAsis (Mac v-2).
In cells transfected with truncated mCAR a primary band at approximately 32 kDa was observed. Immunofluorescence microscopy of permeablized cells revealed a stippled pattern with expression of t-mCAR and t-vCAR isoforms, as compared to a homogenous pattern over the cell surface with expression of the full length CARs (Figure 37). This may represent clustering of the receptor on the cell surface in the absence of a localization signal from the cytoplasmic tail of the protein, or CAR may be sequestered in cytoplasmic pockets and not expressed on the cell surface.

To explore a functional role for the truncated CAR isoform, identified in neonatal A/J mouse cardiac myocytes and Vero cells, virus binding and infectivity were assessed. Expression of truncated CAR isoforms facilitated the binding of radiolabelled CVB3 to CHOP cells, as did expression of full length receptors (Figure 38). Murine DAF does not bind radiolabelled CVB3. The ability of t-CAR to bind virus suggests that the short isoform is expressed on the cell surface. However, CHOP cells expressing t-mCAR or v-mCAR were not susceptible to productive CVB3 infection (Figure 39). Viral progeny production was as low as or lower than in untransfected cells.
Figure 36: Expression of coxsackie-adenovirus receptor (CAR) proteins in nonsusceptible cells. Anti-CAR detects t-mCAR1 at about 32 kDa (trunc CAR), and full-length mCAR1 (CAR) at 46 kDa in whole cell lysates from transfected CHOP cells. Twenty-four hours following transfection cell monolayers were lysed and analyzed by western blot.
Figure 37: Immunofluorescent detection of truncated and full-length mCARs expressed Chinese hamster ovary cells. Expression of mCAR and truncated CAR in permeabilized nonsusceptible cells were detected with anti-mCAR polyclonal antibody. The fluorescence pattern was homogenous in mCAR1 and mCAR2 transfected CHOP cells. In contrast, a stippled pattern was observed following transfection with t-mCAR1 and t-vCAR clones, possibly representing clustering of receptor on the cell surface or sequestration in the cytoplasm. Original magnification X250.
Figure 38: Binding of radiolabelled coxsackievirus B3 (CVB3) to nonsusceptible cells expressing isoforms of coxsackie-adenovirus receptor (CAR). Binding of $^{35}$S-methionine-labeled CVB3 ($^{35}$S-CVB3) was greater than that of control in CHOP cells transfected with mCAR1, t-mCAR1, t-vCAR1, and mCAR2 with a substitution at aa 111 (mCAR2[111]) ($p \leq 0.01$). Transfection of murine decay accelerating factor (mDAF) did not increase binding capacity of $^{35}$S-CVB3 above control (lipofectamine treated CHOP cells). Viral binding is expressed as mean % of control ($n = 3$ per group).
Figure 39: Coxsackievirus B3 (CVB3) infection of nonsusceptible cells expressing isoforms of coxsackie-adenovirus receptor (CAR). Viral progeny production 24 hours following infection of transfected CHOP cells with CVB3 at an moi of 1 pfu/cell. Cells expressing mCAR1, mCAR2 and human CAR (hCAR) produced up to $1 \times 10^6$ pfu/2.5X$10^5$ cells, while cells expressing t-mCAR and t-vCAR did not produce virus above control levels. Controls included cells transfected with antisense CAR (reverse; rCAR), and cells treated with lipofectamine alone (nil). Viral titres are expressed as mean log pfu CVB3/2.5X$10^5$ cells ± SEM (n = 3).
6. DISCUSSION

The interaction of virus with host cellular receptors is an important first step in the initiation of infection. The establishment of a mouse model system for exploring host susceptibility to CVB infection confirmed that severe myocarditis develops in A/J mice, while the myocardium of C57BL/6 mice is minimally affected. In this study, the level of myocardial expression of CAR, the functional receptor for CVBs, has been correlated with the development of severe myocarditis and a higher cardiac viral load. Two isoforms of CAR have previously been identified in the human, the rat and the mouse (Fechner 1999). Findings described here indicate that CAR may exist as additional isoforms, possibly with different roles in the infectious process. The results are consistent with the hypothesis that CAR expression is one determinant of host susceptibility to CVB3 myocarditis.

The levels of specific mCAR RNA in murine organs was greatest in liver, kidney and heart, higher than in brain, spleen and pancreas. Previous studies show highest mCAR expression in the liver (Bergelson 1997a; Tomko 1997; Fechner 1999). In both myocarditis-susceptible and myocarditis-resistant mice examined here, expression of 46 kDa mCAR protein differs from RNA, following the pattern brain > heart > liver and kidney > pancreas and spleen. The abundant expression of 46 and 44 kDa mCAR proteins in the hearts of A/J mice may be important in establishing CVB3 infection of the hearts of these mice, promoting a repetitive cycle of virus entry and replication, leading to high cardiac viral titres and severe myocarditis as compared to C57BL/6. Relative expression of mCAR1 and mCAR2 was consistent between mouse strains. Perhaps differences in isoform expression between organs represents tissue-specific roles for the receptor.

Discrepancies in mCAR expression pattern as compared to previous studies may reflect differences in the strain and the age of the mice. The receptor is known to
be abundant in the hearts of newborn rats, but barely detectable in the hearts of adult rats (Ito 2000). As well, decreasing levels of CAR expression have been demonstrated in mouse brain (Honda 2000) and skeletal muscle (Nalbantoglu 1999) with increasing age. Susceptibility to CVB infection is known to change with age in humans. Newborns are particularly sensitive to multiorgan disease affecting the brain, heart and liver, while in older individuals the frequency of infection decreases, with serious disease mainly targeting the heart (Opavsky 1998).

The observation that CVB3 infection of the C57BL/6 mouse causes severe pancreatitis, but little myocarditis, cannot be explained by CAR expression levels. Pancreatic CAR protein expression was equivalent, and less abundant than in hearts, of both strains. CVB infection is known to target the acinar cells, but spare the islets of Langerhans (Vuorinen 1989; Vella 1991; Arola 1995). Recently, it has been demonstrated that while CAR is expressed at high levels in the acinar cells, very little signal is evident in the islet cells, indicating that within the pancreas, CAR expression does correlate with virus tropism (Mena 2000). The severe destruction and inflammation of pancreatic acinar tissue in CVB3 infected C57BL/6 mice may depend on additional factors, such as the affinity of CVB receptors for the virus, the expression of coreceptors, or host intracellular responses to infection. Expression of IFN-γ in the pancreas (Horwitz 1999) and the nitric oxide response to CVB3 infection appear to play a protective role in pancreatic disease.

