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COXSACKIEVIRUS EXPERIMENTAL HEART DISEASES

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1. ABSTRACT

Many microorganisms, particularly viruses, can cause myocarditis, an inflammatory disease of the heart. The frequency of and major factors that contribute to this disease, including a pronounced gender (male) bias, age and genetic background parameters are discussed, along with signs and symptoms of disease in infants to adults. Individuals with acute disease generally recover without sequelae; the chronic form can develop into idiopathic dilated cardiomyopathy and death can follow. Among viruses most frequently associated with cases in the U.S., the coxsackieviruses group B (CVB) are major etiologic agents. The association between the CVB and disease is based on detection of viral RNA in heart biopsy specimens by polymerase chain reaction assays. Excellent CVB−, particularly coxsackievirus B3 (CVB3)−, mouse models of the disease have identified mechanisms of induction and establishment of chronic myocarditis. CVB3-murine models share many biologic parameters of the acute and chronic diseases in humans, and show that cardiopathologic alterations result from virus-induced and immunologic reactions in heart tissues. Several immune responses to a CVB3 infection that become cardiopathogenic, instead of protective, are discussed in an attempt to explain why immunosuppressive treatments are not effective. Bed rest and supportive therapy are the current treatment for patients with myocarditis.

2. INTRODUCTION

Some forms of heart diseases are induced by microorganisms, especially by viruses. One of these diseases is viral myocarditis. Myocarditis is an inflammatory disease of the heart muscle that affects infants to adults and can result in complete recovery, chronic disease leading to dilated cardiomyopathy or death. Among the many viruses that cause myocarditis, group B coxsackieviruses are most frequently associated with this disease in the U.S. Excellent coxsackievirus-mouse models of myocarditis, representing both acute-resolved and chronic forms of the disease, exist and provide important insights into the molecular and cellular mechanisms of the cardiopathologic processes. These animal models share many histologic, virologic and immunologic parameters found in human cases. This communication will focus on data derived from many research laboratories using coxsackievirus models of myocarditis in an attempt to define the origin, mechanism(s) of pathology and treatment of the disease.

3. COXSACKIEVIRUS EXPERIMENTAL HEART DISEASES

3.1. Causes of myocarditis

Myocarditis is an acquired form of inflammatory disease of the heart first described over 150 years ago (1).

In general terms, this disease involves infiltration of the major heart muscle (the myocardium) with a mixture of lymphocytes, macrophages and plasma cells into focal areas containing necrotic myocytes, and myocytes undergoing degeneration in response to an infection or a toxic pharmacologic agent. Clinically, the disease manifests in acute or chronic conditions, and the chronic condition is thought to be a precursor state to many cases of idiopathic dilated cardiomyopathy (IDC, 2-4). There are many infectious agents that can induce myocarditis, including more than a dozen bacterial, protozoal and fungal pathogens and at least 17 viruses (5). Viruses most frequently associated with cases of myocarditis in children and adults in the U.S. and Europe include the enteroviruses and adenoviruses type C (6, 7), and to a lesser extent, cytomegalovirus and parvovirus B19 (1, 8). Hepatitis C virus has recently been linked to myocarditis and other heart diseases in patients in Japan (9). The pathogenic association is made by detection of viral genomic sequences in endomyocardial biopsy specimens via polymerase chain reaction (PCR) assays.

3.2. Frequency of myocarditis

Myocarditis is underdiagnosed (10). Frequency of myocarditis has been reported to range from a low of 4-5% in young men dying of trauma to a high of 16-21% in children succumbing to sudden death (5). In a large multicenter Myocarditis Treatment Trial in the U.S. that used a strict histologic analysis of 5 endomyocardial biopsies per heart (Dallas criteria), an incidence of 9% was found (11); however, imprecise parameters such as interobserver discrepancies in histologic evaluations and a wide variation in results among specimens suggest that by using this criteria, at most, only 50% of all true cases of myocarditis are identified (5). Given that the focal lesions are randomly distributed throughout the myocardium, endomyocardial surface biopsy samples certainly do not provide optimum tissue samples for detection of viral genomes by PCR. Clinically, myocarditis occurs predominantly in males (2/3 cases). Myocarditis most often occurs in females during the third trimester of gestation (peripartum period) (2, 12). This gender bias also holds for mice, with adolescent/adult males being highly susceptible to coxsackievirus-induced myocarditis, whereas virgin females are relatively resistant, as will be discussed subsequently (12, 13).

3.3 Clinical presentation of myocarditis

Within the initial 10-14 days of life, some features of the disease in newborns are unique, and beyond this period, symptoms are rare (14). Frank myocarditis often presents abruptly with respiratory distress, tachycardia, cyanosis, jaundice and diarrhea. In addition, temperature instability, arrhythmias, hepatomegaly and signs of peripheral circulation problems can be detected (14). Infants with myocarditis often simultaneously develop meningoencephalitis, pneumonia, hepatitis, pancreatitis or adrenalitis, and the degree of involvement is severe (14). In the adolescent or adult, the signs and symptoms of myocarditis are quite similar to those of other cardiovascular diseases: many times there is an influenza-like illness, chest pain, fever, heart failure, pulmonary edema, palpitation and lymphadenopathy (15). All or most of these symptoms may be absent in individuals less than 40 years of age and yet contribute to sudden unexpected death (15).

