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Pathological and Genetic Analysis of Host Susceptibility to Cardiovirulent Coxsackievirus B3 Infection in Mice

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PATHOLOGICAL AND GENETIC ANALYSIS OF HOST SUSCEPTIBILITY TO CARDIOVIRULENT COXSACKIEVIRUS B3 INFECTION IN MICE

By

Rim Lejmi Mrad

Thesis submitted to the Faculty of Graduate Studies and Research
of
University of Ottawa
in partial fulfillment of the requirement for the degree of Master of Science

Faculty of Medicine
Department of Biochemistry, Microbiology and Immunology

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Our file Notre référence
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ABSTRACT

Nearly fifty percent of North American myocarditis cases are associated to coxsackievirus group B, type 3 (CVB3) infection. CVB3 infection of mice provides a useful model to study pathogenic mechanism of myocarditis. The objective of this study is to test the hypothesis that susceptibility, during the acute CVB3 infection, is under polygenic control including H2 as well as the non H-2 genes.

To identify differential parameters of the disease, and if they are influenced by the *H-2* haplotype, several phenotypic traits were characterized. Three inbred strains of mice and two congenic strains were infected with CVB3. Differences in survival, body weight loss, quantification of myocarditis and quantification of sarcolemmal disruption were found by comparing three sets of mice sharing the same H-2 haplotype but not the same background. It was determined that host susceptibility to CVB3-induced myocarditis is mainly controlled by "background" genes.

Moreover, because there is a naturally occurring variability among inbred mice, ten inbred strains of mice were used for the genetic analysis of four CVB3-induced phenotypes: survival, body weight loss, heart viral load and quantification of sarcolemmal disruption. It was concluded that the strains could be divided into three groups: the highly resistant, the resistant to intermediate strains and the highly susceptible strains. This phenotypic data on commonly used and genetically diverse inbred mouse strains sets up the platform for a detailed analysis of the genetic basis of susceptibility to CVB3.

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for his constant motivation, support and encouragement, and to my wonderful baby Rami for being a patient foetus while I was in the biohazard room. Their unfailing support, devoted understanding, and cooperation were a continuous source of inspiration in my pursuit.

DEDICATION

This thesis is dedicated to my parents for their never-ending support and encouragement.

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LIST OF ABBREVIATIONS

Ab	Antibody
AMP	Amyopathic
ANOVA	Analysis of Variance
bp	Base Pairs
CAR	Coxsackie-Adenoviral Receptor
CD	Clusters of Differentiation
CDNA	Complementary Deoxyribonucleic Acid
CIM	Chronic Inflammatory Myopathy
$CVB1_T$	Coxsackievirus B1 Tucson
CVB3	Coxsackievirus group B3
DAF	Decay Accelerating Factor
DAPC	Dystrophin Associated Protein Complex
°C	Degrees Celsius
DEPC	Diethyl Pyrocarbonate
Des	Desmin
DMD	Duchenne Muscular Dystrophy
ddH_2O	Double Distilled Water
DMEM	Dulbecco Minimal Essential Medium
DNA	Deoxyribonucleic Acid
DNTP	Deoxyribonucleotide triphosphate
EBD	Evans Blue Dye
ERK-1/2	Extracellular signal-regulated kinases 1 and 2
FBS	Fetal Bovine Serum
Gapdh	Glyceraldehydes phosphodehydrogenase
H-2	Histocompatibility-2
H&E	Haematoxylin and Eosin
HLA	Human Leucocyte Antigens

IDC	Idiopathic Dilated Cardiomyopathy
IFN	Interferon
INOS	Inducible Nitric Oxide Synthase
IRES	Internal Ribosomal Entry Site
Kbp	Kilo base pairs
Kda	Kilo Dalton
Lck	Lymphocyte protein tyrosine kinase
MEM	Minimal Essential Medium
MHC	Major Histocompatibility Complex
Min	Minute
µM	Micromolar
MOPS	3-[N-Morpholino]propanesulphonic acid
MP	Myopathic
N	Amine/N-terminus of proteins
NK	Natural Killer cells
Nm	Nanometer
NO	Nitric Oxide
NTR	Non Translated Region
OCT	Optimum Cutting Temperature
ORF	Open Reading Frame
P	Protein
P	Probability
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
p.i.	Post infection
Pro 2A	Protease 2A
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SCID	Severe Combined Immunodeficiency
SNPs	Single Nucleotide Polymorphisms
TNF	Tumor Necrosis Factor
VP	Viral Protein

INTRODUCTION

I. PATHOGEN: COXSACKIEVIRUS GROUP B TYPE 3 (CVB3)

1. Coxsackievirus Discovery and Classification

In 1947 Dalldorf and Sickles [Dalldorf et al., 1948] isolated two unknown viruses from fecal specimens in Coxsackie, New York, during a poliomyelitis outbreak (poliovirus was the first discovered enterovirus). Histological studies of these two viruses lead to isolation of coxsackie A group viruses. The first isolation of what would become known as the group B coxsackieviruses (CVB) occurred shortly thereafter [Melnick et al., 1949]. By the end of the 1950s, the CVB had been implicated as a significant childhood agent of myocarditis [Disney et al., 1953].

Coxsackieviruses are in the Enterovirus genus of the family Picornaviridae. Enteroviruses enter the body primarily through ingestion, and are stable enough at low pH to establish a primary infection in the gastrointestinal tract. Subsequently, other organs can be infected, depending on the strain of virus and other factors that are not yet well understood. Unlike other enteroviruses with limited host range, the coxsackie group B viruses can infect mice, primates and humans. Evidence also suggest that the virus can infect other hosts as reflected by infection of cultured rat cardiomyocytes [Badorff et al., 1999] and African green monkey kidney cells, among other cells used as a models for CVB3 infection, like HeLa cells which were used in this project. This ability of cosackieviruses to infect rodents (as demonstrated in this project) has greatly aided in

understanding the pathogenesis of enteroviruses-induced disease. Focal-oral transmission of the CVB is the primary mode of transmission, as it is for other enteroviruses. Coxsackie A viruses cause widespread muscle inflammation (myositis) often accompanied by flaccid paralysis, while the CVB induce focal inflammatory lesions in neural tissues, muscles (especially the myocardium), pancreas and liver. Immunological reactivity to defined sera was originally used to classify the coxsackie A viruses into 23 serotypes and the coxsackie B viruses into 6 serotypes. The official abbreviations are "CV-AX" and CV-BX", where "X" is a number. However most of the literature uses the abbreviations "CVAX" and "CVBX". The latter is adopted in this document.

2. Coxsackievirus Life Cycle

Coxsackie group B viruses are small RNA viruses that have icosahedral protein coats made up of 60 identical units, each of which consist of four structural proteins VP1-VP4. The interaction between viruses and constituents of the plasma membrane are critical components in the initiation of the viral life cycle. Host cell receptors are not consistently expressed in all cell type. Viruses may also require co-receptors and other factors on the surface of the cells that aid in the stabilization and the interaction of the virus with the appropriate receptor. The Coxsackie-Adenoviral Receptor (CAR) allows internalization of the coxsackieviral genome after attachment and is a critical step for viral infection [Bergelson et al., 1997]. In addition, the Decay Accelerating Factor (DAF) serves as a coreceptor by significantly increasing the binding efficiency of

coxsackievirus onto the DAF-CAR receptor complex to permit uncoating of viral genome and facilitate its internalization by CAR [Liu et al., 2001] (Figure 1). After binding to the Coxsakie and Adenovirus Receptor (CAR) present on myocytes and other host cells, viral genome enters the cell.

The coxsackievirus genome consists of a single positive stranded RNA genome of 7433 nucleotides, which encodes a large single open reading frame (ORF), are flanked by a 5'- Non Translated Region (5'NTR) and a polyadenylated 3'-NTR. The 5'-NTR contains a type I Internal Ribosomal Entry Site (IRES) which enables 5'cap independent translation initiation of the viral polyprotein [Molla et al., 1992]. Due to its positive strand organization, the CVB3 RNA genome can be translated immediately by host ribosomes to produce viral monocistronic polyprotein. The translated polyprotein as illustrated in Figure 2 consists of three major regions (P1, P2, and P3) with the P1-region containing all four structure proteins (VP1, VP2, VP3 and VP4) which form the viral structural capsids. P2 and P3 contain non-structural proteins. During the translation process, the P1-region is quickly cleaved off by the protease 2A (Pro 2A) before translation can proceed any further. The protease 3C, subsequently cleaves the larger protein fragments into smaller functional polypeptides. Viral genome replication is facilitated by an RNA-dependent polymerase (3Dpol) and involves negative (−) strand RNA intermediates [Xiang et al., 1997] which serves as a template for the production of multiple positive-strand virus genome. Only (+) RNA genomes are packaged into capsids, which are released by lysis of the host cell. A schematic model of Coxsackievirus life cycle is illustrated in Figure 3.

Figure 1: Collaboration of CAR and DAF receptors to permit uncoating and internalization of viral genome.

[Liu et al., 2001].

DAF: Decay Accelerating Factor
CAR: Coxsackie and Adenovirus Receptor

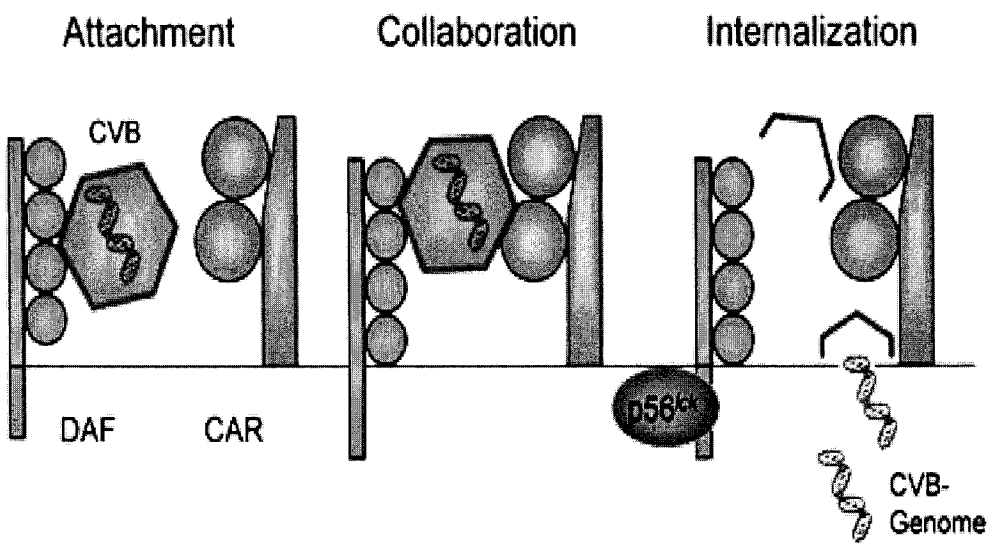

Attachment
Collaboration
Internalization
CVB
DAF
CAR
p56lck
CVB-
Genome

Figure 2: Schematic representation of coxsackievirus B3 polyprotein.

The CVB3 polyprotein consists of the capsid proteins VP1-VP4, the non-structural proteins protease 2A (Pro2A), 2B, 2C, 3A, VPg and protease 3C (Pro3C), and the viral RNA polymerase (3DPOL).

[Badorff et al., 1999].

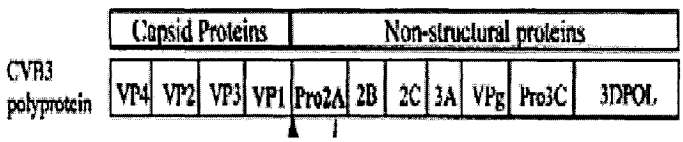
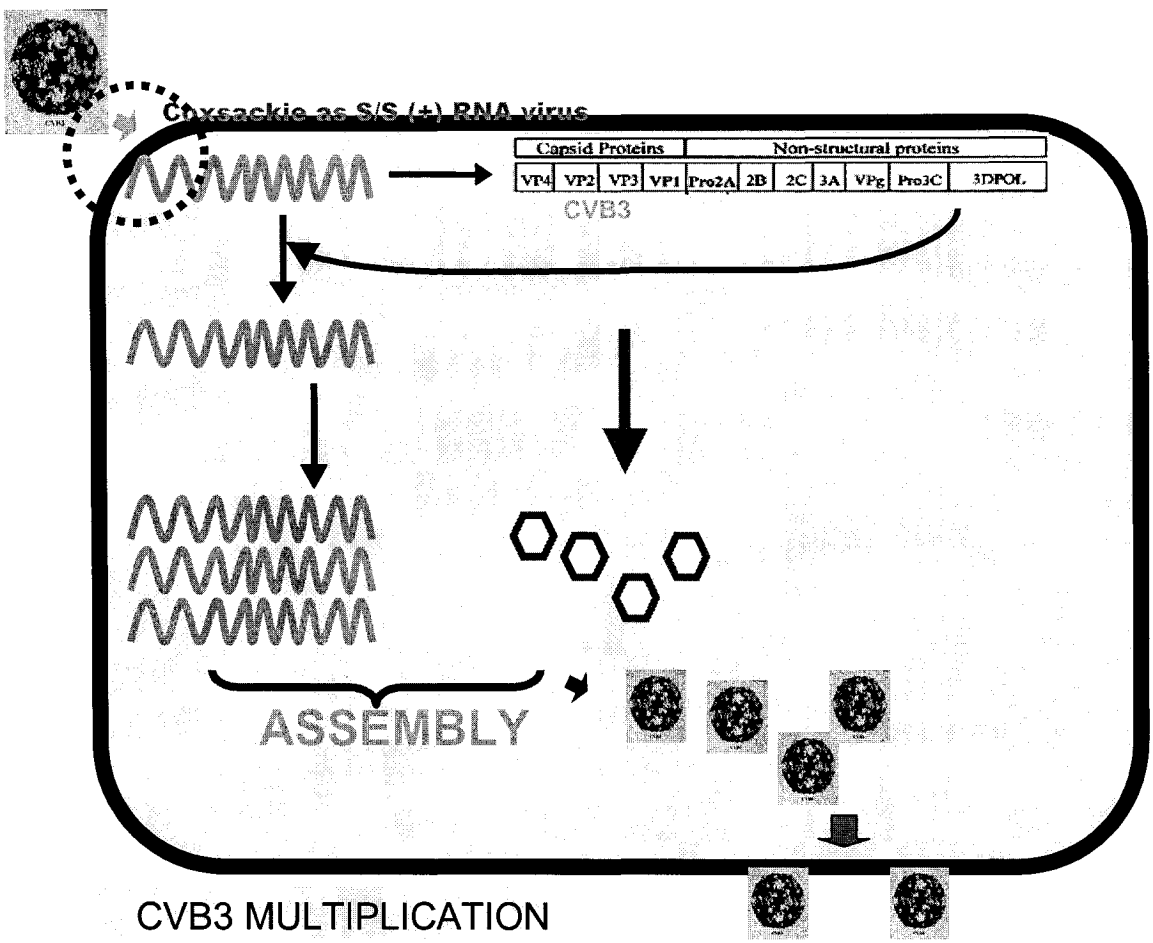

Capsid Proteins
Non-structural proteins
CVB3
polyprotein
VP4
VP2
VP3
VP1
Pro2A
2B
2C
3A
VPg
Pro3C
3DPOL

Figure 3: Schematic model of the CVB3 life cycle.

CVB3 multiplication occurs entirely in the cytoplasm. Infection is initiated by the attachment of CVB3 to the specific cellular receptors, CAR and DAF, which result in conformational changes in the virion, allowing CVB3 RNA to be released into the cytoplasm. CVB3 RNA is translated into a polyprotein. In addition, viral RNA serves as a template for the synthesis of minus-strand RNA, which is used as templates for the synthesis of new plus strands of RNA. Plus-strand RNA and capsid proteins assemble into progeny virions, which are released by cell lysis.



CVB3 MULTIPLICATION

3. Coxsackievirus as a Human Pathogen

At least 70 % of human population has come into contact with CVB3 [Melnick et al., 1996]. The clinical presentation varies from completely asymptomatic in the majority of patients to severe congestive heart failure with a poor prognosis. In children, acute forms of viral myocarditis are more common, often present in fulminant multi-organ disease (pancreatitis and meningitis), whereas in adults, chronic forms are observed more frequently with only a small percentage of patients with fulminant myocarditis [Martin et al., 1994]. The predominant symptoms in patients with myocarditis and reduced cardiac function are shortness of breat and thoracic pain, often in combination with pericarditis and ventricular arrhythmia which can cause syncope or sudden death. Fever is rarely observed in adults [McCarthy et al., 2000]. Children can have severe vomiting, headaches, be very tired and complain of stomach aches, all of which unfortunately are symptoms that are common to numerous childhood diseases.

There is substantial evidence that myocarditis and subsequent myocardial destruction progresses to idiopathic dilated cardiomyopathy (IDC) [Manolio et al., 1992; Hufnagel et al., 2000, Pankuweit et al., 2000, Martino et al., 1994]. IDC is a chronic condition characterized by the enlargement of the heart chambers, impaired myocardial and ventricular function leading ultimately to heart failure. CVB3 can be detected in the hearts of as many as 30-50% of patients with IDC [Martino et al., 1994]. With 750,000 new cases of heart failure diagnosed each year and 250,000 deaths per year; the impact

of the disease on morbidity and mortality, as well as economic Figures is remarkable [http://www.americanheart.org/].

II. MYOCARDITIS

Myocarditis is defined as heart disease in which there is inflammation of the myocardium associated with myocardial cell necrosis [Aretz et al., 1987]. There are therefore two components to the diagnosis: interstitial inflammation and myocyte damage. Myocarditis is the most common cause of acquired heart failure in children and is regarded as an important predisposing condition of dilated cardiomypathy in adults [Liu et al., 1996]. Yet despite its importance in both the pediatric and adult populations, the mortality remains high and there is a lack of effective treatment available.

The most common established etiological agents of myocarditis are viruses. Liu et al., in 1996 listed most common viruses involved in myocarditis (see Table 1). RNA viruses predominate, with picornaviruses being the most commonly identified agents. Coxsackieviruses B have been shown to be the causative agents of about 50% of all cases of established myocarditis [Huber et al., 1998]. Although the mechanisms by which CVB3-infection can cause myocarditis are not completely elucidated, it is clear that virus determinants of virulence as well as host susceptibility factors contribute to disease.

1. Virus Determinants of Virulence

Table 1: Etiological agents in myocarditis.

[Liu et al. 1996].