Identification of t-CAR across species is consistent with an isoform that may have an important role in CVB3 infection and in normal cell function. The truncated clones may represent alternative splicing of a primary transcript from the CAR gene. Four isoforms of the poliovirus receptor, also a member of the Ig superfamily, are membrane-bound (PVRα and PVRδ) or soluble (PVRβ and PVRγ), and are believed to be the consequence of alternative splicing (Mendelsohn 1989; Koike 1990). Investigation of the functional role of truncated CAR in the pathogenesis of CVB3 has
resulted in the interesting observation that t-mCAR and t-vCAR, while able to bind CVB3, do not confer susceptibility to productive viral infection. Perhaps dimerization with receptors having a cytoplasmic component is required for productive infection. However, it has been shown that genetically engineered CAR which lacks the cytoplasmic and/or transmembrane domains is capable of mediating viral infection (Wang 1999).

The truncated CAR may be capable of being released in a soluble form, acting as a natural inhibitor of CAR-ligand interaction. Soluble receptors have been used experimentally to inhibit viral infection in vitro (Deen 1988; Fisher 1988; Hussey 1988; Traunecker 1988; Martino 2000). The interaction between receptor and virus may induce conformational changes in the virus thereby inactivating it, or the soluble receptor can compete with the membrane-anchored receptor for virus binding (Damico 2000). Soluble hCAR (ending at PPSNK), produced as a fusion protein with a rabbit immunoglobulin Fc segment, inactivates CVB3 (Martino 2000) (Chapter 4). Soluble receptors for retroviruses such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and avian sarcoma and leukosis virus (ASLV) can also enhance infection (Allan 1990; Clapman 1992; Damico 2000). Soluble CD4 can promote HIV infection by binding with the viral envelope protein gp120, inducing a conformational change that facilitates viral-chemokine coreceptor interactions (Wyatt 1998). Interestingly, a Vero cell factor has been described (Konishi 1991) that restores infectivity to CVB3 virions previously neutralized by antibody, possibly by inducing a conformational change in the virus. Perhaps this factor identified in Vero cells is soluble truncated CAR.

Recent work has also demonstrated an important role for the cytoplasmic tail of hCAR targeting the protein to the basolateral membrane surface in polarized airway epithelial cells. Tailless CAR constructs migrate to apical and basolateral surfaces, whereas replacement of the transmembrane and intracellular domains with a GPI
anchor targets the protein to the apical cell surface (Pickles 2000). The stippled pattern observed with immunofluorescent microscopy possibly represents clustered expression of the truncated isoform of CAR on the cell surface. Control of CAR mobility suggests the cytoplasmic region interacts with intracellular signalling pathways, possibly influencing cytoskeletal protein dynamics. Four tyrosine residues are located in the cytoplasmic domain of CAR (Bergelson 1997a; Tomko 1997; Tomko 2000). The most likely candidate for tyrosine phosphorylation is Y269, with the required upstream sequence in the highly conserved RKKRREEKY fragment. Thus, although the extracellular portion of CAR is the only region necessary for infection, the remainder of the molecule may modulate infectivity, possibly by interacting with Src-family kinases and activating the ERK1/2 signalling cascade following receptor crosslinking by CVB3, as discussed in Chapter 4.

The binding site of CVBs on CAR has not yet been localized. Mutagenesis of 4 sites in D1 CAR do not prevent binding of CVB3 (Tomko 2000). Consistent with this, the recurring amino acid substitution at 111, located in the D1 region, identified here, did not prevent CVB3 binding. An mCAR antiserum capable of blocking CVB3 infection, binds to the second domain of CAR, D2 (Tomko 2000), which leads to the speculation that the CVB3 binding-site of CAR is in D2, or at the interface between the two immunoglobulin domains. Site-directed mutagenesis and crystallographic analyses have shown that adenoviruses, which are responsible for diverse infections of the respiratory system, the intestinal tract, the eye and also the heart, attach to D1 CAR via their fiber head protein (Bewley 1999; Roelvink 1999; Freimuth 2000; Tomko 2000). Based on crystal structure analysis, domain 1 of CAR is thought to form a homodimer in its interaction with the adenovirus fiber (van Raaij 2000). Kinetic studies have suggested that three CAR molecules bind simultaneously to one fiber head protein (Lortat-Jacob 2000). Virus-receptor interactions have been characterized at the level of the binding site for both the poliovirus receptor and the rhinovirus receptor.
ICAM-1, both Ig superfamily members. In both cases, the N-terminal region of the receptor has been shown to be important for virus binding. Binding of virus is mediated by interaction of the N-terminal domains of these receptors with structures in the viral canyon (Giranda 1990; Zibert 1992).

The link between CAR, the CVB receptor, and CVB3 pathogenicity and cardiotropism has been examined in susceptible (A/J) and resistant (C57BL/6) mouse strains. Differences in receptor distribution, the quantity of CAR expression, and the expression patterns of specific isoforms may determine the severity and the pattern of disease following CVB3 infection. The truncated CAR isoform may be important in the establishment of CVB3 infection. The specific role of individual CAR isoforms in the activation of intracellular signal transduction pathways an interesting opportunity for further investigation.
CHAPTER 6
SUMMARY AND MAJOR CONCLUSIONS

1. HOST SUSCEPTIBILITY TO MYOCARDITIS IS DEPENDENT ON THE RESPONSE OF \( \alpha\beta \) T CELLS TO COXSACKIEVIRAL INFECTION (CHAPTER 3)