3.4. Viruses most frequently associated with myocarditis

The ubiquitous enteroviruses cause an enormous number of infections in the U.S. human population every year (~10 million) and yet, 50-90% of these infections are asymptomatic, resulting in a transient carrier individual that can readily spread this virus to additional susceptible hosts (15-17). About half of these enterovirus infections are attributed to the coxsackieviruses group B (CVB), serotypes B1-B5 (CVB1-CVB5), but not B6 (CVB6) in the U.S. (14-17). Infections of individuals with a CVB can result in no illness or in a wide variety of illnesses that range from the nuisance variety of the common cold or transient diarrhea, to severe pharyngitis, aseptic meningitis, pleurodynia and then to more serious diseases such as myocarditis, pancreatitis, perhaps diabetes mellitus, hepatitis and transient paralysis (18). Some diseases induced by the CVB result in deaths of individuals from fetal to advance age (18). It is not understood why some individuals' infections do not result in any illness and it is only through detection of an anti-CVB IgM antibody response that we know the infections occurred, whereas other individuals acquire minor to major life-threatening or uncommonly, fatal disease (15). The CVB are widely distributed in the environment, with humans the natural host. These viruses are readily available to humans in the environment because they are very stable in nature, especially in water (18). Depending on the CVB serotype and socioeconomic level of an individual, serosurveys have found that 18-94% of humans by age 30 have antibodies to at least one (rarely CVB6), and sometimes two or three of the serotypes B1-B5 (19-22). Infection of individuals by CVB1-CVB5, with or without illness or serious disease, is readily detected by assays of sera for antiviral antibodies (13, 17, 23, 24). However, in populations with known CVB infections, 5 to 12% of these individuals may have a myocardial involvement, as detected by an electrocardiogram (15). Enteroviruses have long been proposed to be the etiologic agents in up to 50% of the cases of myocarditis in most studies from the U.S. and Europe (27, 8, 25). Not surprisingly, several studies in all age groups which used PCR assays containing primers that would detect any of the above viruses failed to detect any viral nucleic acids, suggesting additional viral etiologies may be possible (6, 7). However, in one European registry (Marburg Myocarditis Registry), RT-PCR assays found enteroviral involvement to occur only 3% of the time, whereas PCR data on adenovirus and parvovirus B19 involvement was much higher, i.e., 5-20 and 10-30% of all cases were positive for genomic sequences of these viruses, respectively (8). In other studies, adenovirus and enterovirus involvement in myocarditis was of equal importance, or adenoviruses were slightly more commonly detected (5, 7). Neonatal myocarditis is most frequently caused by the CVB and mortality among infants with myocarditis is generally reported to be quite high, i.e., 30-50% (14). The use of reverse transcriptase (RT)-PCR assays of endomyocardial biopsy tissues for enteroviral

RNA generally found an average of 20-35% of the samples from patients with myocarditis to be positive for enteroviral genomes (2, 6, 8). It must be noted that the majority (80-90%) of adult patients with myocarditis recover (15, 25). The data from several studies suggest that about a third of patients with IDC have either inflammation or the presence of enteroviral RNA sequences in the myocardium, with the latter being detected by RT-PCR (15). The importance of detecting viral RNA in heart tissues is demonstrated by the findings of one study: 26% of those IDC patients with evidence of enteroviral involvement in the myocardium died within 6 months, whereas only 3% of patients with enterovirus-negative biopsy results died (15). Thus, although CVB infections of the upper respiratory or gastrointestinal tracts are common and can lead to a wide variety of diseases (14-16), infections of the heart are uncommon but can lead to serious diseases for which the outcome can be death (15, 25).

3.5 Coxsackieviruses (CVB) and the heart

Like all enteroviruses, CVB virions (infectious virus particles) are small nonenveloped icosahedral particles of about 30nm in diameter that are composed of a capsid containing four proteins surrounding a single-stranded RNA genome of ~7400 nucleotides (6, 16, 26). The RNA genome contains a small protein (virus protein genome, VPg) of 2400 D that is covalently linked to the 5′ terminus. Approximately 740 nucleotides downstream of the 5′ terminus, an authentic AUG codon signals the start of a single open reading frame. Translation of the genome yields a precursor protein (polyprotein) that is processed by viral proteases translated and released from the polyprotein. At the 5′- and 3′- termini of the genome are nontranslated regions, i.e., 5′- and 3′-NTR, respectively. The 5′-NTR has sequences required for translation-initiation, i.e., the internal ribosome entry site or IRES, and regions with significant secondary structure that are required for synthesis of complementary, virion and viral messenger RNA. In conjunction with the VPg precursor as primer, sequences in both 5′-NTR and 3′-NTR, the RNA-dependent RNA-polymerase and virus-encoded accessory proteins become involved in viral RNA synthesis. The CVB and the adenoviruses bind to a cell surface receptor that is readily detected on tissues of the heart and other organs; the shared receptor belongs to the immunoglobulin super family and is known as the coxsackievirus-adenovirus receptor (CAR, reviewed in 6). CAR appears to be the major receptor for the CVB, although at least one other potential receptor, decay accelerating factor (DAF) has also been described (27). The CVB are classified within the family Picornaviridae and the genus Enterovirus. A reorganization of the family has taken place within the past two years due to new molecular data on genome construction and base composition, sharing of amino acid identity in viral proteins, sharing a limited range of host cell receptors, sharing a limited natural host range and sharing significant degrees of compatibility in replication processes (26). The genus enterovirus is now divided into 8 Species and includes 64 human enteroviruses; the previous six CVB serotypes are now classified within the Human Enterovirus B Species which contains 36 serotypes (26).

3.6 Origin of the CVB

Several strains of coxsackieviruses in groups A and B were isolated during 1948-50 from cases of children hospitalized during a small outbreak of paralytic disease in Coxsackie, New York, from cases with aseptic meningitis or fever in New Haven, Connecticut or from fecal samples obtained from children with no disease in several cities (18). Tissue culture methodology wasn't available to the pioneer medical researchers Dalldorf or Melnick who isolated these viruses in suckling mice where inflammatory pathology was noted in several organs, but focal inflammatory lesions were found in the heart and pancreas (1). Recognizing that many of these new viruses were not associated with poliomyelitis, in 1957 the Committee on Enteroviruses classified these viruses into new coxsackie A or B and ECHO virus groups. Subsequently in 1962, an International Subcommittee on Virus Nomenclature was established to classify animal viruses into major families on the basis of biochemical and biophysical properties (28). Family and genus status for these viruses, i.e., Picornaviridae and Enterovirus, were accorded in 1973 and 1976, respectively (18, 29). Many human studies confirmed a highly likely role for the CVB as etiologic agents for numerous cases of myocarditis (30, see reviews 2, 5, 6, 12, 15, 16, 18, 25, 31). Simultaneously, and as a result of the early virus isolation studies using mice to detect viruses, many basic researchers identified cardiovirulent CVB strains and employed the latter viruses in murine models that mimicked human heart pathology in several aspects. Among the CVB, CVB3 was the serotype most frequently associated with myocarditis, initially by serologic data and later by molecular detection of viral genomes (RT-PCR) data (2, 5, 12, 15-18, 25).