Viral
- Coxsackievirus B and A
- Echovirus
- Influenzavirus A and B
- Adenovirus
- Human immunodeficiency virus
- Herpes simplex virus type 1 and 2
- Cytomegalovirus
- Mumps virus
- Rubella virus
- Hepatitis C virus
- Epstein-Barr virus
- Varicella zoster virus

Parasitic
- Chagas' disease (*Trypanosoma cruzi*)
- Taxoplasmosis

Toxic Agents
- Chemotherapeutic agents
- Radiation therapy
- Physical agents

Bacterial
- Lyme carditis (tick borne spirochete)
- Diptheria
- Extension of bacterial endocarditis

Hypersensitivity Reactions
- Drugs
- Acute rheumatic fever
- Allergies

It has been shown that sequencing of multiple clones and viruses identified five candidate determinants that were strictly conserved in myopathic viruses (coxsackievirus B1 Tucson ($CVB1_T$)) with: one located in the 5'-Non Translated Region (NTR), three in the VP1 capsid, and one in the 3C protease. The myopathic $CVB1_T$ causes chronic inflammatory myopathy (CIM) consisting of hind limb weakness and inflammation. It has been also indicated that there are at least two determinants of inflammation and one additional determinant of weakness (Tyr-87 and/or Val-136:candidate determinants of weakness) encoded by myopathic $CVB1_T$ [Tam et al., 2003].

2. Pathogenesis and Mechanism of CVB3-Induced Myocarditis

In analogy to many other virus-mediated illnesses [Oldstone, 1998], both direct viral effects as well as the host's inflammatory response contribute to the pathogenesis of viral heart disease.

2.1. Direct Effect of Coxsackievirus B3

On one hand, coxsackievirus B3 has direct cytopathic effect on cardiomyocytes. On the other hand, the coxsackieviral protease 2A cleaves dystrophin which directly increases sarcolemmal permeability.

2.1.1. CVB3-Direct Myocytolysis

The direct effects of cardiotropic viruses (example, the CVB3-CG strain used in this project) towards destruction of myofibers can be observed in animal models [Rabin et al., 1964; Woodruff et al. 1980 , Matsumori et al., 1988] . Extensive cardiac necrosis is observed in immunosuppressed mice, even though the expected mononuclear cell infiltrate in the heart is abolished [Woodruff et al., 1974]. Likewise, Severe Combined Immunodeficiency (SCID) mice also develop severe and predominant virus-induced myolysis with persistent viremia in comparison to infected non-SCID controls [Chow et al., 1992].

In addition, Knowlton and co-workers demonstrated the direct viral effect in culture and in vivo. In cultured cardiomyocytes, infection with coxsackivirus B3 induces a direct cytopathic effect and cell death [Wessely et al., 1998a]. In mice transgenic expression of CVB3 genomes in the heart is sufficient to induce dilated cardiomyopathy [Wessely et al., 1998 b]. Both effects occurred independently of an immune response and demonstrate that coxsackieviral proteins can principally cause myocyte damage and heart disease.

2.1.2. Enteroviral Protease 2A Disrupt Dystrophin

Dystrophin is a 427-KDa subsarcolemmal cytoskeleton protein which contains, as represented in Figure 4.a, four domains: an N-terminal domain, a rod domain consisting of spectrin-like repeats, a cystein rich domain and a carboxy-terminal domain.

As diagrammed in Figure 4.a., the dystrophin molecule has a N-terminal actin-binding domain followed by 24 spectrin-like triple helical repeats, with four hinge regions. The C terminus of dystrophin contains a cystein-rich domain that binds to dystroglycan followed with a region that associates with dystrobrevin and syntrophin [Sweeney et al., 2000].

Dystrophin is believed to stabilize the sarcolemma by attaching the actin cytoskeleton to the extracellular matrix through the dystrophin-associated glycoprotein complex [Xiong et al., 2002] (see Figure 5.b.). This connection forms the major pathway for transmitting the forces generated in the muscle sarcomeres to the extracellular connective tissues [Sweeney et al., 2000]. In addition to that, this connection protects muscle cells from contraction-induced damage [Badorff et al., 1999]. In fact, dystrophin provides mechanical support to the sarcolemma.

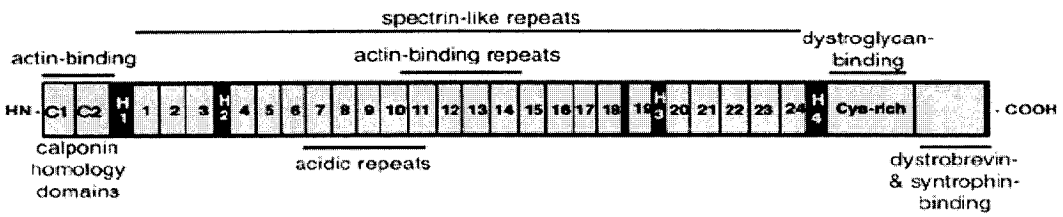
The loss of dystrophin in humans results in loss of the entire protein complex shown in Figure 4.b. and causes a progressive lethal muscle-wasting disease known as Duchenne muscular dystrophy (DMD) [Sweeney et al., 2000].

During CVB3 infection the viral protease 2A is expressed and directly cleaves dystrophin, which separate the actin-binding domain from the β-dystroglycan-binding domain. This leads to a morphological disruption of dystrophin and other components of the dystrophin-associated protein complex (DAPC). Based on what it is known from muscular dystrophy studies, this directly increases sarcolemmal permeability and decreases the transmission of mechanical force. These abnormalities trigger a cascade of events that ultimately contributes to the pathogenesis of CVB3-induced cardiomyopathy [Badorff et al, 2004] (Figure 5).

Figure 4: Schematic structure and interactions of dystrophin

(a)-**Domains of the dystrophin molecule**. The N-terminus contains the primary actin-binding site, whereas the C-terminus contains the b-dystroglycan, dystrobrevin, and syntrophin-binding sites. The N- and C-terminal domains are connected by 24 spectrin-like repeats, some of which have been shown to bind actin. The four ''hinge'' regions are denoted H1–H4 (are accessible to proteolytic cleavage [Badorff et al., 1999]).
(b)- **Schematic representation of dystrophin interactions**
The N-terminal actin-binding domain of dystrophin in purple is associated with the cortical actin. The C-terminal domain associates with b-dystroglycan and with a- and b-syntrophin and dystrobrevin.
[Adopted from Sweeney et al., 2000].

(a)


spectrin-like repeats
actin-binding
actin-binding repeats
dystroglycan-binding
HN-
C1
C2
H1
1
2
3
H2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
H3
20
21
22
23
24
H4
Cys-rich
-COOH
calponin homology domains
acidic repeats
dystrobrevin- & syntrophin-binding

(b)

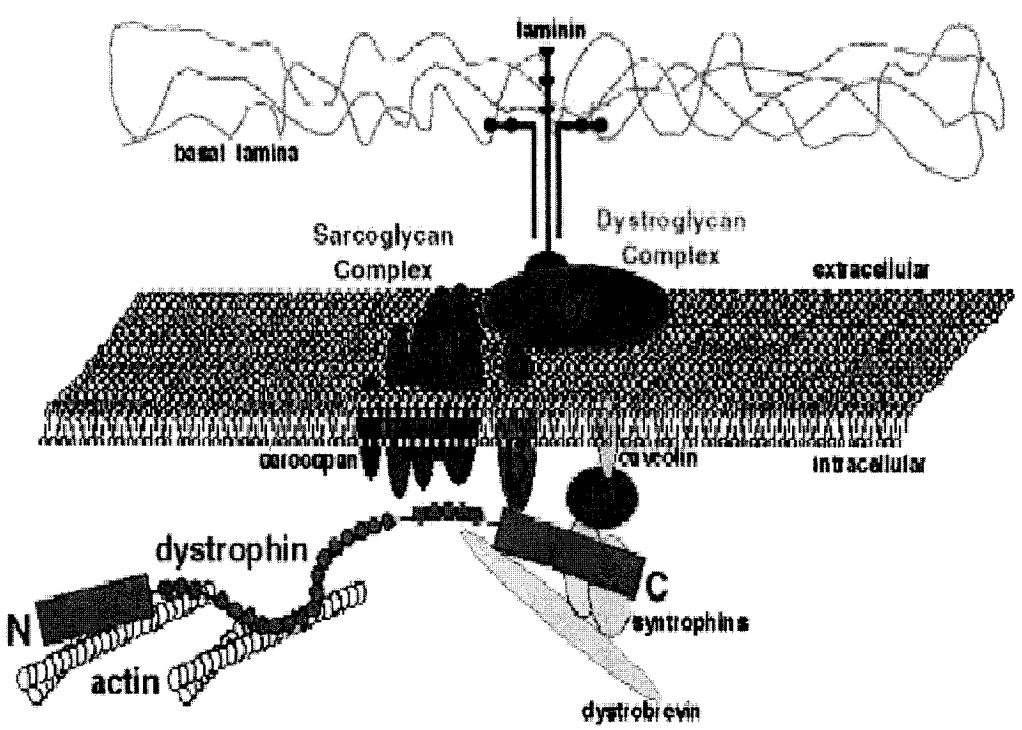
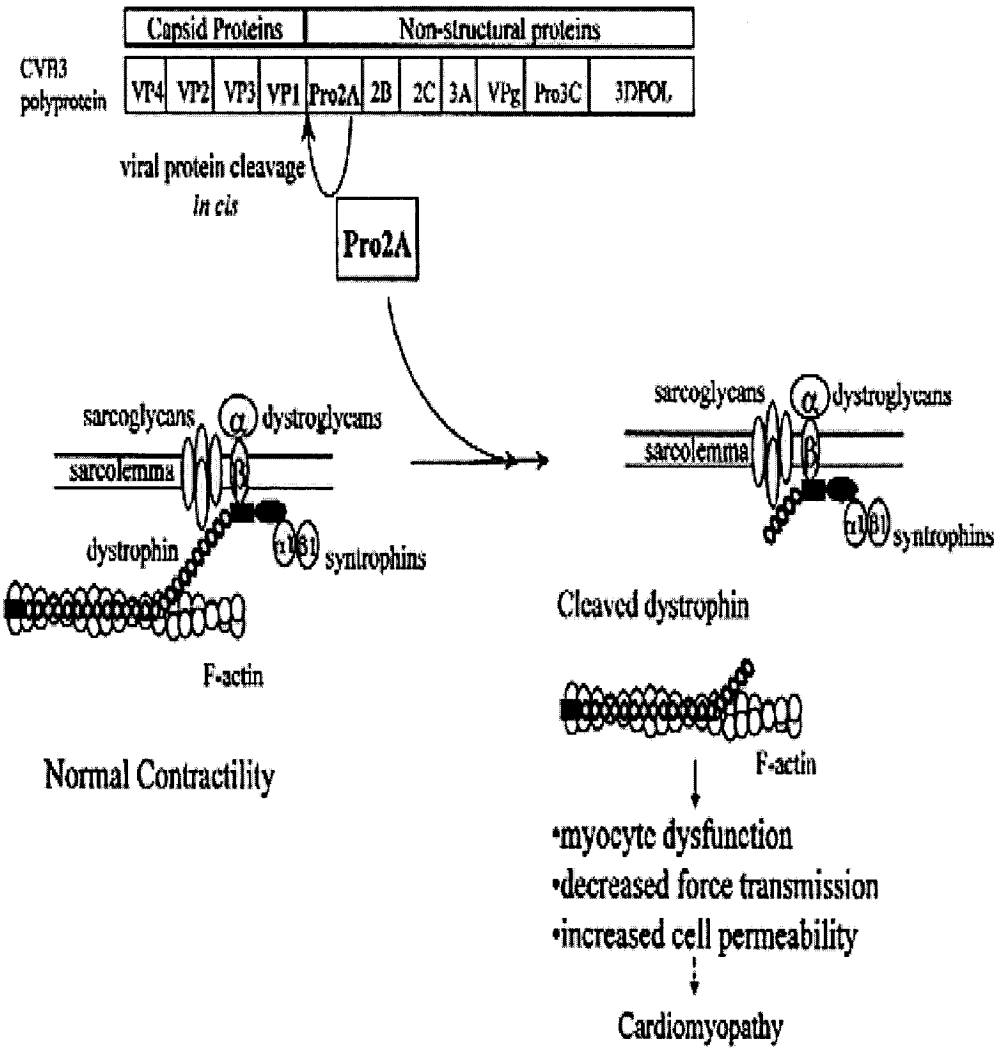

laminin
basal lamina
Sarcoglycan Complex
Dystroglycan Complex
extracellular
sarcoglycan
caveolin
intracellular
dystrophin
C
N
syntrophins
actin
dystrobrevin

Figure 5: Role of dystrophin in Coxsackievirus-induced cardiomyopathy: dystophin disruption-induced pathogenesis.

[Badorff et al., 1999].


Capsid Proteins
Non-structural proteins
CVB3 polyprotein
VP4
VP2
VP3
VP1
Pro2A
2B
2C
3A
VPg
Pro3C
3DPOL
viral protein cleavage
in cis
Pro2A
sarcoglycans
α
dystroglycans
sarcolemma
β
dystrophin
α1β1
syntrophins
F-actin
Normal Contractility
sarcoglycans
α
dystroglycans
sarcolemma
β
α1β1
syntrophins
Cleaved dystrophin
F-actin
•myocyte dysfunction
•decreased force transmission
•increased cell permeability
Cardiomyopathy

This mechanism seems to operate in human infection. In fact, Knowlton and co-worker extended their findings to a patient with dilated cardiomyopathy due to a coxsackievirus B2 myocarditis, and they found that, in vitro, coxsackievirus B2 protease 2A cleaved human dystrophin which may contribute to the pathogenesis of human enterovirus-induced dilated cardiomyopathy [Badorff et al, 2004].

2.2. Immune Response

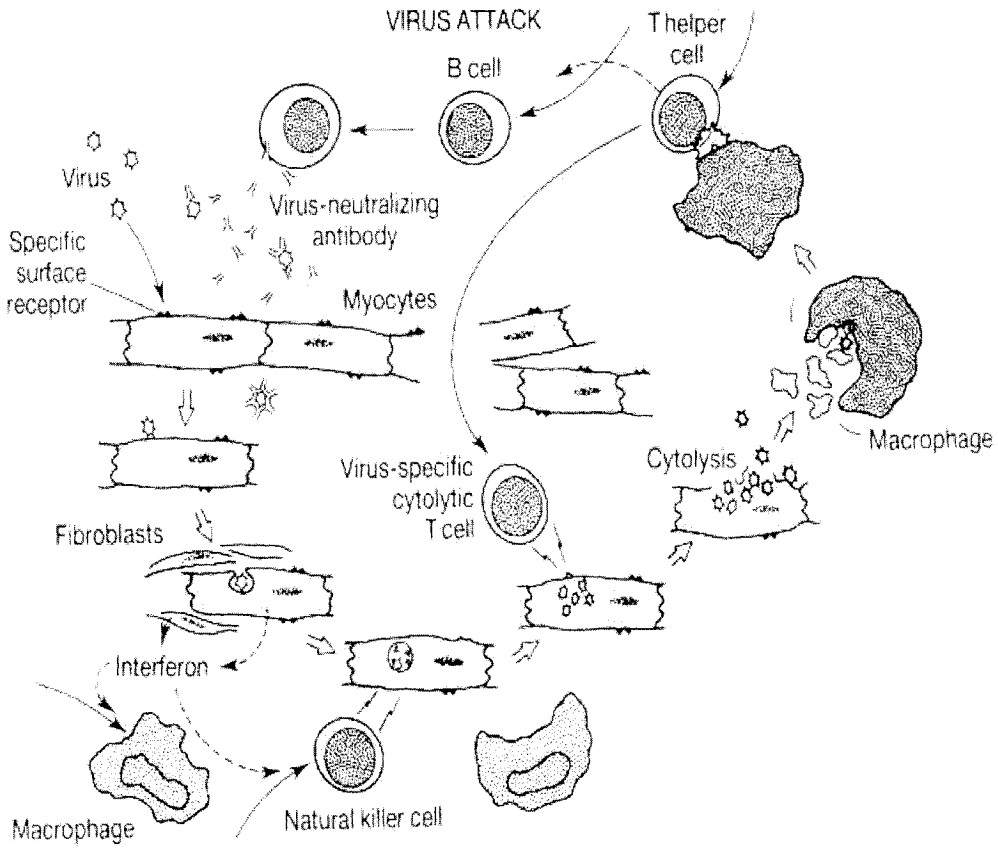
Whereas some elements of the immune response attenuate viral replication and protect the myocardium, others contribute to the development of myocardium infiltration and necrosis. The host inmmune defense mechanisms are summarized in Figure 6.

2.2.1. Innate Immunity

Innate immunity constitutes the first line of defense, providing a rapid response to microbes. The innate immune system includes epithelial barriers, phagocytic cells, natural killer cells, the complement system and cytokines. Natural killer cells (NK) and macrophage are first to arrive at the site of infection in the myocardium. Natural killer cells (NK) [Godeny et al., 1986] and macrophage [Lodge et

Figure 6: Host defense mechanisms to viral infection.

Activated macrophage may stimulate immunity response by presenting virus antigens and presenting cellular antigens engulfed during phagocytosis of virus. Both cellular and humoral immunity response occurs. Cytolytic T cells directly lyse myocytes. [Banatvala, 1993].


VIRUS ATTACK
T helper cell
B cell
Virus
Virus-neutralizing antibody
Specific surface receptor
Myocytes
Macrophage
Cytolysis
Virus-specific cytolytic T cell
Fibroblasts
Interferon
Macrophage
Natural killer cell

al., 1987] are protective of CVB3 induced myocarditis. NK cells appear to limit viral replication as evidenced in NK-cell-deficient mice treated with anti-serum against NK cells [Godeny et al., 1986]. However, there is no further contribution of NK cells to lesion pathology because they interact only with virus-infected myofibers [Kawai,1999].

2.2.2. Adaptive Immunity

Adaptive immunity is a slower, yet highly specific response mediated by T and B lymphocytes that confer effective and long-lasting protection against infection. Adaptive immunity is based on the generation of a large repertoire of antigen-recognition receptors by somatic gene rearrangement. Adaptive immunity is characterized with specificity, immunological memory and self/nonself recognition.

In severe combined immunodeficiency (SCID), mice lack both T and B cell functions and CVB3 induces severe disease [Chow et al., 1992]. In contrast, a decrease in myocardial damage was noted in severe models of depletion of T lymphocytes including nude mice [Lodge et al., 1987; Huber, 1997 and Huber et al., 1983]. More recently, using knock out technology, it was shown that the severity of myocardial damage was magnified in CD8-/- mice but attenuated in CD4-/- mice, consistent with a pathogenic role for $CD4^+$ lymphocytes [Opavsky et al., 1999]. However, double knock out mice CD4-/- CD8-/- or mice deficient for the T-cell receptor β chain, presented an increased benefit [Opavsky et al., 1999] indicating that the development of myocarditis requires virus to initiate the process and components of adaptive immunity for disease

progression, and is dependent on the predominant T-cell type available to respond to CVB3 infection.