In this study, the role of the T lymphocyte in the pathogenesis of group B coxsackievirus (CVB) myocarditis was investigated. The findings demonstrated differential susceptibility to myocarditis in gene-targeted knockout mice lacking CD4 (CD4\(^{-/-}\), CD8 (CD8\(^{-/-}\)), both coreceptors (CD4\(^{-/-}\)CD8\(^{-/-}\)), or the T cell receptor \( \beta \) chain (TCR\(\beta^{-/-}\)) as compared to control mice with functional T cells. Elimination of both CD4\(^{+}\) and CD8\(^{+}\) T cell populations protected mice from virally-mediated heart disease and death, demonstrating that both CD4\(^{+}\) and CD8\(^{+}\) T cells determine host susceptibility to CVB3 infection. Removal of all \( \alpha\beta^{+}\)TCR T cells from the host response in TCR \( \beta^{-/-}\) mice afforded protection as well. The severity of myocarditis was magnified in CD8\(^{-/-}\) mice, but attenuated in CD4\(^{-/-}\) mice, consistent with a pathogenic role for CD4\(^{+}\) lymphocytes. However, severity of myocardial disease was independent of cardiac viral load. Cytokine expression, however, differed depending on the T cell subset available to respond to infection. In mice with no CD4\(^{+}\) T cells (CD4\(^{-/-}\)CD8\(^{-/-}\) T cell mice and CD4\(^{-/-}\) T cell mice), interferon-\( \gamma \) (IFN-\( \gamma \)) and interleukin-6 (IL-6) expression were elevated. In addition, tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) expression was decreased in CD4\(^{-/-}\)CD8\(^{-/-}\) mice. Finally, natural killer (NK) cell infiltration in the myocardium was associated with elevated IFN-\( \gamma \) expression and cardioprotection. The presence of TCR\(\alpha\beta^{+}\) T cells enhances host susceptibility to myocarditis. CD4\(^{+}\) and CD8\(^{+}\) T cell subsets may
influence the pathogenesis of myocarditis by associated specific cytokine expression patterns.

2. INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS AS DETERMINANTS OF HOST SUSCEPTIBILITY TO GROUP B COXSACKIEVIRUS INFECTION (CHAPTER 4)

In this study, the mechanism by which p56<sup>Lck</sup> (Lck), the Src-family protein tyrosine kinase necessary for normal T cell activation and development, determines host susceptibility to CVB3 myocarditis was investigated. In addition, the contribution of extracellular signal-regulated kinases 1 and 2 (ERK1/2), downstream of Lck, in the pathogenesis of the disease was examined. The findings demonstrated that the ERK1/2 cascade is a determinant of host susceptibility to CVB3 infection and disease. At the time of initial virus-Jurkat T cell interaction the ERK1/2 cascade was rapidly activated in an Lck-dependent manner. In addition, in T cells and cardiomyocytes optimal CVB3 replication was dependent on MEK1/ERK1/2 activation. Infection of A/J mice with cardiovirulent CVB3 led to acute and severe myocarditis with high cardiac viral titres, whereas minimal myocardial inflammation and necrosis occurred in C57BL/6 mice. The severity of myocarditis correlated with activation of the ERK1/2 signalling pathway early in the course of infection. ERK1/2 phosphorylation was greater in the hearts of myocarditis susceptible A/J mice. These data demonstrate that CVB3 influences host cell function and self-promotes viral replication via the ERK1/2 cascade, downstream of Lck.
3. THE COXSACKIE-ADENOVIRUS RECEPTOR IS ASSOCIATED WITH DIFFERENTIAL SUSCEPTIBILITY TO CVB3 INFECTION AND MYOCARDITIS (CHAPTER 5)

In this study, the expression of the coxsackie-adenovirus receptor (CAR) was investigated as a determinant of host susceptibility to CVB3 myocarditis. Characterization of CVB3 infection of two murine strains showed that A/J mice were susceptible to myocarditis, whereas C57BL/6 mice developed pancreatitis as the primary site of disease. Viral titres were higher in the hearts of A/J mice, but similar between strains in other organs. Analysis of CAR RNA by RNase protection assay revealed that in both A/J and C57BL/6 mice mCAR RNA expression was highest in liver, heart and kidney. Expression of CAR protein was greater in the myocardium of A/J as compared to C57BL/6 mice, correlating with the increased cardiac viral titre and more severe myocarditis. An intense 44 kDa band, consistent with a glycosylation product, was identified in the A/J hearts. Sequence analysis of CAR yielded a clone encoding a truncated protein common to both A/J myocytes and Vero cells. The truncated isoforms from mouse (t-mCAR1) and Vero cells (t-vCAR) are 99.2% homologous and diverge from the full length molecule at the start of the transmembrane region, with a 24 amino acid segment completing the protein. Also, a recurring amino acid substitution in the first immunoglobulin loop CAR was identified in the mCAR2 isoform. Truncated CARs expressed in nonsusceptible cells bind radiolabelled CVB3, but do not mediate productive infection, suggesting that in addition to the level of mCAR expression, specific isoforms of CAR may determine tissue tropism and the pattern of disease.
CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

Exploration of the pathogenesis of viral myocarditis over the past several years has targeted both the role of the immune response and the nature of the virus. A number of factors that influence the disease process have, as a consequence, been identified. The investigations presented in this thesis have focused on three determinants of host susceptibility to group B coxsackievirus (CVB3) myocarditis: the T lymphocyte-mediated immune response; virus-triggered intracellular signal transduction; and, the coxsackie-adenovirus receptor (CAR). In this section the interactive and complex relationship between these three factors will be discussed. Specific concepts arising from the studies presented will be highlighted in the context of the host's vulnerability to coxsackieviral infection of the heart.

1. The T lymphocyte role in susceptibility to viral myocarditis is multifaceted

The T lymphocyte is important to the current understanding of the pathogenesis of viral myocarditis. Although classically considered to be protective in the context of viral infection, the T cell can exacerbate myocardial disease in murine models of CVB3 myocarditis as first demonstrated in 1974 by Woodruff and Woodruff (Woodruff 1974). Before the adaptive T cell is stimulated by MHC-restricted antigen presentation, the innate immune response is activated as the host’s the first line of defense. Elements of the innate immune response which participate in CVB3 myocarditis include natural killer (NK) cells (Godeny 1986; Godeny 1987), macrophages, (Bell 1971; Woodruff 1980) and interferon (IFN) (Kandolf 1985; Lutton 1985; Matsumori 1987a; Heim 1992). In the myocardium, cytotoxic CD8+ T cells lyse infected host cells via perforin, (Young
1990; Seko 1991; Seko 1993b), while CD4+ T cells activate effector cells via cytokines and chemokines (Mak 1994; Abbas 1996). CD4+ T cells express two phenotypic cytokine profiles, designated Th1 and Th2 (Rocken 1992; Fitch 1993). Th1 responses, characterized by IFN and tumour necrosis factor-α (TNF-α), are primary elements in the response to most viral infections, including CVB (Huber 1992; Huber 1994a). Other T cell subsets, including TCRαβ+double negative (DN) and TCRγδ+ T cells can also respond to viral infection with cytolytic capacity and cytokine expression (Porcelli 1992; Pfeffer 1994; Nishizawa 1997).