3.7 CVB3-murine models of myocarditis

Several CVB3-murine models have provided a plethora of information about virus-induced changes in cells of heart tissues and the myriad of innate and immune responses to the infection, all of which can contribute to the outcome of the infection (32, 33). Numerous protective host response factors attempt to clear the virus (34) and there may be a successful outcome in some mice: investigators who have worked with CVB3-murine models of myocarditis have repeatedly found that among a large group of male mice of similar age which were inoculated with the same dose of virus, several mice will show minimal to no evidence of inflammatory heart disease, whereas most mice will have moderate to severe myocarditis. Obviously, there are many parameters that affect the outcome of the infection and the investigator can manipulate some, but not all of them. In establishing a CVB3-murine model for studies of myocarditis, the major parameters that determine whether acute-resolving or acute-transiting-to-chronic myocarditis occurs, or death ensues are: murine strain, age and gender of the mouse, nutritional or general health status of the mouse and the cardiovirulence capability of the CVB3 variant. The effect of genetic background of a murine strain on potential outcomes that can result from challenge with a cardiovirulent CVB3 strain is shown in Figure 1. It is not known what innate or immune systems or heart tissue cell defect(s) is(are) present in an inbred strain that determine

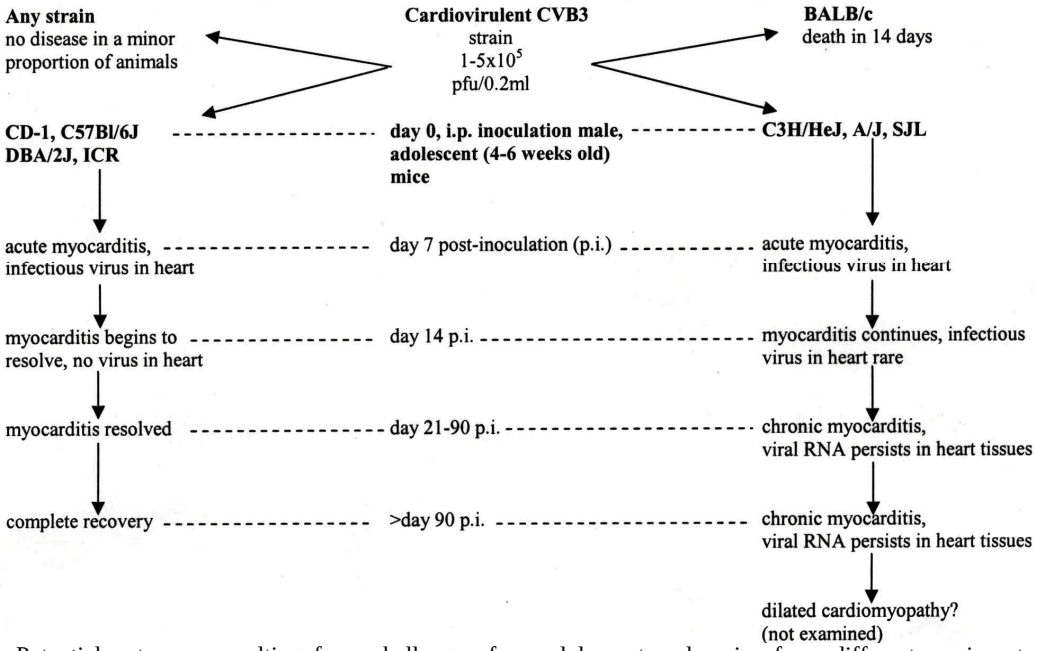

Figure 1. Potential outcomes resulting from challenge of an adolescent male mice from different murine strains with a cardiovirulent CVB3 strain.

whether the virus-induced acute disease is resolved or transits to chronic disease (2, 12, 15). In the acute disease, virus-induced interferon, nitric oxide and natural killer cells form a primary line of defense in reducing virus titers in cells and in limiting the severity of myocarditis (2, 18, 33, 34).

3.8. CVB3 and cardiopathology

Specific immune responses activated against CVB3 virions and infected cells are the likely mechanisms that contribute to clearance of infectious virus from the blood and organs of a virus-challenged mouse (2, 18, 34), although some of these responses may also contribute to cardiopathology (2, 15, 17, 25). Because of differences in genetic background among murine strains, it has been appreciated for years that different immunopathogenic mechanisms are operative in each murine strain inoculated with a cardiovirulent CVB3 to account for the destruction of heart tissues (2, 13, 15, 25, 35, 36). Although some controversy may still exist, most researchers believe that both virus-induced processes and immune responses against the CVB3 infection contribute to the cardiopathologic alterations found in the acute disease. Infectious virus and then subsequently, viral RNA, are cleared from the heart of most murine strains (Figure 1), and most humans (2, 12, 15, 25). In those few murine strains that permit persistence of low level synthesis of CVB RNA in heart tissues, the continued inflammatory responses to new cellular and shared viral/cellular epitopes are thought to be the major contributors to maintenance of the chronic myocarditis, although the persistent viral RNA in heart tissues likely plays a role in continuously directing the inflammatory response toward focal inflammatory lesions that were developed during the acute stage. It must be remembered that for even limited viral RNA synthesis to be maintained in the various heart tissue cells, e.g., myocytes, fibroblasts and perhaps endothelial cells, all viral proteins are produced at a low level and the viral proteases, particularly protease 2A, are toxic to cell proteins (2, 15, 16, 37). As will be discussed later, the persistent synthesis of viral products in infected cells most likely induces production of soluble proinflammatory mediators, the cytokines and chemokines, which stimulate the infiltrating inflammatory cells to produce more of these mediators to maintain the chronic inflammation (15, 38).