2.2.3. Cytokines

Cytokines are soluble protein factors produced by many different cell types that mediate inflammatory and immune reactions. Cytokines are principal mediators of communication between cells of the immune system.

In humans, cytokines and cytokine receptors are important predictors of mortality with advanced heart failure [Deswal et al., 2001]. In mice, manipulation of cytokine levels can alter the development of myocarditis. The proinflammatory cytokines have important roles in the development of chronic inflammatory disease. Tumor Necrosis Factor (TNF) activates endothelial cells, recruits inflammatory cells and enhances the production of inflammatory cytokines. IFN-γ transgene expressed in the pancreas, control viral growth and are myocarditis free [Horwitz et al., 2000]. Interferon-γ can induce macrophages to express inducible nitric oxide synthase iNOS and to produce nitric oxide (NO). Although macrophages might be the major source of NO in the infected heart, cardiac myocytes and lymphocytes can also produce NO from iNOS [Zaragoza et al., 1998]. It has been shown through the literature that Coxsackievirus replicates to high titers in NOS2 $^{-/-}$ mice, that the host lacking NOS2 clears virus more slowly than the wild-type host, and that myocarditis is much more severe in infected NOS2$^{-/-}$ mice. Thus, NO protects the myocardium against damage from CVB3 infection by inhibiting viral replication [Zaragoza et al., 1998].

In contrast, it has been shown that the nitric oxide produced at a high output may act at times as part of the immune defense as an antiviral agent and may be toxic to host tissue [Robinson et al., 1999]. In addition it has been shown that mice lacking the IFN-β gene and infected with CVB3 showed increased susceptibility to infection (70% mortality), and that IFN-β is important in mediating protection against CVB3-induecd myocarditis [Deonarain et al., 2004]. In addition, in 2000 Wessely et al. showed that IFN type I but not IFN type II signaling is essential for the prevention of early death due to CVB3 infection [Wessely et al., 2000].

3. Host Factors that Influence Susceptibility to Viral Myocarditis

The virulence of murine viral infection is increased by malnutrition, exercise, sex and sex hormones and age [Kawai, 1999]. More importantly, it is widely recognized that susceptibility of the host to viral infection is under genetic control [Brinton et al., 1984]. However, it was shown through the literature that susceptibility to viral myocarditis is controlled by both background (nonmajor histocompatibility complex) and H-2 (major histocompatibility complex) genes [Gaunett et al., 1984; Herskowitz et al., 1985; Herskowitz et al., 1987; Kawai 1999 and Martino et al., 1994].

Herskowitz et al., in 1987 showed, during the seven days after CVB3 infection and using six different strains of mice (including three A congenic mice and two B congenic mice), that there were dramatic difference among strains with respect to prevalence and severity of myocarditis. They found that the major histocompatibility complex may control the early response to coxsackievirus B3 myocarditis, but the A background

genome appears to control the characteristic of the late pathogenic changes seen in all three of the A H-2 congenics strains that they used.

Gaunett et al., in 1984, suggested that the major virus susceptibility gene for CVB3 is not present within the H-2 locus. Their selection of mice with specific H-2 genotypes did not reveal an all or none response or show major quantifiable changes in the response to CVB3 infection.

In this project we wanted to study in more detail the acute phase of the disease. In fact, among the many challenges facing clinicians treating patients with acute myocarditis or idiopathic dilated cardiomyopathy IDC are the detection of early disease and the identification of the predominant mechanism of left ventricular dysfunction. In addition, in this study we wanted to identify more than one candidate gene, not only the effect of the major histocompatibility complex genes after CVB3 induced acute myocarditis but also the effect of coxsackievirus B3 on background genes including dystrophin.

In our research for genetic determinants for susceptibility to cardiovirulent CVB3 in the acute phase of the disease, quantifiable pathological parameters-survival, body weight loss, viral load and quantification of damaged myocytes determined by histopathological analysis –were identified for linkage analysis.

3.1 The major histocompatibility complex (H-2)

H-2 complex is the major histocompatibility complex (MHC) of the mouse. In humans MHC complex is called Human leucocyte antigens HLA. Together they are called the major histocompatibility complex (MHC) because the corresponding antigens are major

histocompatibility antigens. The MHC molecules were originally recognized for their role in triggering T cell responses that caused the rejection of transplanted tissue between inbred strains of mice. It is now known that the physiologic function of MHC molecules is the presentation of peptides to T cells. H-2 is a complex of genetic loci, located on chromosome 17; they span approximately 2 cM of genetic length. MHC genes encode for three major classes of MHC molecules:

- Class I: present in all nucleated cells

  Function: presentation of altered self-cells

- Class II: present only on antigen presenting cells APC

  Function: presentation of processed peptides to lymphocytes T helper

- Class III: secreted molecules associated with the immune response such as complement components and tumor necrosis factor TNF

Haplotypes of the H-2 complex are determined by the combination of alleles of the class I (K,D,L,Qa,Tla) genes, class II (Aβ, Aα, Eβ, Eα) genes, and class III (S) genes illustrated in Table 2 (e.g., H-2^b = K^b A^b E^b S^b D^b L^b Qa^a and Tla^b). Class I and class II genes of the H-2 complex are highly polymorphic (with many alleles at each locus); the class III genes are much less. The occurrence among inbred strains of several alleles at individual H-2 loci is often cited as evidence for polymorphism of these loci [Klein et al., 1983]. Among different strains of mice, the following numbers of major alleles at H-2 loci have been found; 11 different alleles at K, 10 at D, 11 at Aβ, 11 at Eβ, 11 at Aα, and 11 at Eα (see Table 2).

In this study, the influence of the H-2 haplotypes on myocarditis was studied by comparison of the disease of the strains of mice sharing the same background but not the

Table 2: Alleles of loci in the H-2 haplotype.

[Jackson Laboratory website: http://jaxmice.jax.org/library/notes/433d.html].

The highlighted H-2 haplotypes (a,b,d,k and q) were used in this project.

	H-2 loci										
	Class I	Class II				Class III	Class I				
	K	A_β	A_α	E_β	E_α	S	D	L	Qa-2	Tla	Qa-1
***H-2* haplotype**											
a	**k**	**k**	**k**	**k**	**k**	**d**	**d**	**d**	**a**	**a**	**a**
b	**b**	**b**	**b**	**b**	**b**	**b**	**b**	**b**	**a**	**b**	**b**
d	**d**	**d**	**d**	**d**	**d**	**d**	**d**	**d**	**b**	**d**	**b**
f	f	f	f	f	f	f	f	f	b	d	b
j	j	j	j	j	j	j	b	b	a	b	b
k	**k**	**k**	**k**	**k**	**k**	**k**	**k**	**k**	**b**	**b**	**b**
m	k	k	k	k	k	k	q	q	a	a	a
p	p	p	p	p	p	p	p	p	b	e	a
q	**q**	**q**	**q**	**q**	**q**	**q**	**q**	**q**	**a**	**b**	**b**
r	r	r	r	r	r	r	r	r	b	b	b
s	s	s	s	s	s	s	s	s	a	b	b
u	u	u	u	u	u	u	d	d	a	a	a
v	v	v	v	v	v	v	v	v	a	b	b
z	u	u	u	u	u	z	z	z	b	b	b

same H-2 haplotype. In contrast, the influence of non-H-2 genes was analyzed by comparison of phenotype of strains of mice sharing the same H-2 haplotype.

3.2. Dystrophin

Knowlton and co-workers showed that dystrophin deficiency increases host susceptibility to coxsackieviral infection. In fact, in dystrophin-deficient mdx mice (having mutation similar to the one existing in humans with Duchenne Muscular Dystrophy) infected with coxsackievirus B3, there were more efficient release of the virus from infected myocytes and subsequent infection of adjacent myocytes than in infected wild type mice [Xiong et al., 2002]. They also indicated that this difference appears to be a result of more efficient release of the virus from dystrophin-deficient myocytes.

In 1999, they demonstrated that in vitro, purified coxsackievirus protease 2A cleaves dystrophin and that dystrophin is cleaved during coxsackievirus infection in infected mouse hearts, leading to impared dystrophin function. In vivo, dystrophin and dystrophin associated glycoproteins α-sarcoglycan and β-dystroglycan are morphologically disrupted in infected myocytes [Badorff et al., 1999].

3.3. The Tyrosine Kinase p56lck

Liu et al., in 2000, showed that the sarcoma family kinase p56lck is an essential host factor that controls replication and pathogenecity of CVB3 in vivo. CVB3 virus was undetectable in p56lck-deficient mice by day 10 post infection (p.i), whereas heterozygous mice had a detectable amount of virus up to day 42 pi. Chimeric re-introduction of p56lck+/+ T-cells into knockout mice restored CVB3-induced pathogenecity and infectious virus to levels found in control littermates indicating that p56lck expression is essential for T-cell activity as well as mediating viral pathogenecity of CVB3 in myocarditis. This sarcoma family kinase is a key regulator of CVB3 pathogenecity and multiplication of it rescues the phenotype and prevents progression of myocarditis to DCM.

Another important candidate is the extracellular signal-regulated kinases 1 and 2 (ERK-1/2) which can influence cell function downstream of Lck. Enhanced ERK-1/2 activation was seen in the hearts of susceptible A/J mice to CVB3 infection. Iin contrast, less ERK-1/2 activation was found in the hearts of resistant C57BL/6 mice after CVB3 infection [Opavsky et al., 2002]. Thus, Liu and co-workers concluded that the ERK-1/2 response might contribute to host susceptibility after CVB3 mediated myocarditis.

4. Mouse Model of Coxsackievirus Infection

Infection of mice with CVB3 mimics virus-induced disease in humans. Development of myocarditis takes three distinct phases [Kawai 1999; McManus et al., 1993 and Chow et al., 1991]:

1. Phase of peak viremia, occurring 2-4 days post infection. This phase is characterized by direct virus induced myocardial injury and death of infected cardiomyocytes.
2. Phase of acute myocarditis, occurring 4-15 days post infection. This phase is the phase of interest for this study (6 days post-infection). It is characterized by the presence of inflammatory infiltration of macrophages [Lodge et al., 1987], and Natural Killer cells (NK) [Godeny et al., 1986] by day 4, followed by the presence of $CD8^+$ T lymphocytes that predominate at day 6 and are then exceeded in number by $CD4^+$ T lymphocytes [Klingel et al., 1992].
3. Phase of chronic myocarditis, occurring 15-90 days post infection. This phase is characterized by the absence of cultivable virus, persistent myocardial damage and importantly, a process of tissue reparation and remodeling of the remaining myocytes, particularly in the left ventricle. This pathology is similar to human dilated cardiomyopathy [Kawai 1999], [Martino et al., 1994].

Inbred strains:

An inbred strain is one that has been maintained by sibling (sister x brother) mating for 20 or more consecutive generations. At 20 generations, on average at least 98.6% of the loci in each mouse are homozygous. Each inbred strain is also isogenic (genetically identical) because all individuals trace back to a common ancestor in the twentieth or a subsequent generation. Because of the availability of a large number of genetically defined strains, the mouse lends itself to the study of the host genetic control of susceptibility to CVB3 infection. In addition, inbred strains of mice differ markedly in susceptibility to CVB3. It has been shown through the literature that strains of the C57BL background are resistant to CVB3 infection. They rapidly eliminate cardiac viral

load and present mild transient myocarditis. The strains of DBA background are intermediate strains. They eliminate viral load despite a phase of acute myocarditis. The highly susceptible strains are strains of the A background that sustain high viral loads for longer periods and progress into the chronic phase [Gauntt et al., 1984; Herskowitz et al., 1985 and Herskowitz et al., 1987].

In this study, three inbred strains A/J (H-2^a), C57BL/10 (H-2^b), 129/sv (H-2^b) mice and two congenic A.BY (H-2^b) and B10.A (H-2^a) mice were used to identify differential parameters of the disease and their influence on the H-2 haplotype. The influence of non-MHC genes was demonstrated by comparison of phenotype of strains of mice sharing the same H-2. In contrast to the influence of H-2 genes on myocarditis was demonstrated by comparison of the disease of the strains of mice sharing the same background but not the same H-2 haplotype (H2 congenic strains).

Congenic strains:

By definition a congenic strain is produced by placing an H-2 gene from various genetics sources onto a standard inbred strain background and repeating backcrosses to an inbred (background) strain, with selection for a particular marker from the donor strain. A strain developed by this method is regarded as congenic when a minimum of ten backcross generations to the background strain has been made. Congenic mice are genetically identical to the receiver parent except for the H-2 haplotype.

Because there is a naturally occurring variability among the following inbred strains, including A/J (H-2^a), C57BL10 (H-2^b), 129 (H-2^b), Balb/c (H-2^d), DBA/2 (H-2^d), C3H (H-2^k), Balb/k (H-2^k), MaMy (H-2^k), AKR (H-2^k) and FVB (H-2^q), these were used in this project to study CVB3 induced phenotypes.

Phenotypic data on commonly used and genetically diverse inbred mouse strains could sets up the platform for a detailed analysis of the genetic basis of susceptibility to CVB3 (i.e. the identification of the chromosomal regions regulating the phenotypic traits).

OBJECTIVE

The objective of this study is to:

1. Characterize phenotypic traits in different strains of mice after CVB3-induced acute myocarditis.
2. Test the hypothesis that susceptibility, during the acute CVB3 infection, is under polygenic control including H-2 as well as the non H-2 genes.

MATERIALS AND METHODS

1. MICE

Two inbred strains of mice A/J (H-2^a), C57BL10 (H-2^b) and 2 congenic strains of mice B10.A (H-2^a) and A.BY (H-2^b) were purchased from the Jackson Laboratory. The following strains were purchased from Charles River Laboratory: 129/sv (H-2^b), Balb/c (H-2^d), DBA/2 (H-2^d), C3H (H-2^k), Balb/k (H-2^k), MaMy (H-2^k), AKR (H-2^k) and FVB (H-2^q). Mice were subsequently maintained at the animal facility at the University of Ottawa in agreement with guidelines and regulations of the Canadian Council of Animal Care. Mice were 7 to 9 weeks of age when received at the animal facility at the University of Ottawa and 8 to 10 weeks of age at the time of infection. They were injected intraperitoneally with 5×10^3 plaque forming units (PFU) of CVB3-CG adjusted for the weight of the mice and euthanized by CO2 on day 6 post infection. Animals were weighed daily and observed for changes in fur characteristics, level of activity and edema. 1% BW of 1% Evans Blue Dye (EBD), purchased from Sigma, was injected intraperitoneally to our mice 16 to 24 hours before sampling.

2. VIRUS

Stock coxsackievirus B3-CG was generously provided by Dr. Peter Liu (Heart and Stroke Center for Cardiovascular Research, University of Toronto) and stored at -80°C. Virus was grown in HeLa cells and titered by plaque assay.

3. TISSUES ACQUISITION

The weight of each mouse was recorded daily. Mice were sacrificed at day 6 post-infection. Hearts were removed, weighed and washed in Phosphate Buffered Saline (PBS). Sagittal sections of each heart were made. The anterior section of the heart was kept in PBS and on ice, and then was embedded on O.C.T. compound (Tissue-Tek, Sakura Finetek USA, Cat. N. 4583) for histopathological analysis. The posterior section of the heart was cut transversally, the top part was weighed and placed in 2 volumes of 1 X MEM (Gibco/BRL) for viral titer assay and the bottom part also was weighed and snap frozen in liquid nitrogen for extraction of RNA.

4. CELL LINES AND VIRAL TITERS

a. HeLa Cell Culture

HeLa cells were provided generously by Dr Dimock (BMI department, University of Ottawa) and were grown in Minimum Essential Media (MEM) containing 1% of penicillin/streptomycin and 10% of Fetal Bovine Serum (FBS). Cells were incubated

until more than 90% confluence was reached, harvested with 1% trypsin, centrifuged, resuspended in media, counted using a hemocytometer and seeded into a new flask(s).

b. Heart Viral Load

The heart tissues were weighed, homogenized in MEM medium and then subjected to 3 cycles of freezing and thawing to release the virus CVB3 from the homogenates. Cellular debris was removed by centrifugation and the titer of the virus in the supernatant was determined by standard plaque-formation assays on HeLa monolayers.

5. EXTRACTION OF TOTAL RNA

Total RNA was extracted from heart tissues using Trizol (GIBCO-INVITROGEN, Burlington, ON, and Cat.N.15596). Briefly, frozen heart samples (around 25 mg) were homogenized in 500 µl of Trizol, incubated for 5 min at room temperature (RT) followed by the addition of 0.1 ml of chloroform per 500 µl of Trizol Reagent. The samples were shaken vigorously and incubated at RT for 3 min. Samples were then centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was removed into a fresh tube and 0.25 ml of isopropyl alcohol per 500 µl of Trizol used was added. The samples were incubated at RT for 10 min then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed once with 500 µ of 70% ethanol per 500 µl of Trizol used. The samples were vortexed and then

centrifuged at 7,500 x g for 5 min at 4°C. The supernatant was discarded and the RNA pellet was allowed to dry briefly and then resuspended in 20µl of DEPC (Diethyl Pyrocarbonate) water (RNase-free water) (200µl of DEPC in 1000ml of ddH_2O (double distilled water)). The concentration of the total RNA yield was determined by spectrophotometry at A_{260}. RNA samples were run on 1% agarose MOPS ((3-[N-Morpholino]propanesulphonic acid) formaldehyde gels (41.5 ml DEPC, 2,7 ml formaldehyde, and 5 ml 10 X MOPS) to check the degradation and equal loading.

6. GENE EXPRESSION BY RT-PCR

Gene expression was detected by semi-quantitative RT-PCR. Briefly, 1 µg of total RNA was reverse transcribed using random primers and superscript II reverse transcriptase according to the manufacturer's protocols (Life Technologies). Heart cDNA of CVB3-infected mice were then used as a template for PCR. Each 25µl PCR reaction contained 10µMof each primer pair, 2.5 mM of each dNTP (regular) and Taq polymerase. Samples were analyzed in a thermal cycler as follows: 95°C/30 s, 55°C/30s, 72°C/1 min (for some primers, the annealing temperature is not 55°C as listed in the Table 3). Gene specific oligonucleotides for the PCR step were (Primer sequences, used for gene expression analysis on coxsackievirus B3 infection, were listed in Table 3). The final PCR products were compared using ethidium bromide-stained agarose gel electrophoresis.

Table 3: Primer sequences used for gene expression analysis of several candidates genes on coxsackievirus infected mice.