In this thesis, gene-targeted knockout of specific T cell subsets illustrates the multiple ways the T cell response determines host susceptibility to CVB3 myocarditis. The elimination of T cells expressing CD4, CD4 and CD8 coreceptors, and the TCR-β chain, p56^{Lck} (Lck) protein tyrosine kinase all protect mice from CVB3 myocarditis (Opavsky 1999; Liu 2000). In association with increased protection of the heart and infection-related death in CD4+ and CD8+ double knockout mice, a differential effect on the cytokine response is observed (Opavsky 1999). With only αβ+DN T and γδ+T cells available, an environment of elevated IFN-γ, elevated interleukin-6 (IL-6) and attenuated tumour necrosis factor-α (TNF-α) is created. This cytokine profile is consistent with the established protective effect of IFNs (Heim 1992) and IL-6 (Kanda 1996), and the detrimental effect of TNF-α (Finkel 1992; Lane 1992; Yamada 1994; Freeman 1998) in the course of viral myocarditis. The altered cytokine pattern is early in the course of CVB infection, prior to any evidence of infiltrating T lymphocytes in the myocardium. However, NK cells are present in the heart at this time, and more abundant in the hearts of CD4^{-/-} and CD4^{-/-}CD8^{-/-} mice, in association with protection from myocarditis. In the absence of the T cell response, NK cells mediate viral resistance with prolonged activation (Kos 1996). NK cells are activated in response to IFNα/β produced by infected cells, such as myocytes. In addition, IL-12 is a major NK cell stimulatory factor. Perhaps circulating levels of cytokines, or other T cell-related
events removed from the myocardium influence early protective mechanisms within
the myocardium itself, or those that target the site of viral replication, such as NK cells.
NK cells themselves regulate the specific immune response through cytokine
secretion, and also directly kill virally-infected cells by the perforin and granzymes
(Kos 1996). CVB3 infection of myocarditis-resistant Lck knockout mice following
depletion of NK cells results in increased mortality and heart disease, demonstrating
the protective role of NK cells (personal communication, T. Martino). In the absence of
CD4+ T cells, the balance of destructive and protective elements of the immune
response to CVB3 infection is shifted, creating an antiviral environment with elevated
IFN-γ and myocardial targeting by NK cells. It is tempting to hypothesize that an
interrelationship exists between the innate and specific immune responses, such that
the subset of T cells available to respond to viral infection can influence the intensity of
and protection provided by the innate response.

In mice with gene-targeted knockout of Lck survival with CVB3 infection is
100%, no myocarditis develops and viral replication is impaired (Liu 2000).
Attenuation of CVB3 replication in the organs of infected Lck knockout mice, and in
JCaM (Lck negative) T cells was particularly interesting. CD45 protein tyrosine
phosphatase (PTP) activates Lck by dephosphorylating its regulatory domain.
Consistent with the resistance to CVB3 infection observed in Lck knockout mice,
CD45−/− mice are protected from CVB3-related mortality and myocarditis. Moreover, the
ability of CD45 to modulate the cytokine-activated JAK/STAT pathway has further
implications (Irie-Sasaki 2001). This intracellular mechanism, present in T cells, B
cells, mast cells and macrophages can control the cytokine response to viral infection.
The regulation of proinflammatory, or antiviral cytokine responses is certainly
important in determining susceptibility to myocardial inflammation and dysfunction.
Thus, two regulators of intracellular signalling pathways, Lck and CD45, can
determine host susceptibility to CVB3 infection.
2. The influence of mitogen-activated protein kinase signalling cascades on host susceptibility to CVB3 myocarditis

The ERK1/2 signalling cascade is a determinant of host susceptibility to CVB3 infection and disease. In T cell lines the ERK1/2 pathway is specifically and rapidly activated in an Lck-dependent manner. Src-family kinases promote CVB3 replication in T cells and cardiac myocytes. As well, CVB3 replication in T cell lines and myocytes depends upon activation of the ERK1/2 pathway. In A/J mice, severe myocarditis and high viral load in the myocardium were correlated with early and persistent activation of the ERK1/2 signalling pathway. ERK1/2 activation was weaker in myocarditis-resistant C57BL/6 mice. A reciprocal activation of the stress-responsive p38 MAPK pathway was observed in the mouse models.

The rapid Lck-dependent activation of ERK1/2 by CVB3 occurs in T cells at the time of initial virus-receptor interaction. But, the ERK cascade may play a role in viral replication at any time from viral entry to progeny release. ERKs mediate upstream signals from growth receptors, integrins, and cytokine receptors via several pathways. Multiple events in the course of the viral life cycle may depend on ERK phosphorylation of viral or host proteins. Initial ERK activation may have immediate effects on viral entry, probably involving an endocytotic pathway, or induce gene expression which modulates viral replication and/or host cell function hours to days later. Perhaps ERK activation inhibits gene expression of host defense systems. For example, crosstalk between the ERK1/2 cascade and the cytokine-responsive JAK/STAT system may direct downstream gene expression, influencing the host's response to CVB3 infection. Interaction between MAPK pathways and JAK/STATs is supported in IFN signalling (David 1995; Arora 1999). A hypothetical mechanism for CVB3 activation of cell signalling pathways in the T cell and the myocyte is illustrated in figure 40.
Figure 40: A hypothetical mechanism for CVB3 activation of cell signaling pathways in the T cell and myocyte. The T cell receptor is engaged by the antigen presenting cell (APC) in an MHC-restricted manner. CD4/8 coreceptors act in concert to activate Lck. αβ+ T cells with functional LCK and CD45 are essential to the T cell response, which contributes to severe myocarditis by proinflammatory cytokine elaboration and cytotoxicity. CD45 also regulates cytokine activation of the JAK/STAT pathway. If CD45 dephosphorylation of JAK is prevented, the antiviral effects of IFN-α are increased. CVB3, possibly by interaction with its receptors CAR or DAF, activates the ERK1/2 cascade in an LCK-dependent manner. Full-length CAR isoforms have a tyrosine phosphorylation site in the cytoplasmic domain, and crosslinking of DAF can activate LCK. Both LCK in T cells and Src-family kinases in myocytes are important for optimal viral replication. In addition, ERK1/2 activation also promotes CVB3 replication and is correlated with increased cardiac viral levels and severe myocarditis. p38MAPK activity is reduced in hearts with more severe myocarditis. Crosstalk between ERKs and JAK/STATs may modulate the response to CVB3 infection at a cellular level. Downstream ERKs direct gene transcription, and in this way may influence host cell function and promote viral replication contributing to the pathogenesis of myocarditis. Arrows indicate proposed events initially triggered by CVB3 infection.
Just as control of the balance between protective and destructive elements of the immune response is relevant at a systemic level, the balance between cell signalling pathways at the intracellular level may determine the severity of myocarditis. Activation of the ERK1/2 signalling cascade promotes CVB3 infection and disease, while activation of the IFN-responsive JAK/STAT system protects from disease. The manipulation of these intracellular signalling pathways in altering host susceptibility to viral infection may have therapeutic potential.