In experiments designed to elucidate the molecular basis for inducibility of myocarditis by the CVB genome, most studies focused on coxsackievirus B3 (CVB3) as the virus most frequently associated with the disease in children and adults (2, 7, 14-16, 25). Using naturally-occurring variants, mutants or chimeric molecular constructs generated between cardiovirulent and noncardiovirulent CVB3 strains, cardiovirulence in the CVB3 genome for adolescent males in several murine models has been mapped to several different single nucleotides in the 5′-NTR, to an unknown sequence in the 5′-NTR or to VP2 in the capsid region (reviewed in 1, 6, 16). It must be noted that only a small portion (<20%) of naturally-occurring strains of CVB3 are cardiovirulent for adolescent male mice (18). While virus is rarely isolated from adolescent or adult heart tissues of individuals with myocarditis, coxsackievirus strains are isolated from infants' and young childrens' heart tissues (6, 15, 16, 25); the proportion of these strains that are myocarditic for mice of any age is not known.

3.9. Innate host responses against CVB3 particles

There are several innate responses to virus particles circulating in the plasma phase of the blood

following intraperitoneal or per oral challenge of the host (15). Contact of virus with preB and B lymphocytes can result in minimal replication but more importantly, this interaction could perhaps contribute to immune deviation of the $CD4^+$ Th_0 subset to the pro-inflammatory $CD4^+$ Th_1 subset instead of the humoral immune $CD4^+$ Th_2 subset of cells (15). Direct interaction of particles of a highly cardiovirulent, but not a noncardiovirulent, CVB3 strain with presumed T lymphocytes (B220-negative) resulted in a rapid influx of Ca^{++} into isolated murine myocytes in culture that persisted for longer than 10 min (Huber et al, personal communication). As Ca^{++} influx can upregulate NFkappaB activity in T lymphocytes, enhanced transcription of mRNAs can occur for a variety of cytokines, chemokines and adhesion molecules, including IL-1alpha, IL-1beta, IL-6, IL-12, IL-8, ICAM-1 and molecules that regulate Bcl-x molecules (2, 15). A related picornavirus, rhinovirus, can activate NFkappaB but this activation follows an infection (39). Thus, many events that nonspecifically activate T cells can upregulate NFkappaB activity, which in turn subsequently can induce reactive responses from several components of the immune systems. It was also found that cardiovirulent strain CVB3 particle interactions with normal inguinal lymph node cells could induce an initial polyclonal non-antigen-specific production of cytokines in absence of any virus replication. It was hypothesized that this response is crucial to the subsequent gender-specific and antigen-specific immune responses that effect the final cardiopathologic outcome (Huber et al, personal communication). In fact, lymph node cells from male and female mice behaved differently in response to activation by infectious or ultraviolet light-inactivated CVB3 particles: within four hours, male cells produced primarily the pro-inflammatory cytokine IFN-gamma for activating Th_1 lymphocytes whereas female cells produced the humoral immune cytokine IL-10 that activates Th_2 lymphocytes, but little IFN-gamma (Huber et al, personal communication). The different responses of male and female cells to direct virus-induced signaling probably reflects the affect of sex-associated hormones on virus receptor expression. Androgens (testosterone and progesterone) stimulate a 6-fold increase in virus receptor expression on myocytes and endothelial cells, compared to estradiol (40). Having more receptors per cell should result in stronger signal transduction and enhanced responses in males and pregnant females compared to non-pregnant females. These data may be the earliest host response showing that a CVB3 infection will activate a Th_1 cell response that contributes via pro-inflammatory reactions to developing the moderate to severe myocarditis found in males, whereas the IL-10 induction directs a Th_2 humoral response in females which exhibit minimal to no myocarditis (41). Major receptors for the CVB include both decay accelerating factor (DAF or CD55, 42) and CAR (43), molecules with broad tissue distribution. It is of interest that cross-linking of DAF by antibody leads to several events: phosphorylation of the tyrosine kinase $p56^{lck}$, ZAP70 and the CD3 zeta chain, activation of T cells and production of IL-2 (44), another potential early signaling event that could involve virus particle binding to cells in absence of replication and influence pro-inflammatory events. CVB3 binds primarily to short consensus regions (SCR) 2 and 3 on DAF (42) and activation of $p56^{lck}$, which requires cross-linking of SCR3, is required for efficient replication of CVB3 and development of myocarditis, i.e., knock-out mice lacking $p56^{lck}$ do not develop myocarditis (45).

3.10. Innate host defenses against CVB3-infected cells

Once inside a permissive host cell in the heart (myocyte, fibroblast or endothelial cell), the viral single-stranded positive sense RNA genome is immediately translated into viral proteins. Among the proteins, the RNA-dependent RNA polymerase and several other viral proteins participate in synthesis of a complete negative (complementary) strand of RNA that serves as a template for synthesis of many genomic viral RNA molecules which, after removal of VPg, serve as mRNA. After repeating these cycles several times, large quantities of viral RNA and proteins accumulate and assembly of new virions and other virus particles commences and is exponential for several hours (2). Release of virions from endothelial cells and perhaps myocytes may be a result of virus-induced lysis, whereas the cardiac fibroblasts may not lyse and release is via an unknown mechanism. During replication, a viral process(es) stimulates synthesis of an enzyme, inducible nitric oxide synthetase which produces nitric oxide, an inhibitor of CVB3 replication (46). Production of viral RNA involves a transient double stranded RNA (dsRNA) intermediate which accumulates in infected cells. dsRNA is a powerful inducer of the production of interferons, notable antiviral molecules that can stimulate several antiviral proteins and some of the latter can lead to apoptosis of the infected cell (2). Other proteins activated by dsRNA can activate a major transcription factor (NFkappaB), which can upregulate production of several proinflammatory cytokines (2). Although CVB3 is not an enveloped virus, exocytosis of viral particles and viral peptide fragments to the cell surface attracts NK cells activated by virus-induced interferon and these activated cells then lyse virus-infected target cells (2, 15, 18).