CAR: Coxasakie and adenovirus receptor
DAF: Decay accelerating factor
DES: Desmin
DMD: Duchenne muscular dystrophy
IFN: Interferon
TNF: Tumor necrosis factor
iNOS: Inducible nitric oxide synthase

	PRIMER NAME	LENGTH	SEQUENCES	ANNEALING TEMP	BAND SIZE (bp)
Genes coding for viral receptors	CAR-F	20	5-TACGCTTACAAGACCGATGG-3	55	420
	CAR-R	20	5-ATATGGGACCTTGTCACGG-3	55	420
	DAF-F	20	5-GGAGAGCCTAACACAGGTGG-3	59	125
	DAF-R	21	5-TCTTCGTAACTCTTCGTTGGG-3	57	125
Innate immunity genes	IFNg-F	20	5-ACGCTACACACTGATCTTG-3	55	520
	IFNg-R	20	5-TTCAAATAGTGCTGGCAGAA-3	55	520
	TNFa-F	20	5-GAGCACAGAAAGCATGATCG-3	55	620
	TNFa-R	20	5-CTTCTCCAGCTGGAAGACTC-3	55	620
Cytoskeletal protein genes mutated in cardiomyopathies	DES-F	20	5-TGATGAGGCAGATGAGGAG-3	60	246
	DES-R	20	5-TGAGAGCTGAGAAGGTCTGG-3	60	246
	DMD-F	22	5-CAGCTTTCACACGATGATACTG-3	55	800
	DMD-R	20	5-CTCTCTCATTGGCTTTCCGG-3	55	800

7. HISTOPATHOLOGY

Heart tissues were embedded in OCT compound (Tissue-Tek, Sakura Finetek USA, Cat. N. 4583) and snap frozen in isopentane chilled in liquid nitrogen. Frozen 10 μm sections were cut at -20°C by a cryostat.

- Unstained frozen heart sections were viewed with a fluorescent microscope Olympus BX50. The total area of each cryosection was imaged using an Axio Cam MRm, Zeiss monochrome camera. Evans Blue Dye-positive cells (red fluorescence) were visualized by excitation at 510-550 nm with a barrier filter of 590 nm.
- For H&E staining: Frozen sections were dipped in alcohol for 2 min, washed in running water for 2 min, stained in Hematoxylin (Fisher Scientific, Cat. N. 28-601-02) for 30 s, washed in running water for 2min, washed in 0.2% acid alcohol for 10 s, washed in running water for 2 min, stained blue (1 drop) with the Lithium carbonate (Fisher Scientific, Cat. N. L-119), washed with running water for 2 min, stained with Eosin (Fisher Scientific, Cat. N. 45380) for 10 s, washed several times in 100% alcohol (30 dips in each) and cleared in 4X Toluene (VWR, Cat. N. 07353) for 1 min. The H&E-stained heart sections were viewed with a transmitted light microscopy Olympus BH-2 and a neutral density filter LBD-2N. All the area of each cryosection was imaged using a Pixelink camera (Serial number: 50161).
- For Immunocytochemistry: Unstained frozen sections were permeabilized with 0.3% Triton X-100 in TBS. Dystrophin was visualized by a mouse monoclonal antibody recognizing the mid-rod domain of dystrophin (Dy4/6D3, Novocastra, Newcastle, UK, Cat. N. NCL-DYS1). To localize this mouse primary antibody on mouse

tissues, Vector Fluorecsein M.O.M. immunodetection Kit (Vector laboratories, Cat. N. FMK-2201) which contains Fluorescein Avidin (green fluorescence) was used. To identify coxsackievirus-infected cells, anti-coxsackievirus B3 monoclonal antibody (Chemicon International, Cat. N. 3306) was used and visualized using Alexa Fluor 350 goat anti-mouse (Molecular Probes Cat. N. A-11045) (blue fluorescence). All antibodies were used at 1:200 dilutions. Sections were viewed using the same fluorescent microscope and all the area of each cryosection was imaged using the same monochrome camera as indicated above.

8. QUANTIFICATION OF SARCOLEMMAL DISRUPTION

Digitized images were acquired using a monochrome camera as described above (section 6, first bullet). The images were imported into an image-analysis system "Image Pro-Plus", version 5, (Media Cybernetics, Silver Spring, Maryland). For analysis all images were viewed and captured at 80X magnification.

Gray scale pixel values represent a level of grayness or brightness ranging from completely black (background) to completely white (damaged myocytes). A pixel with a value of 0 is completely black and pixel with a value of 255 is completely white. In total, there are 256 shades of grey, which is more than what the human eye can differentiate.

Thresholding was used to segment an image in order to reduce it to two intensity levels. Initially, the grayscale threshold value was obtained by taking the mean pixel intensity obtained from the field, and only objects having a value above this threshold were

counted. Since the animals were injected with EBD, there is a naturally occurring variability of the intensity of EBD from one heart (section) to another. We assumed that objects having values greater than the mean pixel intensity plus 30 were counted and which represent damaged myocytes.

The area of Evans Blue Dye-positive cells was quantified and the extent of sarcolemmal disruption was expressed as the percentage of the area of damaged myocytes (white fluorescence) compared with the total area of the heart in the microscope field.

9. QUANTIFICATION OF MYOCARDITIS IN H&E STAINED SECTIONS

Myocarditis was identified by the presence of an inflammatory infiltrate associated with injury to cardiomyocytes. True color, 100 X magnification, images from H&E-stained heart sections were captured using Pixelink camera. Automatic quantification of myocarditis was done using the image-analysis system "Image Pro-Plus". The inflammatory infiltrate and associated injured cardiomyocytes (stained Blue with the EBD) were identified by selecting their color range. Threshold value was obtained by taking the mean pixel intensity within the selected color range obtained from the field. Quantification of myocarditis was expressed as the percentage of the area of inflammation and associated injured cardiomyocytes compared with the total area of the heart in the microscope field.

RESULTS

I. IDENTIFICATION OF PARAMETERS ASSOCIATED WITH SUSCEPTIBILITY AND RESISTANCE DURING THE ACUTE PHASE OF CVB3 INFECTION

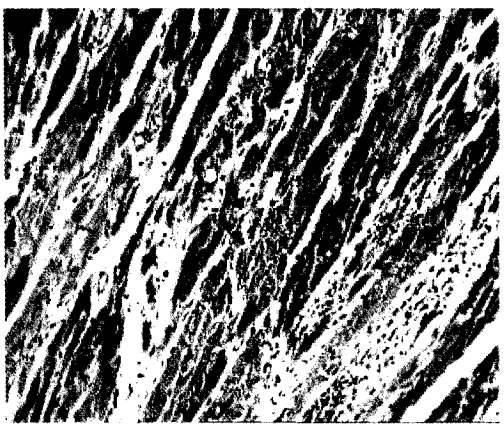
We started to establish the experimental model of CVB3 infection in our laboratory by optimizing the following variables: strains and age of mice, the viral dose and the time point of collecting tissues. Phenotypic traits were characterized when myocarditis was well established to quantify the severity of CVB3-induced disease in the acute phase of the infection. For that, we started to study the histopathology of coxsackievirus B3 myocarditis in A (known to be susceptible to CVB3-induced myocarditis) and C57BL (known to be resistant to CVB3-induced myocarditis) mice at days 4, 6 and 8 after CVB3 infection. At day 4 after CVB3 infection, no gross abnormalities were present in the two chosen strains of mice (data not shown). However, at day 6 after CVB3 infection, presentation of histopathology was dramatically different between A and C57BL mice as seen in Figure 7. Myocarditis was evaluated based on the presence of an inflammatory infiltrate associated with injury to cardiomyocytes. As shown in Figure 7 a, discrete inflammatory infiltrate is observed in the H&E stained myocardial section of C57BL infected mice 6 days post-infection. In contrast, as shown in Figure 7 b, myocardium from A infected mice show intense interstitial infiltrate with disruption of

Figure 7: Heart histopathology in H&E stained myocardial sections from C57BL and A strains of mice at day 6 after CVB3 infection.

a. Discrete inflammatory infiltrate in the myocardium from C57BL infected mice 6 days after CVB3 infection.

b. Myocardium from A mice showing intense interstitial infiltrate with disruption of myocyte architecture.
(Magnification for each case (x120)

a) Hematoxylin-eosin stained myocardial section from the heart of C57BL at day 6 p.i.



b) Hematoxylin-eosin stained myocardial section from the heart of A at day 6 p.i.

myocyte architecture. By day 8 post-infection, the susceptible A mice died and we couldn't look at the histopathology. Myocarditis was well established only at day 6 post-CVB3 infection. That's the reason for characterizing the early phase of CVB3-induced myocarditis during that specific time point. At day six post CVB3 infection, the five different phenotypic traits, including biological (survival and body weight loss) and pathological (quantification of myocarditis, quantification of sarcolemmal disruption and viral load) parameters, were characterized in five strains of mice (three inbred strains A/J (H-2^a), C57BL/10 (H-2^b), 129 (H-2^b) mice and two congenic A.BY (H-2^b) and B10.A (H-2^a) mice) to look for the role of the host's genetic makeup in the infectious process.

II. EFFECT OF H-2 HAPLOTYPE ON CVB3-INDUCED MYOCARDITIS

Previous studies suggested that host susceptibility to CVB3-induced myocarditis is controlled by multiple genes [Wolfgram et al., 1986]. To analyze the effect of this genetic regulation on the expression of CVB3-induced myocarditis we chose five strains of mice for detailed analysis. These strains were selected to provide examples of strains that differ both in genes within the H-2 complex and in the background (non major histocompatibility complex) genes. Three inbred strains A/J (H-2^a), C57BL/10 (H-2^b), 129 (H-2^b) mice and two congenic A.BY (H-2^b) and B10.A (H-2^a) mice were infected with 5.10^3 PFU of the cardiovirulent CVB3. The A.BY H-2 congenic share the same background genes as the A/J strain, but differ in their major histocompatibility complex

genome ((H-2 ^{b}) instead of (H-2 ^{a})). The congenic B10.A also share the same background genes as C57BL/10 strain but differ in the haplotype ((H-2 ^{a}) instead of (H-2 ^{b})). Results are assessed in terms of survival, body weight loss, heart viral load, quantification of sarcolemmal disruption and quantification of myocarditis.

1. Survival

Only 20% of A.BY, 50% of A/J and 87.5% of B10.A survived during the six day study period. In contrast all 129 and C57BL/10 mice survived by day 6 (Figure 8). None of the control animals died.

2. Body Weight (BW) Loss

We recorded the daily BW for each mouse during the 6 days of infection. We observed that the resistant mice keep their BW or slightly decrease; however some resistant mice gain weight while the susceptible mice lose more than 12% (Figure 9).

3. Identification and Quantification of Myocarditis in H&E Stained Sections

Myocarditis was evaluated based on the presence of an inflammatory infiltrate associated with injury to cardiomyocytes. Histologically, the hearts of non-infected mice showed neither cellular infiltration nor myocardial cell necrosis (Figure 10 a). In A.BY and A/J myocardium, necrotic foci appeared in the myocardium with

Figure 8: Survival curves for the five strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection.

Chi-square test indicated that the five strains of mice differ at a p value < 0.0001 (X^2 statistic is 98.44).

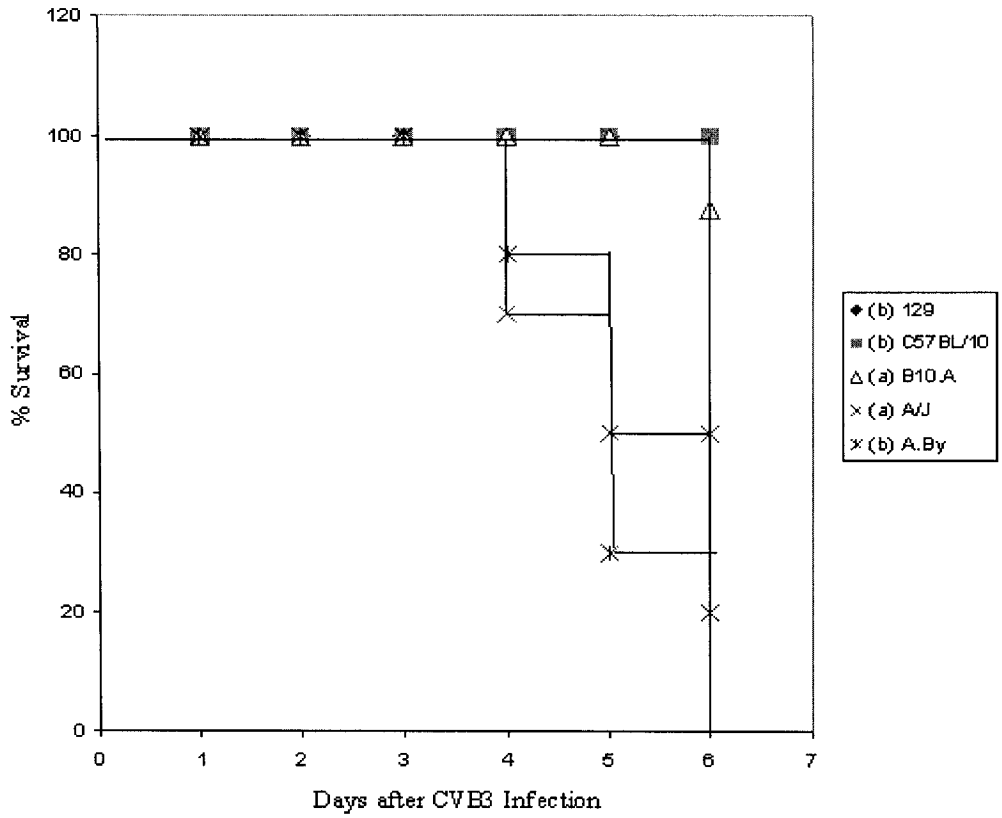

120
100
80
60
40
20
0
% Survival
0
1
2
3
4
5
6
7
Days after CVB3 Infection
(b) 129
(b) C57BL/10
(a) B10.A
(a) A/J
(b) A.By

Figure 9: Body weight loss for the five strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection.

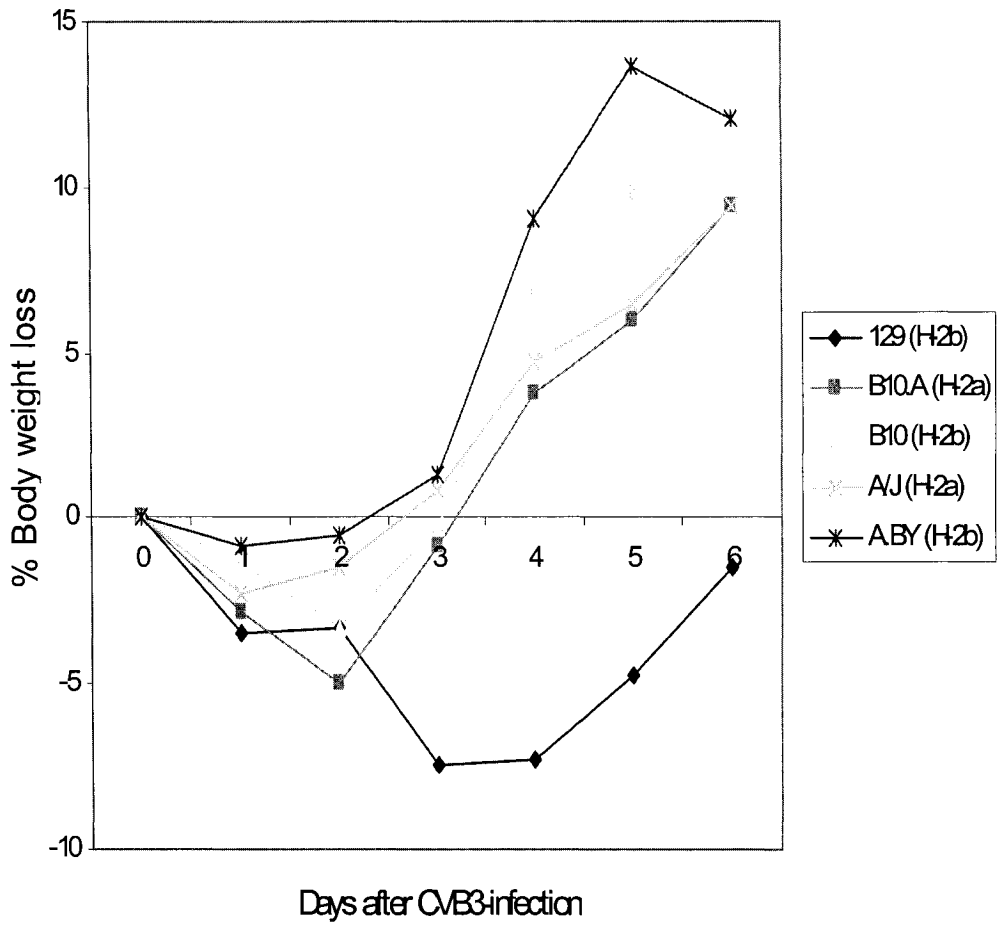
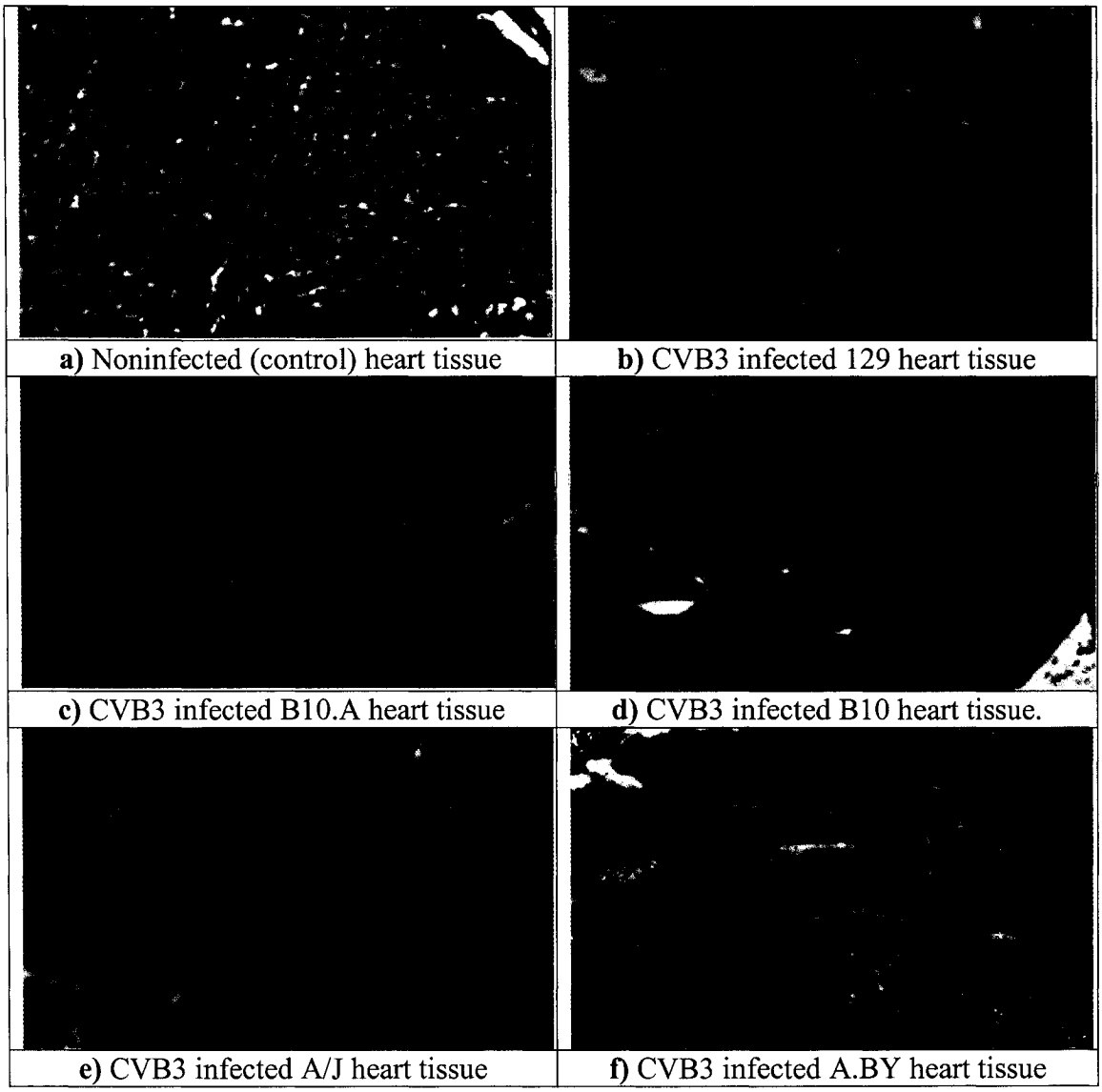