3. The role of the coxsackie-adenovirus receptor in host susceptibility to viral myocarditis

Isoforms of CAR are members of the immunoglobulin (Ig) superfamily. The role of CAR in cell function is unknown, however it has been speculated that given its relationship to other Ig superfamily members it functions in adhesion and intercellular communication (Ito 2000). High levels of CAR expression early in life, decline in adult rodents, suggesting a developmental role for the cell surface protein (Nalbantoglu 1999; Honda 2000; Ito 2000). The highly conserved cytoplasmic domain and the potential tyrosine phosphorylation sites suggest possible key interactions with other intracellular proteins. While the extracellular portion of CAR is required for productive infection (Wang 1999), the influence of the cytoplasmic and transmembrane domains on the efficiency of infection has not yet been determined. It is reasonable to speculate that the cytoplasmic domain, with potential tyrosine phosphorylation sites contributes to infection of the host cell and the, as yet unknown, natural function of the receptor. In polarized cells the cytoplasmic tail of CAR directs the protein to the basolateral membrane surface, suggesting a line between the cytoplasmic domain and signal transduction pathways (Pickles 2000). Downstream activation of ERK1/2 that occurs likely promotes viral replication and direct the host cell response to infection. The truncated CAR isoform (t-CAR) may participate in CVB3 infection and in normal cell
function. The inability of t-CAR to mediate productive infection, despite CVB3 binding, suggests the transmembrane and cytoplasmic domains are necessary, or alternatively, t-CAR may be a primarily secreted protein. Interactions between truncated and full-length CAR isoforms, DAF and other cell surface proteins may impact on CVB3 infection, at the cellular level, or in the whole animal based on differential receptor distribution.

The adenoviral binding site of CAR has been localized to the first immunoglobulin loop of CAR. While the extracellular portion of the receptor is required for productive CVB infection, the specific interaction site(s) between CAR and CVBs has not yet been determined. Cloning of mCAR cDNA from several mouse organs has revealed a number of deduced amino acid mutations. It is known that RNA viruses exist as quasispecies due to the high mutation rate during the course of viral replication (Prabhakar 1982). Passaging of CVB through different cell lines or organs, results in specific virus subsets being selected with variable tissue tropism (Huber 1990). It is tempting to speculate that tissue-dependent amino acid changes in the CVB3 binding site of CAR display differential affinities for select subpopulations of the quasispecies group. A functional test of this mutual-selection hypothesis would separate random mutation from receptor-selection and illustrate a mechanism by which receptor mutations determine disease.

The identification of coreceptors for CVB and other diverse viruses such as HIV and adenovirus, illustrates that a common characteristic of viruses is to take advantage of several cell surface markers, which each may determine host susceptibility. A multicomplex nature of the CVB receptor has long been considered. In human cells DAF has been proven to be a coreceptor for CVB3 infection (Bergelson 1995; Shafren 1995). However, evidence to date has not supported the role of murine DAF (mDAF) in infection (Spiller 2000). Receptors other than CAR and DAF that may participate in the initiation of CVB infection include αvβ6 integrin, which enhances CVB1 infection in a
colon cancer cell line (Agrez 1997). Following CVB cross-linking of DAF or integrins, intracellular signalling pathways may be activated. Cell surface proteins may be clustered with signalling proteins in areas of the plasma membrane referred to as microdomains. In T cells, aggregation of lipid rafts may be important to T cell activation (Janes 2000). In other cell types plasma membrane invaginations termed caveolae share many properties with lipid rafts, but also contain the structural protein caveolin (Janes 2000). DAF, a GPI-anchored protein, has been localized to both lipid rafts and caveolae (Mayor 1994; Janes 2000). The localization of GPI-anchored proteins, Src kinases, protein kinase C isoforms and components of the ERK1/2 signalling cascade in caveolae, has been identified as one mechanism of compartmentalizing signalling molecules (Ostrom 1999; Rybin 1999). Interestingly, GPI-anchored proteins may be excluded from clathrin-coated pit regions that are involved in receptor-mediated endocytosis, one hypothesized mechanism of picornavirus entry into the cell. Alternatively, GPI-anchored proteins may cluster in caveolae only following crosslinking (Mayor 1994). CAR has not been localized in either clathrin coated pits or caveolae, however, given the fluidity of the cell membrane virus binding may induce colocalization between DAF, CAR, other cell surface proteins and tyrosine kinases.

4. The basis for differential susceptibility of mice to myocarditis is multifactorial

Factors which determine host susceptibility to coxsackieviral myocarditis have been explored in this dissertation. The T cell knockout models have illustrated that the T lymphocyte influences the response of the host to viral infection by regulating cytokine responses to infection. Lck activation is correlated with CVB3 infection (Chapter 4) and myocarditis in mice (Liu 2000). However, virus replication proceeds even in the absence of Lck, indicating that alternative Src-family kinases may be involved. By examination of host factors in myocarditis-susceptible A/J mice and
myocarditis-resistant C57BL/6 mice additional factors have been identified. These include the differential expression of CAR in the myocardium, and activation of the ERK1/2 and p38 MAPK signalling cascades. Key determinants of host susceptibility to CVB3 myocarditis are illustrated in figure 41.