3.11. Factors affecting the immune systems response against CVB3-induced myocarditis

Multiple parameters need to be considered when setting up CVB3-murine models of myocarditis. Obviously, murine strain responses to the virus at innate, cellular and immune levels determine whether the acute disease resolves or chronic disease then occurs (18). Infant to young mice less than four weeks of age and BALB/c mice almost always die when challenged with a cardiovirulent CVB3 strain (18), but pediatric models of both acute and chronic myocarditis have been developed using a CVB3 strain that is relatively noncardiovirulent in adolescent mice (18, 25, 33). Gender is a major factor in that male mice of all strains, like humans, develop far more severe CVB3-induced myocarditis than females in an age-matched group, the latter of which generally develop only minimal to no disease (2, 13). The type of $CD4^+$ T cells activated appears to be significant in explaining the gender difference in severity of disease presented: CVB3-infected males primarily activate the Th_1 inflammatory cell response whereas the infected females primarily activate the Th_2

humoral immune response (15, 41). Males also have higher virus titers in cardiac tissues than females, and absolute numbers of Th_1 and Th_2 $CD4^+$ T cells are increased in male and female mice, respectively (15). Hormone differences between the genders are a significant influence on the disease outcome in CVB3-challenged mice (47). Males who experienced castration prior to virus challenge had a severity in myocarditis that resembled that of age-matched females, but testosterone replacement in castrated male mice prior to virus inoculation restored the levels of myocarditis and $CD4^+$ Th_1 cells to those found in normal infected males (47). Conversely, treatment of females with testosterone increased the severity of myocarditis in females to the level experienced by normal males (47). In confirmation that the Th_1 cell response in males plays a significant role in inducing myocarditis, females that were given IFN-gamma or anti-IL-4 antibodies showed an increase in the virus-infected $CD4^+$ Th_1 phenotype and in the severity of myocarditis (2). Males also differ from females in making a selective activation of gamma/delta T cells in heart tissues, a parameter not observed in infected female mice (47). The gamma/delta T cells also effect a positive bias in directing $CD4^+$ Th_0 cells to a predominant Th_1 cell population phenotype, as treatment of infected normal males or testosterone-inoculated infected females with anti-gamma/delta T cell antibodies prevented both deviation to the Th_1 cell response and induction of myocarditis (48, 49). In addition, T cells that express the $Vgamma1^+$ T cell receptor are known to promote the $CD4^+$ Th_2 cell phenotype, whereas $Vgamma4^+$ T cells promote a bias toward the Th_1 phenotype; thus, giving females anti-$Vgamma1^+$ T cell antibodies increases CVB3-induced myocarditis whereas the converse, treatment of males with anti-$Vgamma4^+$ T cell antibodies suppresses virus induction of myocarditis (49). Differential and potentially cardiopathogenic roles for $Vgamma4^+$ T and $Vgamma1^+$ T cells were found, in that only the former cells lysed infected cardiac myocytes (49). It must be noted that male, but not female, mice activate large numbers of $CD8^+$alpha/$beta^+$ T cells that preferentially lyse uninfected syngeneic mouse cardiac myocytes cells in culture, suggesting that they contribute to cardiopathology (15). Finally, it has been shown that transfer of either $Vgamma4^+$ T or $CD8^+$alpha/$beta^+$ T cells into T cell-depleted CVB3-challenged male mice significantly increases myocarditis in a cell dose-dependent manner (15). Data from many studies (reviewed in 10) show that mice placed on protein-restricted, vitamin E- or selenium-deficient diets, or on a hypercholesteremic diet prior to challenge with CVB3 have a significantly increased severity of myocarditis over mice on a balanced healthy diet (50). Similarly, mice inoculated with CVB3 and administered immunosuppressive drugs during the acute phase of disease also have an increased severity in disease over untreated virus-challenged littermates (15, 18).

3.12. Immunological responses to a CVB3 infection

What are the adaptive immunological responses mounted by the host to a CVB3 infection and what factors modify these immune responses? Immune responses to a CVB3 infection in murine strains are mounted quickly via several types of humoral and cell-mediated reactions (2, 15, 18, 25). The humoral immune response to a CVB3 infection is rapid and can be protective or autoimmune in nature (15, 17). A protective role for antibodies has been established in numerous experiments in which passive administration of anti-CVB3 antibodies can significantly reduce the number of myocarditic lesions (17, 18). Relative levels of protection against CVB3-induced myocarditis among several murine strains have been correlated with early and high titer production of anti-CVB3 neutralizing antibodies (25). Another factor that likely contributes to a reduction in severity of CVB3-induced myocarditis in female mice is that females produce anti-CVB3 neutralizing antibodies more quickly and to higher titer than males (17). Females primarily respond to a CVB3 infection with a $CD4^+$ Th_2 T cell response and production of IgG_1 antibodies, whereas males respond with production of a $CD4^+$ Th_1 phenotype and production of IgG_{2a} antibodies (17). It must be noted that not all neutralizing anti-CVB3 antibodies are protective, and this is likely a result of some CVB3 strains sharing epitopes with normal host cell proteins (2, 15, 17). Several in a panel of monoclonal neutralizing antibodies generated against a CVB3 strain were found to participate in complement-mediated lysis of normal mouse heart fibroblasts, bind to the latter cells and induce production of a macrophage chemoattractant, and induce cardio-pathologic alterations in normal mice (17). In some mouse strains, such as DBA/2 animals, humoral immunity and CD4+Th_2 cell responses rather than the CD4+Th1 cell response of BALB/c are pathogenic (51). In this latter mouse strain, cardiac myosin, the probable autoantigen in myocarditis, is deposited outside the myocyte and is available for antibody binding and complement activation (52). Because enteroviruses are non-enveloped, anti-virus antibodies must cross-react to recognize cell surface molecules in order to be pathogenic. Although it is well established that anti-CVB3 antibodies generated subsequent to inoculation of mice with vaccine strains can protect mice against myocarditis induced by a cardiovirulent CVB3 strain, comparative studies between homozygous (nu–/nu–) nude and euthymic (nu–/+) mice clearly established that anti-CVB3 antibodies were insufficient to clear a CVB3 infection and prevent development of myocardial lesions (17). These data suggest that once a CVB3 infection is established, cell-mediated immune responses are also required to clear the virus.