15
10
5
0
-5
-10
% Body weight loss
0
1
2
3
4
5
6
Days after CVB3-infection
129 (H-2b)
B10.A (H-2a)
B10 (H-2b)
A/J (H-2a)
A.BY (H-2b)

Figure 10: Hearts histopathology of the five strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection. Histopathology was evaluated using H&E staining six days after infection. Original magnifications, x100.

a. Noninfected control heart tissue.
b. CVB3 infected 129 heart tissue
c. CVB3 infected B10.A heart tissue
d. CVB3 infected B10 heart tissue.
e. CVB3 infected A/J heart tissue
f. CVB3 infected A.BY heart tissue



a) Noninfected (control) heart tissue

b) CVB3 infected 129 heart tissue

c) CVB3 infected B10.A heart tissue

d) CVB3 infected B10 heart tissue.

e) CVB3 infected A/J heart tissue

f) CVB3 infected A.BY heart tissue

interstitial edema and the presence of an interstitial mononuclear cell infiltrate (Figure 10 b). In B10 and B10.A myocardium, we noticed the presence of only some inflammatory cells (Figure 10 c), whereas, the 129 myocardium appears almost normal (Figure 10 d).

Semi-quantitative analysis showed that the A.BY and A/J mice showed active myocarditis, the B10 and B10.A mice showed mild infiltration and the 129 mice showed almost neither inflammation nor necrosis.

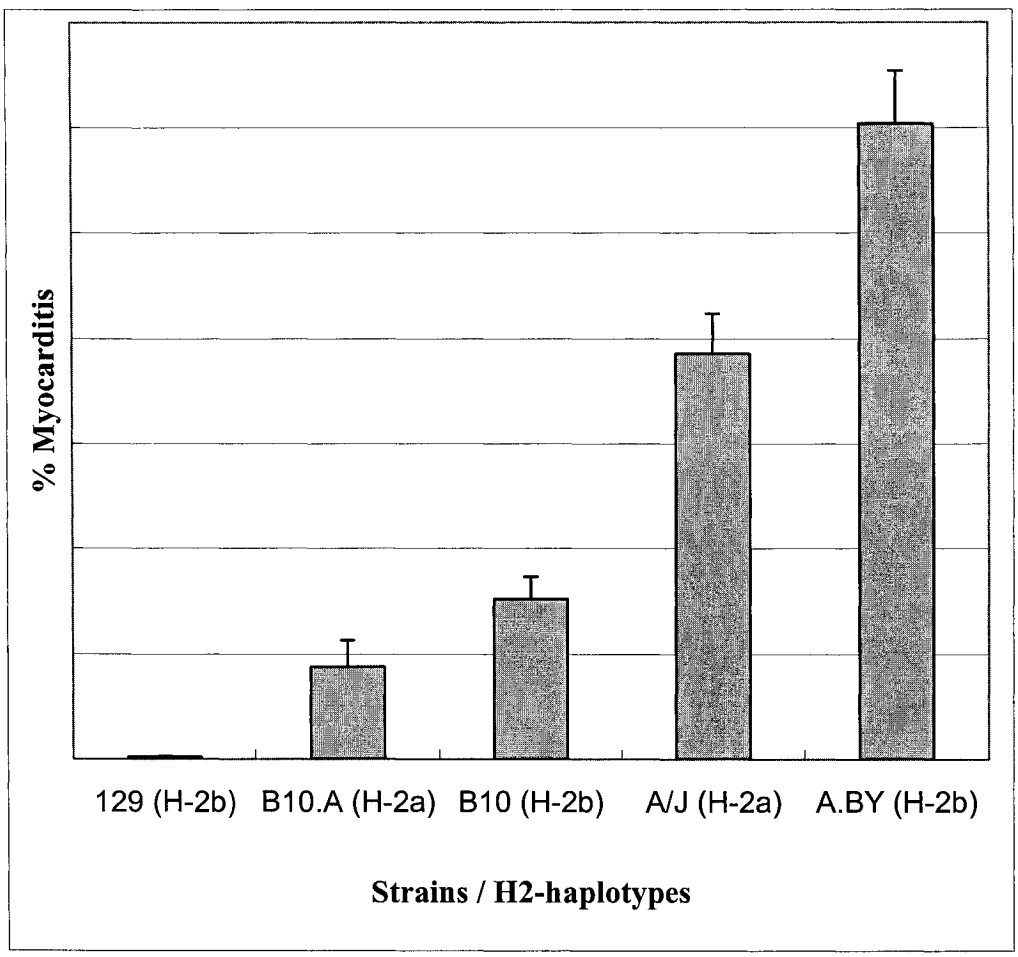
To quantify myocarditis from H&E stained sections, Image Pro-Plus analysis system was used, and myocarditis was evaluated based on the presence of interstitial inflammation and damaged myocytes. As a result, A/J and A.BY showed extensive myocyte interstitial inflammation affecting 2 to 3% of the myocardium. In contrast, although some inflammatory foci are observed in the resistant mice: C57BL/10, B10.A, lesion size were significantly smaller and less than 0.7% of the myocardium. 129 mice showed only 0.008% of myocarditis (Figure 11).

4. Analysis of Disruption of Sarcolemmal Membrane Integrity

Viral infection of cardiomyocytes disrupts the integrity of the sarcolemmal membrane [Badorff et al., 1999]. To assess the sarcolemmal membrane integrity (in vivo) in the selected strains of mice: A/J, C57BL/10, 129, A.BY and B.10, a fluorescent dye EBD was used. EBD is a large molecule that is selectively taken up by cells with disrupted sarcolemmal disruption [Badorff et al., 1999]. The use of EBD as an in vivo marker of

Figure 11: Quantification of myocarditis in H&E stained sections in five different strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection. Data represent mean values (± SEM: standard error of the mean).


% Myocarditis
129 (H-2b)
B10.A (H-2a)
B10 (H-2b)
A/J (H-2a)
A.BY (H-2b)
Strains / H2-haplotypes

plasma membrane permeability of cardiomyocytes facilitates the investigation of the effect of the pathology induced by CVB3.

Cardiac tissue was examined from all injected animals. Uninfected control mice never showed EBD-positive cardiomyocytes (data not shown). In contrast, all infected mice showed EBD-positive lesions (red fluorescence) in the myocardium (Figure 12).The area of the EBD uptake into cardiomyocytes varied from one strains to another. We saw markedly greater uptake of EBD in infected A.BY and A/J mice as compared with infected B10 and B10.A. The 129 mice showed very small EBD-positive area (Figure 13).

As determined by the analysis of variance (ANOVA), the five strains of mice differ at a p value < 0.0001. Moreover, Bonferroni testing indicated that the five strains of mice could be divided into three groups: the A.BY and A/J strains which showed the greatest percentage around 20% of EBD-positive area; the C57BL/10 and B10.A mice which showed around 9% of EBD-positive area; and the 129 mice which showed only 1.44 % of EBD–positive area (Figure 13).

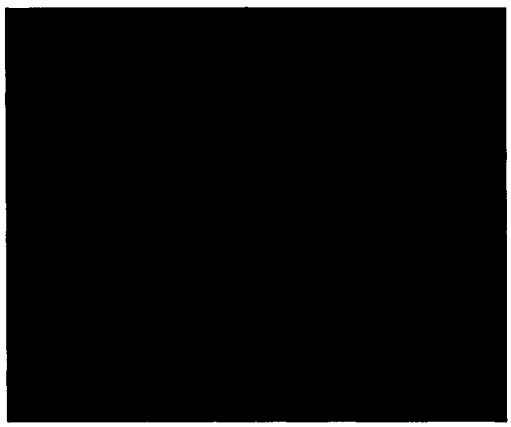
H&E staining of infected myocardium showed that the Evans Blue Dye-positive cardiomyocytes (see arrow in Figure 10 f) were generally located close to areas of active necrosis; they were normal in size and shape and almost free of cellular inflammation. These results suggest that the damaged cardiomyocytes eventually die.

5. Analysis of Heart Viral load

To assess the heart content of infectious CVB3, viral load was measured in the hearts of the infected five strains of mice using plaque assays in HeLa cells. The analysis of

Figure 12: Evans Blue Dye uptake in an A.BY infected mouse.

A.BY mouse was inoculated with 5.10^{3} PFU of CVB3 and sacrificed at day 6 post-infection (This Figure is the same as Figure 20 a). There were many cardiomyocytes that stained positive for Evans Blue Dye (EBD) in A.BY infected mouse. The same result was seen in all infected mice. The only difference is the variation of the percentage of the EBD-positive area between the susceptible and the resistant strain.

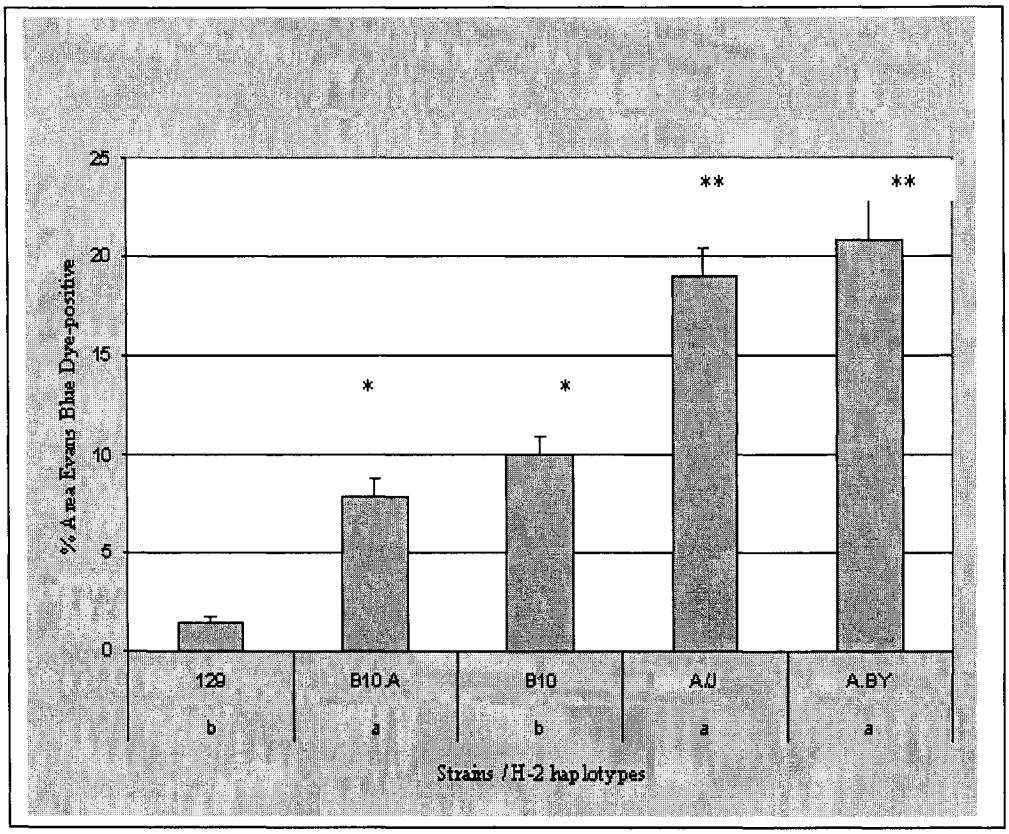


Evans dye uptake (red fluorescence)

Figure 13: Quantification of sarcolemmal disruption in infected heart of five strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection. Data represent mean values (± SEM: standard error of the mean).

* Student's *t* test differences: P>0.05.
** Student's *t* test differences: P>0.05.


25
20
15
10
5
0
% Area Evans Blue Dye-positive
**
**
*
*
129
B10.A
B10
A.J
A.BY
b
a
b
a
a
Strains / H-2 haplotypes

variance (ANOVA) indicated that there were no significant quantitative differences among the five strains of mice in the CVB3 load of their heart tissue. The mean $\log_{10}$ CVB3 PFU/mg of tissue ranged from 2.9 to 4.4 with the SEM ranging from 0.2 to 0.4 (Figure 14). There were no significant strains differences in the ability of CVB3 to infect and replicate in the heart tissues.

6. Conclusion

All these results indicate that the A/J (H-2^a) and A.BY (H-2^b) strains displayed the greatest susceptibility to CVB3 as seen in survival, BW loss, heart viral load, % of myocarditis and the % of EBD-positive cells. In comparison C57BL/10 (H-2^b) and B10.A (H-2^a) had no/low mortality, low heart viral load and low percentage of myocarditis as well as low % of EBD-positive cells. The result indicates also that 129 mice displayed the greatest resistance to CVB3 (see Table 4). Differences in survival, BW loss, quantification of myocarditis and quantification of sarcolemmal disruption were found by comparing three set of mice A/J(H-2^a) and B10.A (H-2^a); C57BL/10 (H-2^b) and A.BY(H-2^b); and 129 (H-2^b) and A.BY(H-2^b). Because these three set of mice share the same H-2 haplotype but not the same background, the main conclusion of these results is that host susceptibility to CVB3-induced myocarditis is controlled by "background" genes. Moreover, our statistical analysis for survival and heart histopathology indicated that the Pearson's correlation coefficient for these traits is highly significant. This result indicate that these two are related and under similar genetic control.

Figure 14: Heart CVB3 titers in five strains of mice.

Three inbred and two congenic were inoculated with 5.10^{3} PFU of CVB3 and monitored for six day after infection.
Viral titers were determined using standard plaque assays on HeLa monolayer. The number of animal examined from each strain was three. Data represent Mean values of CVB3 PFU per mg of heart ± SEM. $P>0.05$ using analysis of variance between the five strains of mice (no significant difference among the five strains of mice).

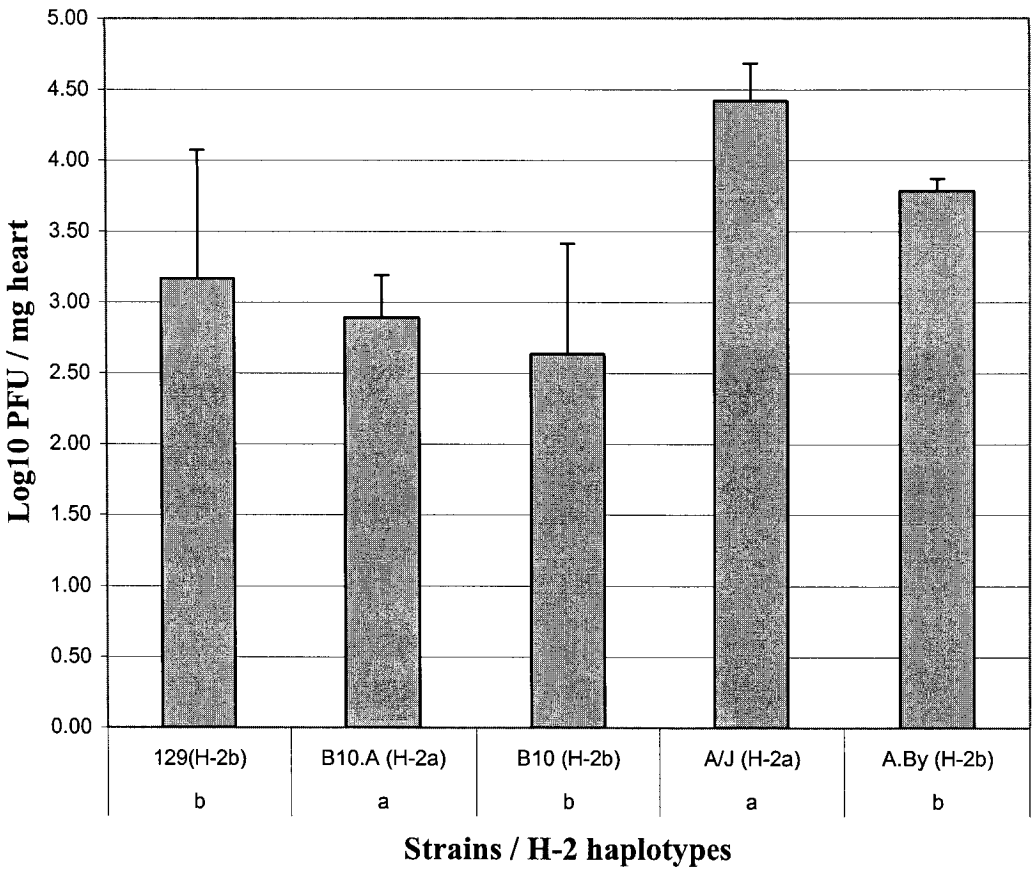

5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Log10 PFU / mg heart
129(H-2b)
b
B10.A (H-2a)
a
B10 (H-2b)
b
A/J (H-2a)
a
A.By (H-2b)
b
Strains / H-2 haplotypes

Table 4: Different phenotypic traits in five strains of mice infected with CVB3.

These strains of mice were inoculated intraperitoneally with 5.10^{3} PFU of CVB3 per mouse and sacrified at day 6 post infection.
Viral titers are Log_{10} values. Numbers in parenthesis indicate the number of mice used.
The results are calculated as mean ± SEM (standard error of the mean).