There are several possible candidates which may contribute to host susceptibility to CVB3 disease. Microarray analysis determined that CVB infection altered the expression of 169 genes in the myocardium (Taylor 2000). Data organized into functional groups illustrated the enormous and diverse impact viral infection has on cell defense, cell structure/motility, cell metabolism, cell signalling, and protein expression genes in the heart. Preliminary assessment of gene regulation by microarray (data not shown) concurs and is consistent with the key roles of the immune response and cell signal transduction investigated in this thesis. For example, modulation of cytokine responses by downregulation of tumour necrosis factor (TNF) receptor precursors and upregulation of interferon regulatory factor 1 gene (IRF-1) indicate self-protective mechanisms. As well, alteration of the gene expression of multiple protein kinases, phosphatases, and associated signalling molecules, supports a complex role for signal transduction pathways in the host response to CVB infection.

All potential determinants of host susceptibility can be studied in isolation, however, consideration of the vast array of proteins as an dynamic process provides valuable information. Perhaps CVB receptors, Lck, and downstream signalling pathways represent an interactive pathway which the virus utilizes to infect a host cell and self-promote its replication. One can imagine that CVB-receptor binding sets in motion activation of signal transduction pathways which subsequently trigger responses in both the T cell and the cardiac myocyte that determine disease susceptibility and resistance. Cell surface expression of CVB receptors themselves may be regulated as suggested by the influence of CVB3 infection on CAR expression.
Figure 41: Key determinants of susceptibility to coxsackieviral B3 myocarditis. The studies presented in this thesis, using mouse models and cell culture systems, have identified several host factors which may define the susceptible and the resistant host.
in the myocardium. Receptor specific activation of Src-kinase and the MAPK pathway activation has yet to be investigated. Patterns of organ tropism may reflect both differences in activation of signalling pathways between organs, and in the specific repertoire of CVB receptor molecules expressed in cells and tissues.

In summary, several viruses have evolved mechanisms to stimulate signal transduction pathways which affect viral transformation of cells, promote viral replication, and regulate host inflammatory responses to infection. In the course of this thesis, a direct link between CVB3 triggered signal transduction and host susceptibility to infection has been demonstrated using cell culture systems and mouse models of susceptibility. Group B coxsackieviruses appear to take advantage of the cell surface proteins CAR and DAF, the Src-family kinase Lck, and components of the ERK1/2 signalling cascade, as well as the host immune response to establish productive infection of the host. Ultimately these experiments will have impact on our understanding of the pathogenesis of myocarditis, identification of those at risk for viral myocarditis, and future therapeutic modalities.

5. Future directions

Future research focusing on the determinants of susceptibility and resistance to coxsackieviral infection can be pursued using both directed and candidate screening approaches. Further exploration of the mechanism of interaction between CVB3, the host immune response and key cellular proteins will continue to advance our understanding of host susceptibility to viral infection. A central question is how does the coupling of the viral receptor and signal transduction pathways influence the pathogenesis of viral infection?

The site of interaction between CVBs and CAR has not yet been determined, although the extracellular portion of the receptor is required for productive CVB infection. Comparison of CAR amino acid sequences across species in relation to their
susceptibility to CVB infection would indicate potential binding regions that could subsequently be targeted with site-directed mutagenesis. The strong developmental expression pattern of CAR and its hypothesized role as an adhesion molecule suggest that gene-targeted knockout of CAR may be embryonically lethal. The function of CAR in the normal host and its endogenous ligand, the protein's influence on signal transduction and its impact on host susceptibility to CVB3 infection could be elucidated by the construction of conditional transgenic mice with organ-targeted knockout of the receptor.

By taking advantage of the established model of myocarditis-susceptible A/J mice and myocarditis-resistant, pancreatitis-susceptible C57BL/6 mice mechanisms of tissue tropism can be investigated. Comparing primary cardiomyocyte and pancreatic cells (and cell lines) represents a simple system which could be developed to examine susceptibility. The targeted approach would analyze receptor isoform expression using RNA probes and isoform-specific anti-CAR antibodies. Infection of cardiomyocytes from susceptible and resistant mice with wildtype cardiovirulent CVB3 versus transfected viral cDNA will distinguish receptor-mediated from post-receptor requirements for productive infection. The roles of signal transduction molecules and downstream regulation of gene transcription could reveal how ERK1/2 activation ultimately leads to myocyte destruction and viral proliferation. The candidate screening approach would apply cDNA microarray technology to primary cell lines of different susceptibility (ie myocyte vs pancreas; A/J vs C57BL/6 myocyte) to compare differential host gene regulation in response to CVB infection at the cellular level. Key genes upregulated and down regulated would be highlighted for further investigation.

Vascular endothelial cells (VECs) are the portal of entry for viral infection of any target organ, and would provide a valuable model for the study of susceptibility to CVB3. As passage of CVB3 through the heart increases the infectivity of cardiac VECs, while virus isolated from the liver targets hepatic VECs (Huber 1990), indicating some
selection factor(s) are operative. The hypothesized role of CAR as an adhesion molecule may have particular relevance in endothelial cells. Following the establishment of a VEC system for heart, liver, and pancreas, multiple factors can be examined. Differential CAR isoform expression, location, or differential VEC responses to CVB infection (either dependent or independent of CAR) may have a significant influence on organ tropism.

An observation made during the course of the signal transduction studies in Jurkat cells merits further exploration. Jurkat cells, which express both DAF and CAR viral receptors, are not 100% permissive to CVB infection. The cell surface transducer of virus triggered activation of the ERK1/2 signalling cascade is not yet determined. The identification of DAF and/or CAR negative subsets by flow cytometry and the establishment of selected cell lines could form the basis for determining the connection between CVB3 and Lck, and the role of CAR and DAF in viral activation of ERK1/2.