Cell-mediated immune responses to a CVB3 infection are a significant factor in development of heart disease in the murine host. Inflammatory mononuclear cells in focal association with degenerating or necrotic myofibers are a hallmark of coxsackievirus-induced myocarditis in murine models and in human cases of the disease (12, 13, 15, 25). The lesions in the murine and human myocardia share many features in histology, composition of infiltrating cells and random distribution in the myocardium. The acute murine myocardial lesions contain macrophages, $CD4^+$ T (likely Th_1) lymphocytes, natural killer cells, $CD8^+$ T cells and gamma/delta T cells (15). Several types of host cells within or at the edge of each myocardial lesion are infected with CVB3 and include

myocytes, fibroblasts and perhaps some endothelial cells, in both the acute and the chronic diseases. Initially, it is likely that the infiltrating inflammatory cells are responding to the focal infections in a beneficial protective role for the host. Numerous studies in CVB3-challenged euthymic mice of several strains subsequently suggested that the continued presence of the inflammatory cells in the focal lesions most likely contribute to the pathology found in heart tissues (13, 15, 18, 31, 33). Several studies by Huber and colleagues (2, 15) showed that the types of CVB3-induced T lymphocytes involved in cardiopathologic reactions were murine strain-specific, e.g., in BALB/c mice, it is the $CD8^+$ T cells that serve as the major effector cell in cardiac tissue injury, whereas in DBA/2 mice, this cardiotoxic role is played by $CD4^+$ T cells, along with deposition of IgG autoantibodies in heart tissues (35, 36). Surprisingly, within the $CD8^+$alpha/beta T cell receptor (TCR+) cell populations from CVB3-challenged BALB/c mice, a major cardiopathologic autoimmune subset of cytotoxic T cells preferentially was found to lyse uninfected syngeneic target cells in culture. It was only this latter subset, and not the subset that lysed virus-infected target cells, that could transfer cardiopathologic alterations to uninfected normal mice (2, 15, 53). Viral epitopes recognized by T cells have been identified in linear sequences of the largest capsid polypeptide (VP1) of three that comprise the surface of the CVB3 capsid (54). Immunization of mice with peptides representing several of these epitopes prior to challenge with a cardiovirulent CVB3 showed that myocarditis was either reduced or exacerbated, showing that T cell immunity can be protective or cardiopathologic (54). As cited above, data from several studies showed that events or factors that effected an immune deviation toward the $CD4^+$ Th_1 type of inflammatory cell during a CVB3 infection exacerbated the heart disease. Recent studies in the last few years detected a new type of T lymphocyte, the gamma/delta T cell, which accumulates at inflammatory sites and can modulate susceptibility to diseases by enhancing or suppressing the inflammatory response (49, 55). In the CVB3-infected mouse, gamma/delta T cells concentrate in virus-infected heart tissues and contribute to establishing the Th_1 cell phenotype, perhaps by selectively inhibiting the Th_2 cell response (54, 55). Treatment of CVB3-infected male or infected and testosterone-treated female mice with antibody to gamma/delta T cells resulted in a shift in the T lymphocyte phenotype, from Th_1 to Th_2, and a significant decrease in severity of myocarditis (47). Activated gamma/delta T cell subpopulations may also act via regulation of the MHC class II IE antigen that plays a role in susceptibility of certain hosts to myocarditis induced by CVB3 (56). In follow-up studies of the latter CVB3-BALB/c model, it was shown that subsets of gamma/delta T cells have different roles in these animals: the $Vgamma1^+$ subset contributes to resistance to disease, likely via production of IL-4 and suppression of $CD4^+$ Th_1 cell responses, whereas the $Vgamma4^+$ subset induces susceptibility to myocarditis (49, 55). Unlike $CD8^+$ alpha/beta T cells, the $Vgamma4^+$ T cell subset cannot transfer myocarditis to uninfected mice (56.). Table 1 summarizes the types and subsets of T cells induced during a CVB3 infection of a murine host with acute or chronic myocarditis, and indicates a predicted role for each type/subset of T cell in protection or cardiopathology.

3.13. Cytokines involvement in CVB3-induced myocarditis

Cytokines are always produced in any inflammatory setting, and the type and quantity of cytokines induced during a CVB3 infection of cells in heart tissues, and from the concomitant infiltrating inflammatory cells responding to the foci of infection, varies with the gender and genetic background of the murine strain (32, 38, 57). Cytokine expression in heart tissue cells of mice with CVB3-induced myocarditis was deduced to include predominantly IL-1alpha, IL-5, IL-6 and IL-7, whereas the inflammatory leukocytes infiltrating the heart predominantly expressed IL-1beta, IL-2, IL-3, IL-4, IL-10, TNF-alpha, TNF-beta and IFN-gamma (58). CVB3 infection of mice results in induction of several cytokines during the acute and chronic stages of infection, i.e., IFN-gamma, TNF-alpha, IL-1 and IL-6 (59). Similar kinds of cytokines are produced in CVB3-infected cultured human monocytes (60) or human myocardial fibroblasts (61), or in patients with myocarditis or dilated cardiomyopathy (62, 63). Experimentally, exogenous administration of cytokines to CVB3-challenged mice can significantly effect the outcome of whether mice develop more severe myocarditis or not at all (2, 15, 32, 57). Thus, addition of IL-1 or IL-2 to infected mice can exacerbate the disease (64) or in the case of a pediatric CVB3-murine model, a murine strain that is resistant to development of CVB3-induced chronic myocarditis can be induced to develop disease when inoculated with IL-1 or TNF-alpha (65). In CVB3-infected male mice, expression of TNF-alpha and IFN-gamma by $CD4^+$ Th_1 cells promoted infiltration of T cells and macrophages into virus-induced myocardial lesions, and also upregulated expression of intercellular adhesion molecule 1 (ICAM-1, 66). Cytokine production by gamma/delta T cell subsets is also significantly influential in directing the type of CD4 Th cell phenotype (Th_1 or Th_2), and the susceptibility of mice to CVB3-induced myocarditis (67).