Strain	H-2 haplotype	% Survival	% Weight loss	Sarcolemmal disruption	Heart viral titer	Myocarditis
129	b	100 (10)	-1.477 (10)	1.44% ± 0.27 (10)	317 ± 0.9 (3)	0.001% ± 0.003 (3)
B10.A	a	87.5 (11)	9.431 (11)	7.88% ± 0.86 (11)	2.891 ± 0.3 (3)	0.44% ± 0.12 (3)
C57BL/10	b	100 (12)	11.536 (12)	9.96 % ± 0.93 (12)	2.63 ± 0.78 (3)	0.76% ± 0.10 (3)
A/J	a	50 (9)	9.391 (9)	18.92 % ± 1.51 (9)	4.42 ± 0.26 (3)	1.93% ± 0.18 (3)
A.BY	b	20 (6)	12.061 (6)	20.88 % ± 1.98 (6)	3.78± 0.083 (3)	3.02% ± 0.25 (3)
ANOVA		ND	ND	P = 0 (< 0.05)	P = 0.24 (>0.05)	

III. SURVEY OF TEN INBRED STRAIN OF MICE

Because there is a naturally occurring variability among the following inbred strains, including A/J (H-2^a), C57BL10 (H-2^b), 129 (H-2^b), Balb/c (H-2^d), DBA/2 (H-2^d), C3H (H-2^k), Balb.k (H-2^k), MaMy (H-2^k), AKR (H-2^k) and FVB (H-2^q), we choose to use them for the genetic analysis of four CVB3 induced phenotypes: survival, BW loss, heart viral load and quantification of sarcolemmal disruption.

1. Survival

During the six days study period; we observed that all 129, DBA/2, MaMy, FVB, Balb.k, AKR, C57BL/10, C3H mice survived and only 72.72% of Balb/c survived. In contrast, only 50% of A/J mice survived by day 6 post-infection (Figure 15).

2. Body Weight Loss

For the BW loss, we demonstrated that 129, DBA/2 and AKR mice gain weight even at day 6 post-infection. Most of the resistant strains start to loose weight by day 5 post-infection. However, the susceptible strain A/J starts to loose weight by day 3 post-infection and reach 9.67% of body weight loss by day6 post-infection. In contrast, 129 mice, the highly resistant strain gain 1.47% of their body weight (Figure 16).

Figure 15: Survival curves for ten inbred strains of mice

Mice were inoculated with 5.10^{3} PFU of CVB3 and monitored for six days after infection. The number of mice used at six days after CVB3 infection was 10 for 129 mice, 11 for DBA/2, 6 for MaMy, 9 for FVB, 6 for Balb/k, 10 for AKR, 8 for Balb/c, 12 for C57BL/10, 11 for C3H and 9 for A/J.

Chi-sq test indicated that there is no significant difference between the ten strains of mice ($p > 0.05$ and $X^2=39.77$).

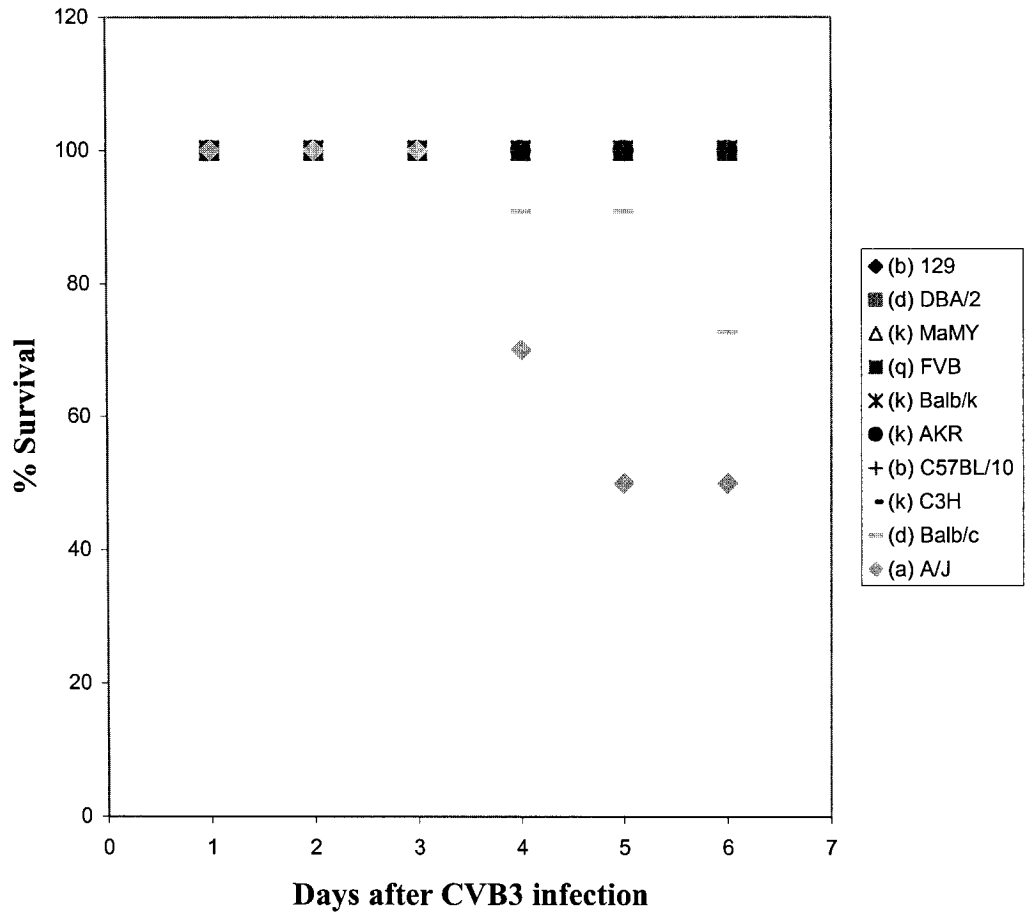
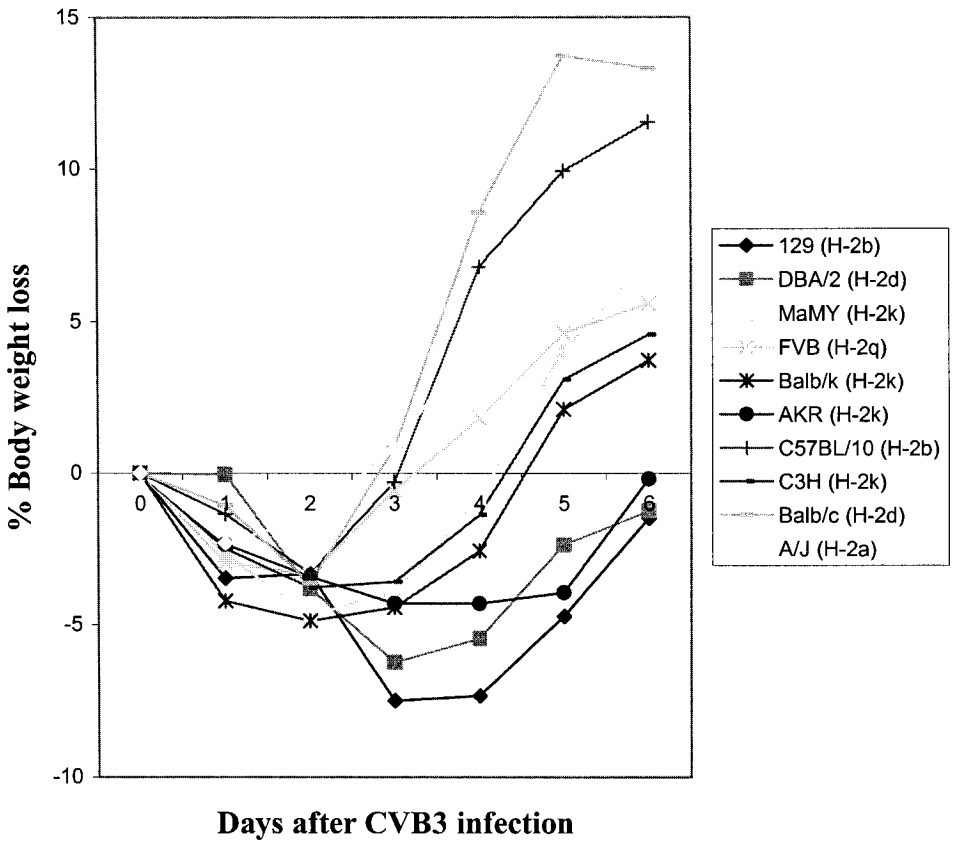

120
100
80
60
40
20
0
% Survival
0
1
2
3
4
5
6
7
Days after CVB3 infection
(b) 129
(d) DBA/2
(k) MaMY
(q) FVB
(k) Balb/k
(k) AKR
(b) C57BL/10
(k) C3H
(d) Balb/c
(a) A/J

Figure 16: Body weight loss for the ten inbred strains of mice infected with 5.10^3 PFU of CVB3.

Mice were inoculated with 5.10^3 PFU of CVB3 and monitored for six days after infection. The number of mice used at six days after CVB3 infection was 10 for 129 mice, 11 for DBA/2, 6 for MaMy, 9 for FVB, 6 for Balb/k, 10 for AKR, 8 for Balb/c, 12 for C57BL/10, 11 for C3H and 9 for A/J.


15
10
5
0
-5
-10
0
1
2
3
4
5
6
% Body weight loss
Days after CVB3 infection
129 (H-2b)
DBA/2 (H-2d)
MaMY (H-2k)
FVB (H-2q)
Balb/k (H-2k)
AKR (H-2k)
C57BL/10 (H-2b)
C3H (H-2k)
Balb/c (H-2d)
A/J (H-2a)

3. CVB3 Infection Increase Sarcolemmal Disruption

As Determined by the analysis of variance (ANOVA), the ten inbred strain of mice differ at a p value < 0.0001. Bonferroni test showed that the ten inbred strains could be divided into three groups: the A/J mice which showed the greatest percentage of EBD-positive area; the C3H, C57BL/10, Balb/c, AKR, Balb.k, FVB and MaMy mice which showed the intermediate percentage of EBD-positive area; and the 129 and DBA/2 showed the lower percentage of EBD–positive area (Figure 17).

4. Heart Viral Load

The analysis of variance (ANOVA) indicated that there were no significant quantitative differences among the ten inbred strains of mice in the CVB3 load of their heart tissue. The mean $\log_{10}$ CVB3 PFU/mg of tissue ranged from 2.47 to 4.42 with the SEM ranging from 0.08 to 0.96 (Figure 18). There were no significant strains differences in the ability of CVB3 to infect and replicate in the heart tissues.

Figure 17: Quantification of sarcolemmal disruption in ten inbred strains of mice infected with 5.103 PFU of CVB3.

Mice were inoculated with 5.10^{3} PFU of CVB3 and monitored for six days after infection. The number of mice used at six days after CVB3 infection was 10 for 129 mice, 11 for DBA/2, 6 for MaMy, 9 for FVB, 6 for Balb/k, 10 for AKR, 8 for Balb/c, 12 for C57BL/10, 11 for C3H and 9 for A/J. Data represent the mean (±SEM). ANOVA showed a significant difference among the ten inbred strains of mice ($p < 0.0001$).

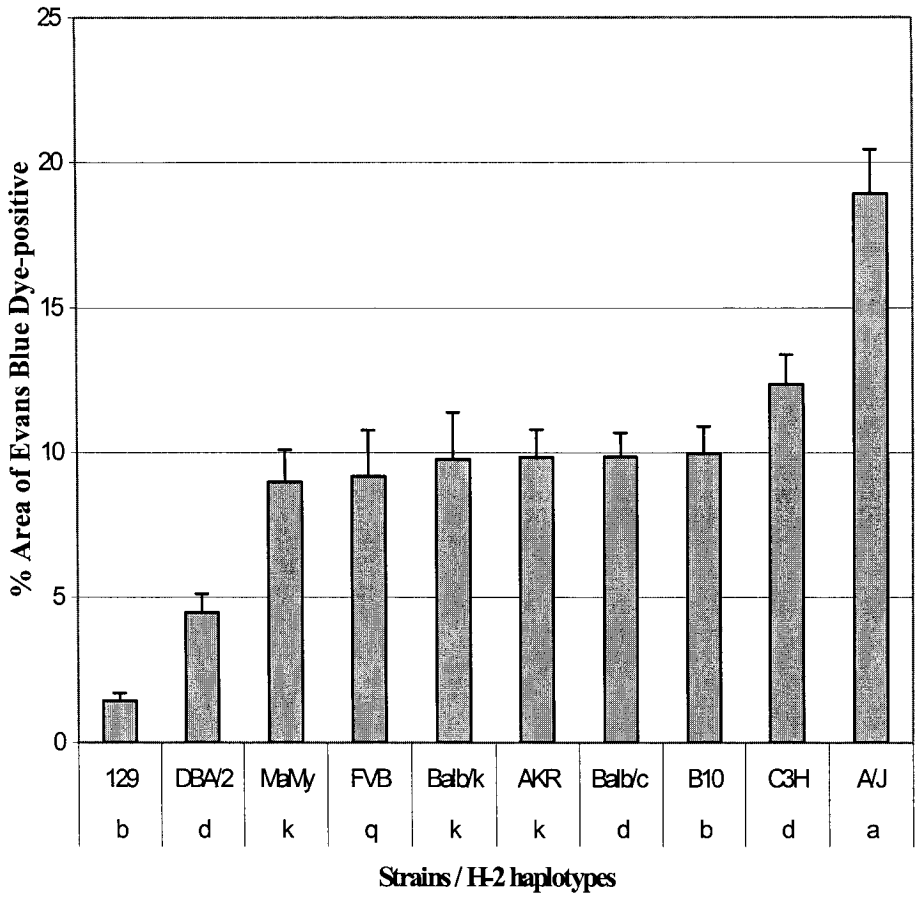
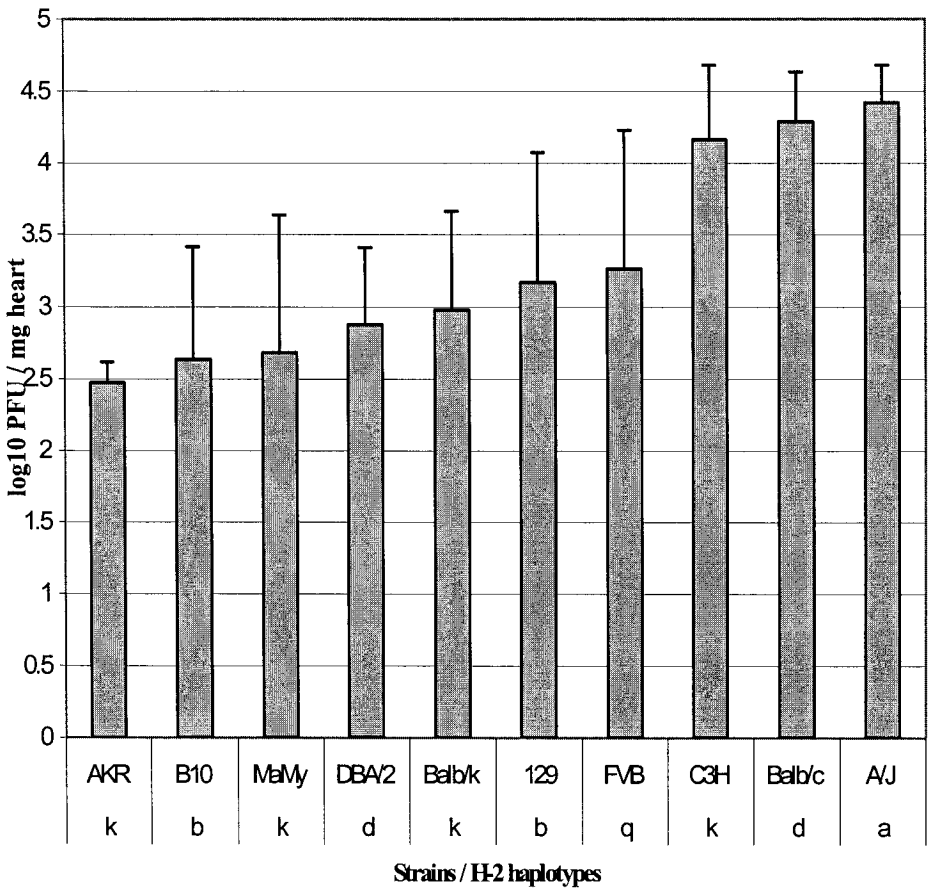

25
20
15
10
5
0
% Area of Evans Blue Dye-positive
129
b
DBA/2
d
MaMy
k
FVB
q
Balb/k
k
AKR
k
Balb/c
d
B10
b
C3H
d
A/J
a
Strains / H-2 haplotypes

Figure 18: Heart viral load in ten inbred strains of mice.

Mice were infected with 5.10^3 PFU of CVB3 and monitored at 6 days post-infection. The number of mice used is 3 for each strain. Data represents the mean (±SEM). There is no significant difference among the ten inbred strains ($p > 0.05$).


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
log10 PFU / mg heart
AKR
k
B10
b
MaMy
k
DBA/2
d
Balb/k
k
129
b
FVB
q
C3H
k
Balb/c
d
A/J
a
Strains / H-2 haplotypes

5. Conclusion: Wide range of host susceptibility to the cardiotropic CVB3 induced acute myocarditis in ten inbred strains of mice:

The present data showed that different inbred strains of mice displayed different responses to CVB3 infection (see Table 5). The A/J mice displayed the greatest susceptibility to CVB3 infection as seen in mortality, high percentage of body weight loss and the highest percentage of sarcolemmal disruption. MaMy, FVB, C3H, Balb/k, AKR, Balb/c and B10 mice were considered as the resistant to intermediate strains showed intermediate phenotypic traits. The 129 and DBA/2 mice displayed the greatest resistance to CVB3 infection as they showed no mortality, no body weight loss (gain weight) and almost no sarcolemmal disruption.

Taken together, these findings indicate the important contribution of the genetic background to CVB3 infection. This phenotypic data on commonly used and genetically diverse inbred mouse strains sets up the platform for a detailed analysis of the genetic basis of susceptibility to CVB3, i.e. the identification of the chromosomal regions regulating the phenotypic traits.

IV. FUNCTIONAL PARAMETERS OF CVB3-SUSCEPTIBILITY WITH SPECIAL EMPHASIS IN DYSTROPHIN

1. At the RNA Level

Numerous markers of heart disease identified in human and animal studies have also been recognized to be altered during CVB3 infection. To test the expression of

Table 5: Different phenotypic traits in ten inbred strain of mice after CVB3 induced acute myocarditis.

These strains of mice were inoculated intraperitoneally with 5.10^3 PFU of CVB3 per mouse and sacrified at day 6 post infection.
Numbers in parenthesis indicate the number of mice used.
The results are calculated as mean ± SEM (standard error of the mean).