In Jurkat cells and cardiac myocytes ERK1/2 activation is important for optimal viral replication. In the hearts of myocarditis-susceptible A/J mice ERK1/2 are activated in association with elevated cardiac viral load. Activation of the JAK/STAT signalling cascade (Irie-Sasaki 2001) and a cytokine expression of elevated IFN-γ and IL-6 (Opavsky 1999) are protective. The in vitro approach to explore crosstalk between JAKs and MAPKs in the response to CVB infection would include the manipulation of MAPK or JAK pathways by activators and inhibitors. The in vivo approach would follow up in vitro results, using gene-targeted knockout or kinase inhibitors to confirm the role of specific elements and downstream targets in host susceptibility. A MEK1 inhibitor has been identified which inhibits activation of the ERK1/2 cascade in colon tumours implanted in mice (Sebolt-Leopold 1999). This compound has low toxicity and would be a valuable in following up the role of ERK1/2 in the pathogenesis of CVB3 myocarditis. A valuable research direction would be the therapeutic gene
targeting of T cell subsets to inhibit proinflammatory cytokine responses detrimental to the host, such a TNF-α, and to promote a cytokine profile including the cytoprotective effects of IL-6 and the antiviral effects of IFNs. The JAK/STAT signalling pathways may be a point amenable to such intervention, or alternatively a chemotherapeutic approach.

In conclusion, a repertoire of specific host cell factors can direct susceptibility to CVB infection. Continued investigation of determinants of host susceptibility to CVB3 infection will be exceptionally valuable and lead to better understanding of the disease and more precise targeting of prevention and therapy.
CHAPTER 9

APPENDIX

CD45 PROTEIN TYROSINE PHOSPHATASE REGULATION OF
GROUP B COXSACKIEVIRAL REPLICATION

1. PURPOSE:

To determine if CD45 protein tyrosine phosphatase can regulate the antiviral
effect of interferon on group B coxsackieviral replication *in vitro*.

Key components of data relevant to this thesis are included in this Appendix and are part of the publication:

2. INTRODUCTION

T lymphocytes play an important role in the pathogenesis of group B coxsackieviral (CVB) myocarditis. Gene-targeted knockout of the T cell specific tyrosine kinase, p56\textsuperscript{Lck} (Lck), which is essential to T cell activation, completely protected mice from CVB3 infection and associated myocarditis (Liu 2000). Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulate intracellular signalling events following T cell activation (Penninger 1993b; Thomas 1999). Phosphorylation of the kinase domain activates the Src kinases, however, phosphorylation of the C-terminal regulatory domain inhibits enzyme activity (Thomas 1999; Alexander 2000). CD45 dephosphorylates the C-terminal tyrosine residue of Lck, a step required for the initiation of kinase activation and subsequent T cell signal transduction (Ostergaard 1989; Sieh 1993; Thomas 1999). Consistent with the role of CD45 as an Lck PTP, gene-targeted knockout of CD45 protected mice from myocarditis and mortality following infection with CVB3 (Irie-Sasaki 2001).

CD45 PTP has been shown to act as a Janus kinase (JAK) phosphatase, with the potential to alter signalling pathways downstream of cytokine interactions with their cell surface receptors (Irie-Sasaki 2001). Following observations that CD45 inhibits the interleukin-3 (IL-3)-activated JAK2-STAT3/5 signalling pathway in bone-marrow-derived mast cell lines, further investigation established the PTP as a JAK phosphatase, able to negatively regulate cytokine receptor signalling in several haematopoietic cell types (Irie-Sasaki 2001). Interferon-\(\alpha\) (IFN-\(\alpha\)) is a known activator of JAK1, however, in the CD45-deficient Jurkat T cells JAK1 is hyperphosphorylated. The JAK/signal transducer and activator of transcription (STAT) network mediates diverse downstream effects of cytokine interactions with their receptors, including responses to viral infection (Rodig 1998).
Both the antiviral activity of IFN-α, -β, and -γ, and the proinflammatory cytokines, tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and lymphotoxin-α (LT-α; TNF-β), have been implicated in the pathogenesis of myocarditis. Interferons have been shown to inhibit CVB3 infection in cell culture and protect mice from myocarditis in vivo (Kandolf 1985; Matsumori 1987a; Matsumori 1988b). In myocarditis-resistant CD4-/- CD8-/- mice, reduced myocardial pathology is associated with a cytokine profile of elevated IFN-γ and reduced TNF-α (Opavsky 1999) (Chapter 3B).

In this study the influence of CD45 on CVB3 infection is explored in a T cell model. Enhanced inhibition of CVB3 replication by IFN-α in T cells lacking CD45 PTP demonstrated one mechanism by which the CD45-/- mice may be protected from myocarditis. CD45 PTP, by its influence on cytokine responses to infection, in addition to its role in T cell activation, is an additional factor which may contribute to host susceptibility to CVB3 myocarditis.
3. MATERIALS AND METHODS

i) Virus

A cardiovirulent strain of CVB3, adapted by Woodruff and Woodruff (Woodruff 1980) and passaged in the laboratory of Dr. Charles Gauntt (Martino 1998) was used. Virus was prepared by passage twice in HeLa cells. Stocks were frozen and thawed three times, clarified by centrifugation and stored at -70°C after titres were determined by plaque assay on HeLa cells.

ii) Cells

Parental wildtype Jurkat cells (J45.01), CD45 deficient (CD45<sup>-/-</sup>) Jurkat cell lines (J-AS-1) (McKenney 1995), CD45<sup>-/-</sup> deficient J-AS-1 cells reconstituted with wildtype CD45 (CD45-recon) and CD45<sup>-/-</sup>-deficient J-AS-1 cells reconstituted with phosphatase-inactive CD45 (D1:C828S mutant) (Felberg 1998) were cultured in RPMI (Gibco BRL, Burlington, Ontario) with 5% fetal calf serum (FCS; Gibco-BRL).

iii) CVB3 infection

Wild type, CD45<sup>-/-</sup>, CD45-reconstituted, and CD45-inactive cell lines were pretreated with human interferon-α (IFN-α; 1000 U/ml; Genzyme) in RPMI+10% FCS for 4 h at 37°C. Then, cells (1 X 10<sup>6</sup>) were washed in serum-free RPMI and incubated with 2 pfu/cell CVB3 in serum-free media for 1 hour at RT. Cells were then washed to remove unbound virus, resuspended in RPMI plus 10% FCS with or without IFN-α (1000 U/ml), incubated at 37°C for 24 h, and frozen at -70°C until titre determination. Cells were frozen and thawed 3 times, cellular debris was removed by centrifugation, and virus was quantitated by plaque assay on HeLa cell monolayers.
Interferon-α phosphorylates JAK1 in wild type Jurkat cells, however, in the absence of CD45 PTP, JAK1 tyrosine phosphorylation was increased (Irie-Sasaki 2001). To determine if CD45 PTP impacts on IFN-α inhibition of CVB3 infection, viral yield in Jurkat cells with and without CD45 was compared. Interferon-α pretreatment and co-treatment decreased viral replication in wild type Jurkat cells by 61% with IFN-α pretreatment and 82% with IFN-α co-treatment. However, the decrease was more profound in CD45⁻/⁻ cells, by 93% with IFN-α pretreatment and 98% with IFN-α co- treatment (Figure 42).