3.14. Autoimmunity in CVB3-induced murine myocarditis

CVB3 infections of mice and humans induce autoimmune reactions in both humoral and cell-mediated immune systems (15, 17, 32). Epitopes shared by surface regions of the three capsid polypeptides and normal cell proteins have been readily demonstrated by showing that during a CVB3 infection of mice, autoantibodies were detected against several cell antigens by cytolytic complement-fixation assays, ELISA titrations, by immunoblot reactions and/or by immunocytochemistry (15, 18, 37). In infected mice, a list of the normal murine cell proteins that share epitopes with CVB3 capsid proteins, as recognized by autoantibodies or autoreactive lymphocytes, is presented in Table 2 (15, 17, 32). An extensive list of host cell antigens to which autoantibodies have been detected in sera of patients with myocarditis or dilated cardiomyopathy is presented in Table 3 (68, 69). Approximately half of the cases of myocarditis and up to 20% of the cases of IDC are associated with CVB

Table 1. Types/Subsets of T Lymphocytes Induced During CVB3 Infections of Mice and a Predicted Role for Each in Protection Against or Contributing to Cardiopathology

Type of T Lymphocyte	Subset	Acute or chronic Myocarditis	Role in protection or cardiopathology
CD4	Th_1	Acute, chronic	Cardiopathology
	Th_2	Acute, chronic	Protection/cardiopathology (DBA/2)
$CD8^+$alpha/beta	Attack normal cells	Acute	Cardiopathology
$CD8^+$gamma/delta	Attack infected cells	Acute	Protection
$CD8^-$gamma/delta	$Vgamma1^+$	Acute, chronic	Protection
	$Vgamma4^+$	Acute, chronic	Cardiopathology

Table 2. CVB3 Infections of Mice Induce Autoreactive Immune Responses to Heart Tissue Antigens

Autoantibodies induced during CVB3 infections of mice to:
• Cardiac myosin (LMM chain)
• Branched chain keto acid dehydrogenase (BCKD)
• Adenine nucleotide translocator (ANT)
• Cardiac sarcolemma
Autoreactive T lymphocytes induced during CVB3 infections of mice to:
• Cardiac Myosin (several epitopes in LMM chain)

Table 3. Autoantibodies induced in humans with myocarditis or dilated cardiomyopathy

Cardiac myosin (alpha and beta isoforms)	Acetylcholine receptor
Laminin	Aconitate hydratase
Branched chain keto acid dehydrogenase (BCKD)	Pyruvate kinase
Adenine nucleotide translocator (ANT)/Ca^{++}channel proteins	Dihydrolipoamide dehydrogenase
beta-adrenergic receptor	Carnitin
Actin	Desmin
Fibrillary proteins	Vimentin
Creatinine kinase	Nicotinamideadenine dinucleotide
Cardiac sarcolemma	Ubiquinol-cytochrome c-reductase
Cardiac myolema	Heat shock proteins 60 and 70
	Muscarinic receptor

infections, and 40 to 100% of the latter patients were found to have autoantibodies to heart tissue antigens (25, 68-70). Antibodies in sera from either type of patient recognized several autoantigens and epitopes on purified CVB3 or CVB4 particles (71, 72). In some biopsy specimens of human heart tissues, autoantibodies were deposited with or without complement components (71). It must be stressed that these data apply only to products (antibodies) of autoimmune reactions and not autoimmune disease. The latter description is reserved for the pathologic consequences of autoimmune phenomena (15). Autoantibodies and autoreactive lymphocytes are frequently found in healthy individuals, i.e., 20% of asymptomatic individuals (15). In CVB3-murine models of myocarditis, anti-cardiac myosin antibodies or T lymphocytes reactive to this antigen were frequently found in the chronic phase of disease (25). Several types of data show that either of these immunoreactants can have cardiopathologic consequences for the host: 1) transfer of either autoantibodies or autoreactive T lymphocytes to normal mice could induce disease, and 2) immunization of normal mice with murine cardiac myosin in complete Freund's adjuvant could induce a myocarditis whose lesions were indistinguishable from those induced by CVB3 (15).

The breakdown in self tolerance in CVB3-induced myocarditis can most likely be explained by one of two major mechanisms: 1) molecular mimicry or 2) a sequestered antigen(s) normally hidden from immune surveillance. The myosin-induced disease likely represents autoimmunity to a normally sequestered self antigen that is released from cells lysing as a consequence of either a virus-induced mechanism(s), a host response (innate [NK cells] or adaptive immunity directed [complement-mediated lysis via antibody-antigen complexes on the cell surface or virus-specific $CD8^+$ T lymphocytes]) against an infected cell (15). Interstitial (dendritic) cells in the heart probably ingest the released cardiac myosin and present portions of this large molecule complexed with major histocompatibility (MHC) class II antigens to CD4 T cells. However, cytokines must be released during expression of the ongoing inflammatory reactions against the infected cells to effect an autoimmune response (15).