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Strain	H-2 Haplotype	Percentage of Survival	Percentage of Body Weight loss	Sarcolemmal Disruption	Heart CVB3 Titer
129/sv	b	100(10)	-1.47 (10)	1.44 ± 0.27 (10)	3.16 ± 0.9 (3)
DBA/2	d	100 (11)	-1.24 (11)	4.47 ± 0.65 (11)	2.88 ± 0.53 (3)
MaMy	k	100 (6)	6.98 (6)	8.97 ± 1.12 (6)	2.68 ± 0.95 (3)
FVB	q	100 (9)	5.58 (9)	9.16 ± 1.6 (9)	3.26 ± 0.96 (3)
Balb/k	k	100 (6)	3.71 (6)	9.75 ± 1.62 (6)	2.98 ± 0.68 (3)
AKR	k	100 (10)	-0.19 (10)	9.82 ± 0.96 (10)	2.47 ± 0.14 (3)
Balb/c	d	72.72 (8)	13.32 (8)	9.84 ± 0.83 (8)	4.29 ± 0.34 (3)
C57BL/10	b	100 (12)	11.53 (12)	9.96 ± 0.93 (12)	2.63 ± 0.78 (3)
C3H	k	100 (11)	4.58 (11)	12.37 ± 1.02 (11)	4.16 ± 0.52 (3)
A/J	a	50 (9)	9.39 (9)	18.92 ± 1.51 (9)	4.42 ± 0.26 (3)

genes known to be involved in the pathological process we have identified several candidate genes including:

1. Genes coding for viral receptors (CAR and DAF),
2. Innate immunity genes (IFN-γ and TNF-α), and
3. Cytoskeletal protein genes mutated in cardiomyopathies (DES and DMD).

In this study we wanted to compare the expression of these candidate genes in two mice with different genetic background, the susceptible A/J mice (H-2^a) and the resistant C57BL mice (H-2^b) at six days after CVB3 infection. The initial event in viral infection is the attachment of the virus to specific host cell receptor. Coxsackievirus B3 makes use of at least two cell membrane proteins: the coxsackie and adenovirus receptor CAR and the decay accelerating factor (DAF or CD55) [Bergelson et al., 1995].

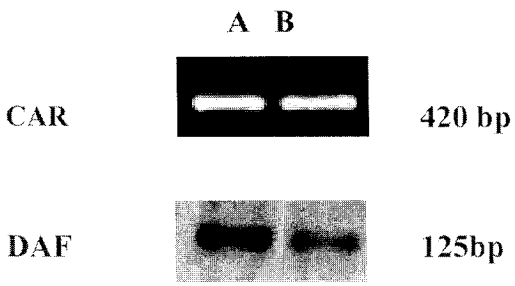
In this study, RT-PCR analysis showed that one of the viral receptors DAF but not CAR was differentially expressed in the susceptible and the resistant strains of mice. DAF was upregulated in the susceptible strain A (Figure 19 a), indicating that DAF, which is a co receptor for cardiovirulent CVB3, may be important in the pathogenesis of CVB3 mediated acute myocarditis.

In addition to that, we found that IFN-γ transcript was up regulated in the B background mice compare to the A background (Figure 19 b). This result suggests that IFN-γ is

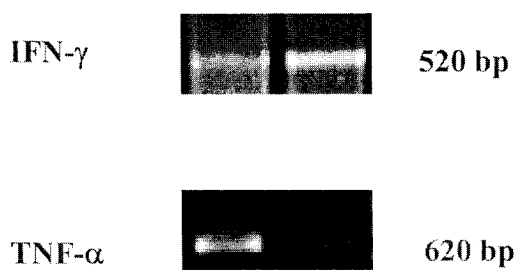
Figure 19: Functional parameters of CVB3-susceptibility with special emphasis in dystrophin using RT-PCR analysis

A: mice having the A background
B: mice having the B background
a. Genes coding for viral receptors:
CAR: Coxasackie and Adenovirus Receptor
DAF: Decay accelerating factor
b. Innate immunity genes:
IFN-γ: Interferon-γ
TNF-α: Tumor necrosis factor-α
iNOS: Inducible nitric oxide synthase
c. Cytoskeletal protein genes mutated in cardiomyopathies:
DES: Desmin
DMD: Ducehenne muscular dystrophy

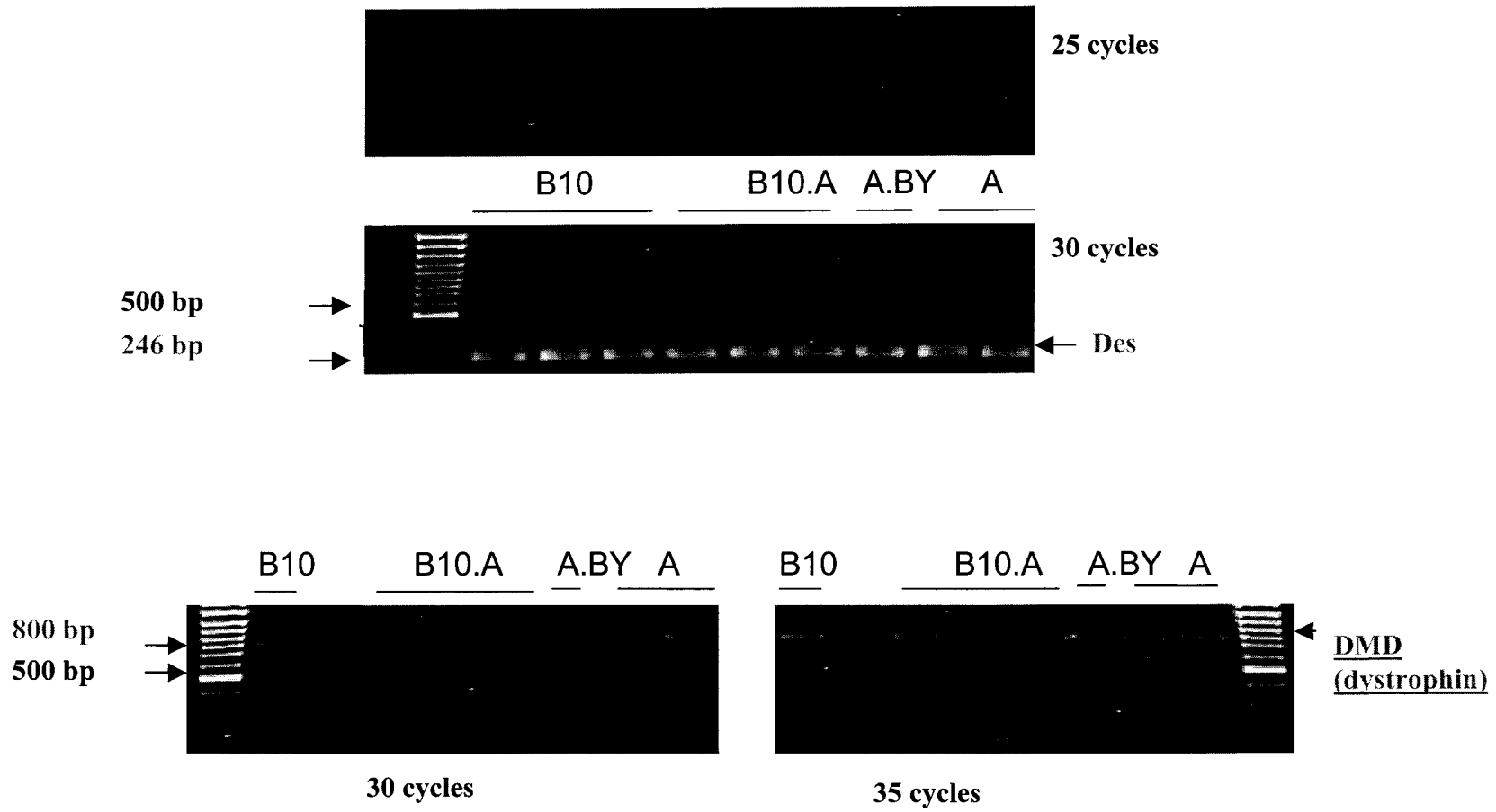
a. Genes coding for viral receptors



b. Innate immunity genes



c. Cytoskeletal genes mutated in cardiomyopathies:



diminished in susceptible mice, resulting in a general delay in the effector phase of the adaptive immune response. In many cases, susceptible mice are deficient in maintaining immunity or generating a memory immune response. In contrast, in resistant mice up regulation of IFN-γ is likely to confer protection against CVB3-induced myocarditis. We also found that TNFα transcript is upregulated in the A background (susceptible mice) compare to the B background (Figure 19 b). This data suggest that this cytokine, TNFα, may contribute to the progression of the disease and may be considered as deleterious to myocarditis in the acute stage of viral myocarditis.

It has been shown through the literature that dystrophin gene is mutated during cardiomyopathies. However, there was no differential gene expression of dystrophin or desmin in the resistant and susceptible strain of mice after CVB3 infection (Figure 19c).

In conclusion, functional parameters of CVB3 susceptibility revealed by differential gene expression of (IFN-γ and TNF-α) Cytokines and DAF receptors point to a complex mechanism of disease that are not easily amenable to genetic analysis.

To narrow down our research for genetic determinants for susceptibility to cardiovirulent CVB3 in the acute phase of the disease, quantifiable pathological parameters-BW loss, viral load and quantification of damaged myocytes determined by histopathological analysis –were identified for linkage analysis.

And because viral protease 2A is known to cleave dystrophin, we wanted to look at the protein level of dystrophin using immunocytochemistry assay.

2. At the Protein Level

To determine whether there was morphological disruption of the dystrophin staining in infected cardiomyocytes, heart tissues of our five infected strains of mice: A/ J ($H\text{-}2^a$), C57BL/10 ($H\text{-}2^b$), 129 ($H\text{-}2^b$), B10.A ($H\text{-}2^a$) and A.BY ($H\text{-}2^b$) were immunostained with antibody against dystrophin Dy4/6D3. The dystrophin staining was disrupted in most susceptible CVB3 infected cells (Figure 20 b); in contrast, dystrophin staining was normal in the uninfected hearts.

Disrupted dystrophin is often accompanied by uptake of EBD in the infected myocardium (Figure 20 d).

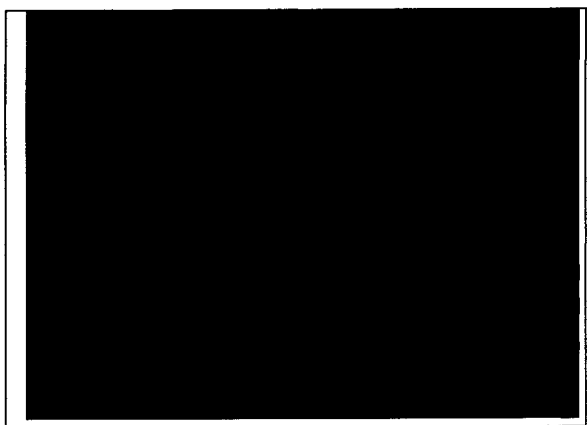
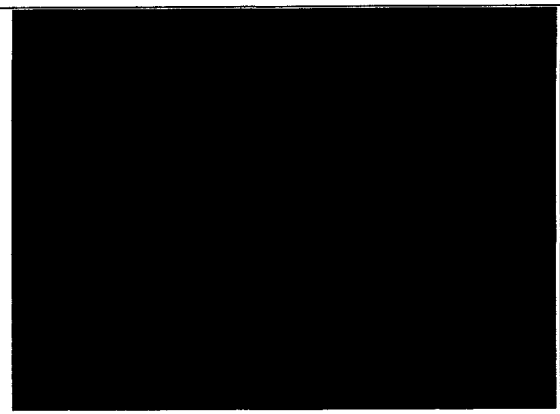
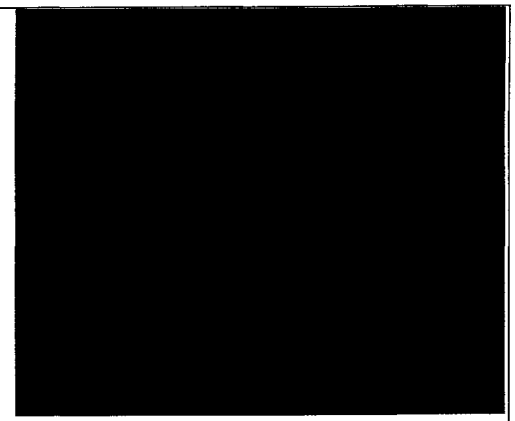
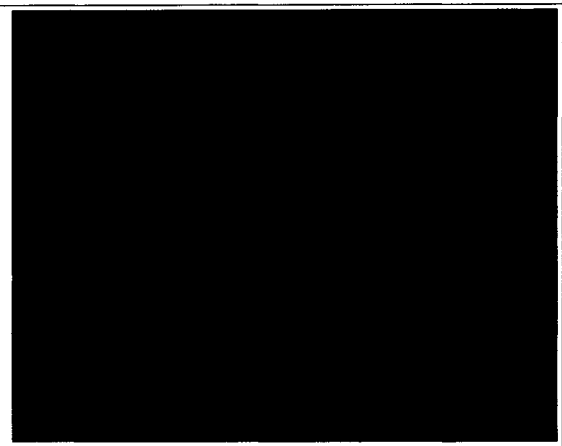
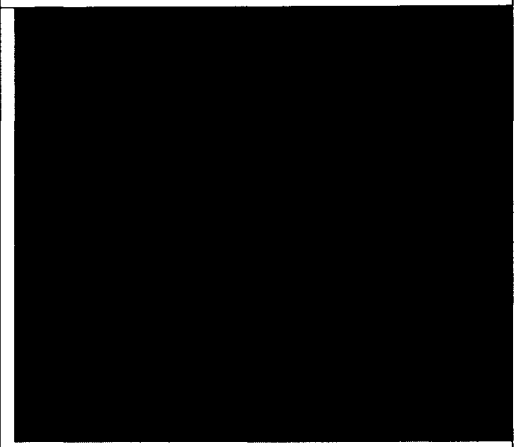
In addition, we consistently saw disruption of dystrophin in the presence of virus in all strain of mice used, even in the absence of the host response as seen in the 129 mice. We conclude that there were no variation in dystrophin at the protein level among the strain of mice used.

Triple labeling of EBD (red fluorescence), anti-CVB3 (blue fluorescence) and anti-dystrophin (green fluorescence) showed a superimposed triple fluorescence (Figure 20 f). This result indicated that the dye uptake occurred specifically in CVB3 infected cardiomyocytes with a disrupted dystrophin staining.

Figure 20: Effects of Coxsackievirus on the disruption of dystrophin and sarcolemmal integrity.

Original magnification (x100).

a. Staining with Evans Blue Dye (red fluorescence), which is a vital dye already injected to all mice 16 to 24 hours prior to sampling,
b. Dystrophin immunostain only (green fluorescence),
c. Coxsackievirus immunostain only (blue fluorescence),
d. Double staining using EBD (red fluorescence) and an antibody against dystrophin (green fluorescence),
e. Double staining using EBD (red fluorescence) and an antibody against coxsackievirus B3 (blue fluorescence),
f. Triple staining with Evans Blue Dye (red fluorescence), antibody against dystrophin (green fluorescence) and antibody against coxsackievirus B3 (blue fluorescence).

		
a) Evans Blue Dye	**b)** Anti-Dystrophin	**c)** Anti-CVB3
		
d) Evans + anti-Dystrophin	**e)** Evans + anti-CVB3	**f)** Evans + anti-Dys + anti-CVB3

DISCUSSION

Myocarditis, which is a disease characterized by inflammation and necrosis of cardiac muscle, often has a viral etiology [Lawson et al., 1990]. Murine models of experimental enteroviral myocarditis provide an excellent avenue for studying the pathogenic mechanisms that recapitulate virus-induced disease in humans.

Myocarditis is a very complex disease [Cooper, 2003]. Complex traits are determined by the combined effects of naturally occurring allelic variation at several genes, together with relatively major influences due to the environment [Fortin et al., 2001]. These traits can be dissected in well-defined inbred strains of mice in which genetic factors have segregated and become fixed during inbreeding and where environmental effects can be controlled [Fortin et al., 2001].

Known genealogic relationship between inbred strains of mice indicates that all inbred strains have an equivalent origin. There is increasing evidence that inbred laboratory mice have been developed with contributions from more than one subspecies of wild mouse (www.informatics.jax.org) including *Mus musculus domesticus, Mus musculus castaneus, Mus musculus musculus* and *Mus musculus molossinus*. The genomes of these strains were predicted to be a "mosaic" of regions with origins in the different subspecies, although these mice are generally thought to reflect predominantly the Mus *musculus domesticus* subspecies, there is some historical contributions from "fancy"

mice bred in Japan and China [Wade et al., 2002] (see Figure 20). In fact this reflects a genetic and environmental variation among the inbred strain of mice.