To confirm the role of CD45 in IFN-α suppression of viral replication, CVB3 titres in CD45⁻/⁻ cells re-expressing functional CD45 (CD45-recon), or re-expressing inactive CD45 PTP (CD45-inactive) were measured. In CD45⁻/⁻ cells, which do not have CD45 JAK phosphatase activity, the antiviral effect of IFN-α is greater than in wild type cells (Figure 43). Pretreatment with IFN-α decreased CVB3 replication by 37% and 27% in wildtype and CD45-recon Jurkat cells, respectively. In contrast, CVB3 replication was more dramatically decreased by 64% in both CD45⁻/⁻ and CD45-inactive Jurkat cells.

The increased inhibitory effect of IFN-α on CVB3 replication observed in T cell lines suggests a novel mechanism by which CD45⁻/⁻ mice may be protected following viral infection. Cytokine activation of the JAK/STAT pathway is amplified in the absence of CD45 PTP dephosphorylation of JAKs (Irie-Sasaki 2001). With the negative regulatory effect of CD45 JAK PTP eliminated, certain cytokine-activated pathways may be enhanced, attenuating viral production, as shown here. JAK1 is a widely expressed tyrosine kinase and activated by several cytokines. Genetic knockout of JAK1 has shown its essential role in responses by class II cytokine receptors (IFNs, IL-10), γc subunit receptors (IL-2, IL-4, IL-7, IL-9, IL-15), and gp130 subunit receptors.
Figure 42: The anti-viral activity of interferon-α (IFN-α) is enhanced in the absence of CD45 PTP. Jurkat cells (wild type) and CD45−/− Jurkat cells were infected with CVB3 at an moi of 2 pfu /cell following 4 h pretreatment with IFN-α (IFN), following 4 h pretreatment with IFN-α plus co-treatment with IFN-α for 24 h post-infection (IFN X 24h), or in the absence of IFN-α (control). Findings shown are expressed as the % change in CVB3 titre from control ± SEM for each cell type (n = 3). For wild type and CD45−/− Jurkat cells *p ≤ 0.0001 for control vs IFN and IFN X 24 h (Student’s T test).
Figure 43: The influence of CD45 on the anti-viral activity of interferon-α (IFN-α) in CVB3-infected CD45⁻/⁻ Jurkat cells is confirmed. Jurkat cells (wild type), CD45⁻/⁻, CD45-reconstituted CD45⁻/⁻ (CD45 recon) and phosphatase-inactive PTP (CD45 inactive) Jurkat cells, were infected with CVB3 at an moi of 2 pfu /cell following 4 h pretreatment with IFN-α (IFN), or in the absence of IFN-α (control). Findings shown are expressed as the % change in CVB3 titre from control ± SEM for each cell type (n = 3). For wild type and CD45-reconstituted cells the reduction in CVB3 titre with IFN-α treatment was not significant, however, in the absence of functional CD45 CD45⁻/⁻ and in phosphatase-inactive CD45 cells a greater reduction in viral titre was observed. *p ≤ 0.005 for control vs IFN in CD45⁻/⁻ and *p ≤ 0.01 for control vs IFN in CD45 inactive cells (Student's T test).
(including IL-6, leukemia inhibitory factor [LIF], and cardiotropin-1 [CT-1]). (Rodig 1998). JAK1-deficient cells do not develop responses to IFNs, such as MHC expression, nitric oxide production, upregulation of ICAM-1 and antiviral activity. Also, no response to gp130 receptor family activation by IL-6, LIF or CT-1, was observed. Thus, enhancement of JAK1 activation in CD45⁻/⁻ cells may have widespread effects.

Gene-targeted knockout of both CD4⁺ and CD8⁺ T cells or all TCR β⁺ T cells protects the heart following CVB3 infection (Opavsky 1999). Targeted disruption of CD45 PTP gene dramatically reduces the number of CD4⁺ and CD8⁺ thymocytes, and lower numbers of peripheral T cells are found (Kishihara 1993). Three to 5% of thymocytes express CD45 PTP of an undetermined isoform. The limited complement of peripheral CD4⁺ and CD8⁺ T cells in CD45⁻/⁻ mice (Kishihara 1993), is a mechanism by which mice lacking CD45 PTP expression may be protected from CVB3 myocarditis.

Multiple cytokines, including IFNs, are produced in the myocardium early following CVB3 infection, (Freeman 1998; Opavsky 1999). Increased IFN-mediated viral attenuation due to enhanced JAK/STAT pathway activation may contribute to resistance to CVB3 myocarditis shown in CD45⁻/⁻ mice (Irie-Sasaki 2001). Certain proinflammatory cytokines, dependent on JAK/STAT signalling to influence cell function, could aggravate myocardial inflammation and increase severity of disease in the absence of CD45 JAK phosphatase activity. However, the combined suppression of the T cell response with lower peripheral T cell numbers and inhibited Lck activation in the CD45⁻/⁻ mice, probably contributes to protection of the myocardium. Manipulation of CD45 activity or conditional knockout in animals with a fully developed complement of lymphocytes may produce different effects in response to cytokine-receptor interactions.

In summary, enhanced inhibition of CVB3 replication by IFN-α in T cells lacking CD45 PTP suggests a mechanism by which CD45 may influence host susceptibility to
viral infection. Three mechanisms may contribute to the resistance of CD45−/− mice to CVB3 myocarditis. The removal of CD45 from the host's repertoire of signalling kinases can limit the numbers of T cells available to respond to viral infection, prevent T cell differentiation and proliferation by inactivating Lck, and enhance T cell responsiveness to IFN-α.
CHAPTER 9
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