Inbred strains of mice are divided into the following seven categories [Beck et al., 2000]:

(A) Swiss mice, derived from either albino Swiss mice or wild mice from Switzerland;

(B) Castle's mice: strain ancestors were originally used in breeding experiments by William Castle (some were also derived from Abbie Lathrop's breeding colonies);

(C) Inbred strains derived from colonies from China and Japan;

(D) Other inbred strains: mice derived from a variety of sometimes unknown sources;

(E) C57-related mice, derived from an original pair of mice bred by Abbie Lathrop;

(F) Inbred strains derived from species or sub-species of wild mice (Unlike these mice, most inbred strains are a mix of *Mus musculus* sub-species, mainly *M. m. domesticus*);

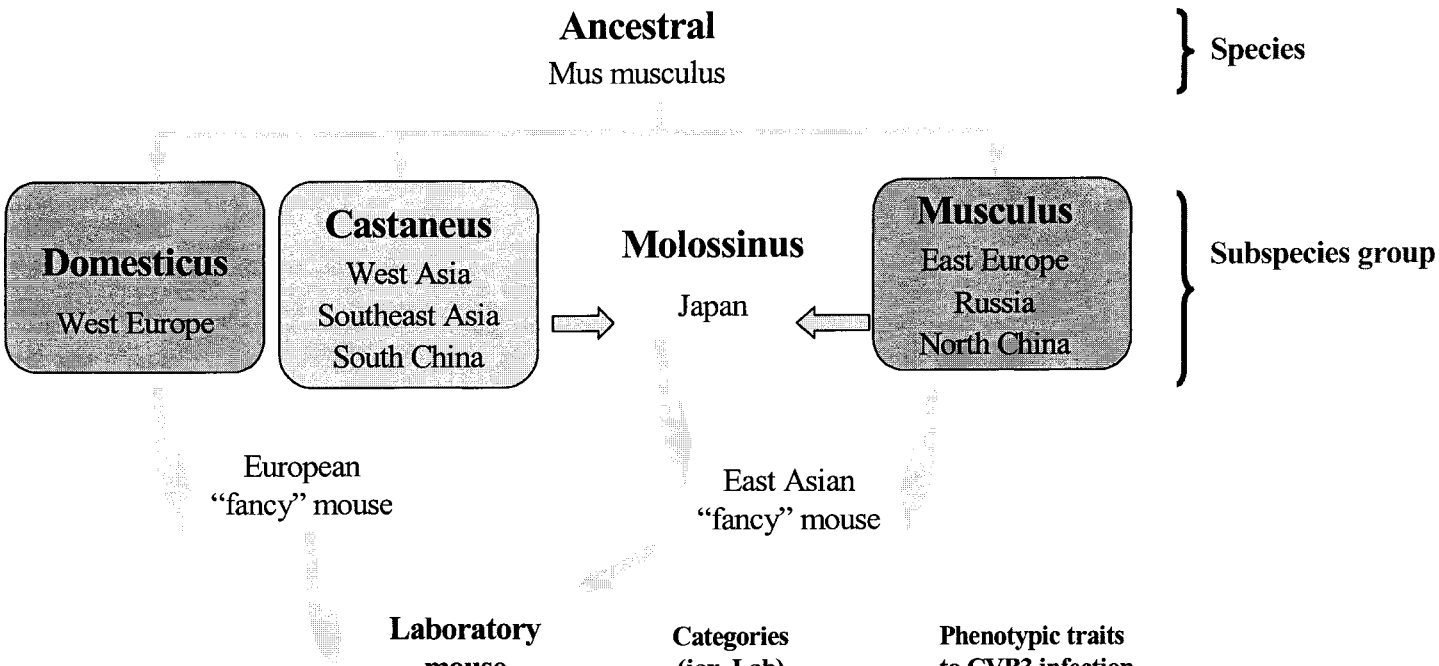
(G) Mice derived from multiple inbred strains. It has been shown that most commonly used strains trace their origins to W. Castle's laboratory at Harvard University and even more strains originate from his supplier A. Lathrop of Granby, Massachusetts [Wade et al., 2002]. The categories of the ten inbred strains of mice used in this study and listed in Figure 21 were adopted from the genealogic chart of inbred mouse strains available on the Jackson Laboratory web site (www.informatics.jax.org).

The inbred strains used in this study are all related by descent and are all originated from a common ancestral, as seen in Figure 21.

For the inbred strains used in this study, there are also thousands of single-nucleotide polymorphisms (SNPs) [http://mouseSNP.roche.com] and many phenotypes ("Mouse Phenome Project", http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home)

Figure 21: History of the inbred strains of mice and the resulting_patterns of phenotypic variation.

Adopted by [Wade et al., 2002] and [www.informatics.jax.org].and modified to reflect our result of phenotypic traits after CVB3 infection of the selected inbred strains of mice



Laboratory mouse	Categories (jax. Lab)	Phenotypic traits to CVB3 infection
129 (H-2b)	castle's mice	highly resistant
DBA/2 (H-2d)	castle's mice	highly resistant
MaMy (H-2k)	C57BL related strains	resistant to intermediate
FVB (H-2q)	Swiss mice	resistant to intermediate
Balb.k (H-2k)	castle's mice	resistant to intermediate
AKR (H-2k)	castle's mice	resistant to intermediate
Balb/c (H-2d)	castle's mice	resistant to intermediate
C57BL/10 (H-2b)	C57BL related strains	resistant to intermediate
C3H (H-2d)	castle's mice	resistant to intermediate
A/J (H-2a)	castle's mice	susceptible

available, which together provide a wealth of information to the understanding of variation among inbred strains of mice. The Phenome Project aims to a systematic phenotypic characterization of a priority list of inbred strains of mice including A/J, C57BL10, 129, Balb/c, DBA/2, C3H, Balb/k, MaMy, AKR and FVB that we used in this study.

Previous studies have shown that several factors including sex, age, dose, viral strains and host genetic background influence myocarditis in inbred strains of mice [Kawai, 1999], [Gaunett et al., 1984], [Herskowitz et al., 1985], [Herskowitz et al., 1987], and [Martino et al., 1994].

In this study, we used a viral dose of 5. 10^3 PFU of the CVB3-CG strain which were inoculated in 8 to 10 weeks old male mice, for six days. Under these conditions, we observed differences that are amenable to quantitative trait loci (QTL) analysis.

In this study, and based on our four phenotypes and specially the quantification of sarcolemmal disruption the strains could be divided into three groups: the highly resistant (like control) strains including 129 and DBA/2 mice, the resistant to intermediate strains including MaMy, FVB, C3H, Balb/k, AKR, Balb/c and B10 mice and the highly susceptible strains of the A background including A/J mice. Taken together, this finding indicates the important contribution of the genetic background to CVB3 infection.

And for the first time, to our knowledge, we found that 129/sv mice displayed the greatest resistance to CVB3 with no mortality, no body weight loss, almost none sarcolemmal disruption and almost no myocarditis.

Studies of host susceptibility to CVB3 infection utilizing inbred strains are relatively few, making comparisons and confirmations between studies difficult. The main problem is the use of the same few strains, but with comparison typically limited to two strains in most of the studies that are C57BL and A strains for which C57BL is the resistant strain and the A is the susceptible to CVB3 infection [Wolfgram et al., 1986] and [Herskowitz et al., 1987]. Gauntt et al., 1984 looked at more strains of mice but they were mainly looking at the differential effect of two variants of CVB3 (CVB3m and ts10R). By comparing many strains in a single study under identical experimental protocols, the present strain survey allows for correlation between phenotypes, such as what we found here in this study: correlation between survivals and histopathology (Pearson's correlation coefficient for these traits was highly significant). Using quantitative trait loci (QTL), genes linked to these phenotypes could be identified.

At the experimental condition used, however, we did not observed an influence of the H-2 genes.

The influence of the major histocompatibility complex (MHC) on myocarditis was studied by comparison of the disease of the strains of mice sharing the same background but not the same H-2 haplotype. In contrast the influence of non-MHC genes was demonstrated by comparison of phenotype of strains of mice sharing the same H-2 haplotypes. The parameters examined were survival, body weight loss, heart viral load, quantification of sarcolemmal disruption and quantification of myocarditis (myocardial pathology) in the five strains of mice. These differed either in the haplotype of the major histocompatibility complex or in their background genes, including three inbred strains A/J, C57BL/10 and 129 mice and two congenic A.BY and B10.A mice.

In this study, we found that A/J (H-2^a) and A.BY (H-2^b) strains displayed the greatest susceptibility to CVB3 as seen in survival, BW loss, percentage of myocarditis and the percentage of sarcolemmal disruption. In comparison, C57BL/10 (H-2^b) and B10.A (H-2^a) had no to low mortality and low percentage of myocarditis as well as low percentage of sarcolemmal disruption.

Because these three set of mice share the same H-2 haplotype but not the same background, the main conclusion of this result is that host susceptibility to CVB3-induced myocarditis is controlled by "background" genes (nonmajor histocompatibility complex) and not H-2 genes (major histocompatibility complex). This finding doesn't support our hypothesis which stated that susceptibility during the acute CVB3 infection is controlled by H-2 as well as the background (non H-2) genes.

Earlier studies performed by Gauntt et al., with CVB3m strain (myocarditic variant of CVB) [Gauntt et al., 1984] agree with our findings since they do not suggest the presence of a major virus susceptibility gene for CVB3 within the major histocompatibility complex. It was shown that mouse strains sharing the same H-2 haplotypes were either highly susceptible or highly resistant to the induction of myocarditis.

However, Noel Rose and co-worker [Wolfgram et al., 1986] and [Herskowitz et al., 1987] found that the major histocompatibility complex, therefore, may control the early response (the first seven days) to coxsackievirus B3 (Nancy strain) induced myocarditis which is in contradiction with our finding. However we have to remember that he used different strain of virus. Our results coincide with Gauntt where he used the same strain of virus. This may suggest that H-2 is virus strain dependent. In addition, Rose and co-

worker found that the A background genome appears to control the characteristic late pathologic changes seen in all the three of the A H-2 congenics strains used including A.BY, A.CA ($H\text{-}2^s$) and A.SW ($H\text{-}2^s$). Their data suggest that one of the properties under MHC control in the early stage of the disease is the time of onset of neutralizing antibody production, which in turn influences viremia, the duration of CVB3 infection in the heart, and the incidence of myocardial disease, they also found that the MHC influences the prevalence and titer of heart-specific autoantibodies [Wolfgram et al., 1986]. All these results depend on:

1. The study stage of the disease (early or late phase of the disease),
2. The parameters of the disease used,
3. The strains of mice, and
4. The strain of the virus.

It would be better if we could increase the number of congenic strains of mice having the A background and the congenic strains having the B background, this will enable more precise determination of the influence of the major histocompatibility complex in the susceptibility to CVB3 induced myocarditis.

At the conditions used, we did not find a correlation between the viral load and the variation in background and H-2 genes suggesting that viral load doesn't seem to be the only determinant of myocarditis. This result is supported by what has been found by Wolfgram et al., 1986; but not by Gauntt et al., 1984.

Using the same conditions, we tried to correlate also functional differences between the A (susceptible strain of mice) and the C57BL (resistant strain of mice), which may

eventually serve as candidate genes on a future genetic analysis. Our results of RT-PCR analysis indicate a differential expression of IFN-γ, TNF-α, indicating an important role of the immune system in the differential response to CVB3 infection in resistant and susceptible mice. In addition to that, we found a differential gene expression of DAF receptors but not CAR. Upregulation of DAF in the susceptible starin may increase binding efficiency of coxsackievirus onto the DAF-CAR complex to permit efficient uncoating of the viral genome.

In conclusion, functional parameters of CVB3 susceptibility revealed by differential gene expression of (IFN-γ and TNF-α) cytokines and DAF receptors point an important mechanism of disease but are not easily amenable to genetic analysis. Therefore, the question now would be whether the upregulation of these genes were mainly expressed by the direct (viral RNA) or indirect (infiltrating cells) effect of the viral at day six-post infection? For this we might need to analyze the profile of these genes expression, in vitro, in cultured cardiac myocytes infected with CVB3 (free of cell infiltration). To narrow down our research for genetic determinants for susceptibility to cardiovirulent CVB3 in the acute phase of the disease, quantifiable pathological parameters-BW loss, viral load and quantification of damaged myocytes determined by histopathological analysis –were identified for linkage analysis.

Here, we are speculating that one of the possible candidates among the background genes is dystrophin gene. The dystrophin gene, which is defective in Duchenne muscular dystrophy (DMD) patients, is the largest known gene, consisting of almost 0.1% of the human genome (2,500 Kbp) and maps to chromosome X. Dystrophin, which belong to the protein family actin-binding proteins, is a large protein responsible

for the linkage of F-actin to the extra cellular matrix. This protein is critical for maintaining the proper function of muscle by preventing contraction-induced injury at the cellular level.

In this study we found that dystrophin protein is morphologically different between susceptible and resistant mice. But we couldn't find any differential gene expression of dystrophin between the susceptible and the resistant strains of mice but this could be because of the small amount of RNA that we used, it would be better to increase the sample size. In case we are limited as is the case here, we could adopt another approach using blood cell-derived RNA [Liew et al., 2004]. Studies using RNA from blood samples in micoarray analysis have successfully detected biomarkers of coronary artery disease [Liew et al., 2004].

But we should remember that mRNA levels do not necessarily correlate with proteins levels, and that post-translational modifications of proteins can significantly alter their function [Liew et al., 2004].

We also found a functional impairment of the dystrophin in CVB3 infected cardiomyocytes. In fact, immunostaining of Evans blue dye-injected hearts for rod domain of dystrophin showed that the dye uptake specifically occur in virally infected cardiomyocytes with a disrupted dystrophin staining pattern.

In fact, we found that Evans blue dye was specifically taken-up by CVB3 infected cardiomyocytes with a disrupted dystrophin staining pattern. This association suggests that dystrophin disruption may play an important role in the observed increase of sarcolemmal permeability.

In addition, we consistently saw disruption of dystrophin in the presence of virus, even in the absence of the host response as seen in the 129 mice. There were no variation in dystrophin, neither at the protein level nor at the RNA level, between the susceptible and resistant strain of mice suggesting that dystrophin doesn't seems to be a good candidate. Nevertheless, based on the known role of the genetic dystrophin deficiency that causes familial dilated cardiomyopathy, it is clear that dystrophin disruption during CVB3 infection may participate in a cascade of events that leads to CVB3 induced myocarditis. This finding supports the results demonstrated by Knowlton and co-workers [Xiong et al., 2002].

In addition to this direct effect of the virus which increases the sarcolemmal permeability we found that host immune system play an important role in the pathogenesis of CVB3-induced myocarditis. In fact, quantification of myocarditis showed that susceptible mice (A/J and A.BY) displayed extensive myocyte inflammation, whereas, only some inflammatory foci were observed in the resistant strains (C57Bl/10 and B10.A) and almost none in 129 mice. The questions now are the following: Which immune cell populations are involved in inducing CVB3 myocarditis? And is there any qualitative immunologic difference between the resistant and susceptible mice? Which cell population is present with the highest percentage in the susceptible strains of mice? Which are the chemical mediators of myocarditis?

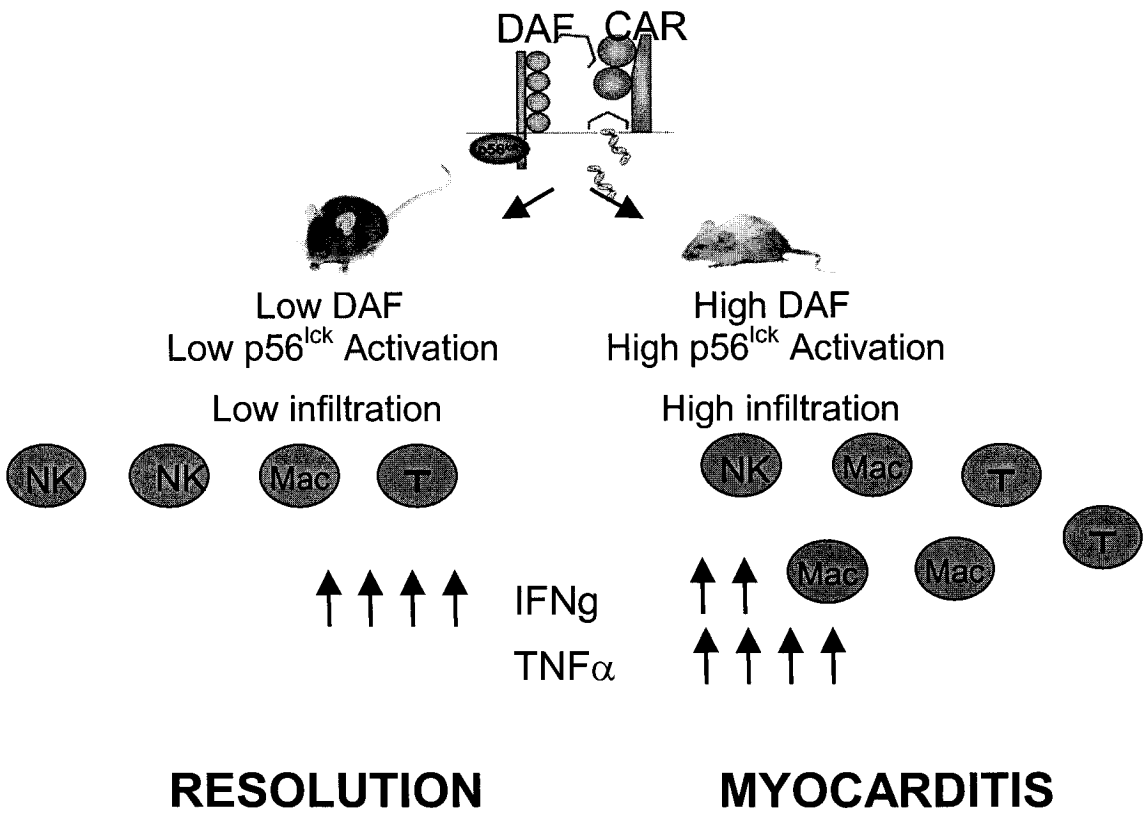
Our functional analysis indicates that variations in IFN-γ and TNF-α gene expression are good candidate genes for CVB3-induced acute myocarditis.

We would like to propose a model of CVB3 mediated acute myocarditis. In this model, shown in Figure 22, both direct viral cardiomyocyte destruction and immune mediated injury play significant role in the pathophysiology of myocarditis. We are speculating that the different level of cytokine in the resistant and susceptible strains of mice may represent different cell population in the two strains. Initial destruction of dystrophin by CVB3 seems to be the same in the resistant and susceptible mice. The high level of IFN-γ observed in the resistant strain is consistent with the protective role of NK cells. The direct cytopathic effect of CVB3 may trigger an initial infiltration of large number of NK cells and some macrophages and T cells. This initial infiltration (doesn't go further) is resolved in the resistant strain of mice.

In contrast, the expression of the high level of DAF in the susceptible mice may cause high $p56^{lck}$ activation which trigger further recruitment of inflammatory cells, in particular T cells. The high level of TNF probably secreted by macrophages as well as the recruitment of large number of T cells following the high activation of $p56^{lck}$, seem to mediate severe myocarditis in the susceptible strains of mice.

In conclusion, our results indicate that inbred strains of mice exhibit varied patterns of susceptibility following infection with coxsackievirus B3. These patterns are under genetic control, which is independent of the major histocompatibility complex. These results suggest a pathogenic link between CVB3 induced acute myocarditis and the host genetic background.

Figure 22: Model for CVB3 mediated acute myocarditis: direct and indirect effect of CVB3 infection.


DAF
CAR
Low DAF
Low p56lck Activation
High DAF
High p56lck Activation
Low infiltration
High infiltration
NK
NK
Mac
T
NK
Mac
T
T
Mac
Mac
IFNg
TNFα
RESOLUTION
MYOCARDITIS

This phenotypic data on commonly used and genetically diverse inbred mouse strains sets up the platform for a detailed genetic dissection of CVB3 enabling the identification of CVB3 susceptibility genes. This should lead to approaches allowing the early diagnosis of myocarditis and the prevention of idiopathic dilated cardiomyopathy (IDC). Genetic analysis are currently undergoing in our Lab at McGill using a genome scan approach to identify host genomic locations containing genes underlying differences in host response mechanisms to CVB3-induced myocarditis. As susceptibility genes begin to be identified in the mouse, we will be better able to understand the genetic basis for human susceptibility to viral myocarditis.

Currently, there is an international effort in order to dissect and identify genes regulating complex and quantitative traits. As a result, over 50 strains are currently being screened as part of the "Mouse Phenome Project" (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home) to rigorously define the remarkable range of phenotypes already available for genetic dissection [Threadgill et al., 2002].

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