Evidence for molecular antigenic mimicry between cardiac myosin and CVB3 capsid proteins has been directly obtained from studies of several monoclonal antibodies (mAb; 2, 15, 73). Neutralizing anti-CVB3 mAb could bind to epitopes on murine or cardiac myosin, induce minimal but reproducible cardiopathologic alterations in normal mice, and significantly exacerbate myocarditis in CVB3-challenged mice (73). These data suggest that autoantibodies to epitopes shared by virus capsid proteins and cardiac myosin do not induce myocarditis, but they could contribute as an accessory factor in exacerbation of

disease (73). In one CVB3-murine model of myocarditis, severity of cardiopathology correlated with titers of eluted anti-cardiac myosin, anti-ANT or anti-BCKD antibodies that had been deposited in heart tissues (74). Murine strains that fail to resolve CVB3-induced acute disease and develop chronic myocarditis produce IgG autoantibodies against cardiac myosin that bind to heart tissues (25). In other studies, several mAbs against a group A streptococcus, an etiologic agent of rheumatic heart disease, could bind human cardiac myosin and neutralize CVB3 (73). These mAbs were used to select escape mutants of CVB3 that were altered in their host range, i.e., the mutants induced more severe disease in murine strains in which the parent virus was only minimally cardiovirulent (73). However, one mAb that could distinguish between cardiovirulent and noncardiovirulent CVB3 selected a CVB3 escape mutant with reduced pathogenicity, showing that not all mimicking epitopes on the virus were associated with increased pathogenicity. There are no convincing data on a role for autoantibodies in cardiopathogenesis in humans (15, 32), although heart tissues from humans or mice with chronic myocarditis contain IgG extensively bound throughout the tissues (73 and the severity of heart impairment in patients with chronic myocarditis was correlated to levels of antibody eluted from heart tissues in one study (75)

Antigenic mimicry also occurs in T lymphocytes isolated from CVB3-infected mice (76). Initial studies by Cunningham et al (77) showed that monoclonal antibodies derived from mice immunized with Group A streptococcus also neutralized CVB3 and reacted with cardiac myosin. One specific monoclonal antibody (clone 49.8.9) distinguished between the myocarditic and non-myocarditic variants of CVB3. Thus a bacteria inducing autoimmune heart disease and a virus inducing autoimmune heart disease must share a common antibody epitope with cardiac myosin, the presumed dominant autoantigen in myocarditis. T cells from CVB3-challenged mice with myocarditis predominantly recognized a peptide (19mer) in the streptococcal M5 protein; this peptide, designated NT4, contains both T- and B-cell epitopes that are cross-reactive with cardiac myosin. NT4 could induce a $CD4^+$ T cell-dependent myocarditis in mice. However, if mice were first tolerized to NT4, they were then partially protected against CVB3-induced myocarditis (15). Finally, $CD4^+$ T cells have been isolated from heart tissues of CVB3-challenged mice with myocarditis and found to respond in culture to CVB3 particles, cardiac myosin and NT4 (76).

3.15. Differences between CVB3-challenged murine strains that resolve acute myocarditis versus strains that develop chronic disease

The identification of murine strains that can transit from CVB3-induced acute to chronic myocarditis, or that can stop the acute disease and completely recover, has permitted investigators to examine mouse heart tissues to assess major changes that occur in either situation. Murine strains that develop only CVB3-induced acute-resolving myocarditis transiently produce only anti-cardiac myosin IgM autoantibodies that minimally bind to heart tissues, whereas murine strains that develop CVB3-induced chronic myocarditis produce anti-cardiac myosin IgG autoantibodies that bind extensively to heart tissues (25). Only those specific murine strains that developed chronic CVB3 myocarditis were identically the only murine strains that could also develop nonviral cardiac myosin- or ANT protein-induced myocarditis (32, 73). The data from those studies reinforce the notion that the genetic background of the mouse directs the type of cell-mediated immune responses which determine whether CVB3-induced chronic myocarditis develops. Those murine strains in which virus infection induces gamma/delta T cells will subsequently promote a Th_1 inflammatory cell response against infected cells in heart tissues, a type of immune response crucial to development of chronic disease (35, 56, 73). Another major parameter that likely contributes to maintenance of chronic inflammation in the heart of select murine strains is the persistent synthesis of viral RNA in absence of infectious virus (37). Viral RNA synthesis has been detected in heart tissues of mice and humans with myocarditis by *in situ* hybridization (78) and by RT-PCR (79). Only murine strains that develop chronic myocarditis in response to inoculation by a cardiovirulent CVB3 develop persistent viral RNA synthesis in heart tissues, whereas murine strains that resolve the disease do not permit this continued synthesis of viral RNA (37, 80). A well-studied noncardiovirulent CVB3 strain ($CVB3_0$) did not persist in heart tissues via RNA synthesis in a murine strain capable of developing chronic myocarditis (37). In studies of persistence of viral RNA synthesis in a transformed mouse cardiac fibroblast cell line, data were generated which suggested that virus-induction of IFN-beta likely played a contributing role in maintenance of viral RNA synthesis at a low level in these cells (37). It was hypothesized that the continued presence of viral RNA, likely at sites of focal inflammation in mouse heart tissues (78), induced production of proinflammatory cytokines that participated in maintaining the inflamed state in the heart (37).

3.16. Myocarditis, prevention and treatment

The myriad of infectious agents and toxic drugs that can induce myocarditis currently precludes any meaningful plans for prevention of myocarditis by a polyvalent vaccine. Earlier clinical trials for patients with myocarditis involved therapy with polyclonal gamma globulin or interferon and the results showed promise (15). A new trial of interferon treatment for patients with myocarditis will be initiated quite soon in Germany (H.-P. Schultheiss, personal communication). A recent multicenter trial involving immunosuppressive therapy for treatment of histologically verifiable myocarditis (Dallas criteria) in U.S. patients was found to be without any significant benefit (11). Currently, bed rest and supportive therapy is the recommended treatment for patients with myocarditis. Murine-CVB3 models of myocarditis have taught us that multiple mechanisms of immunopathology contribute to myocarditis, depending on the strain of mouse involved (15), and that immunosuppressive therapy by drugs such as cyclosporin A only prove beneficial in mice with specific types of immunopathogenic mechanisms of myocarditis (81). At this time, one viable target inviting treatment is the low level persistent synthesis of CVB3

RNA in heart tissues of mice and humans, i.e., an inhibition of the RNA-dependent RNA polymerase that is synthesized continuously at a low level in these cells (37). Unfortunately, unlike the anti-reverse transcriptase drugs available to people infected with HIV-1, there are no experimental drugs available that specifically target enteroviral RNA polymerases. Such drugs could offer great promise for therapy of myocarditis and likely for some cases of dilated cardiomyopathy and these drugs could potentially have low cytotoxicity, as normal eukaryotic cells do not possess such an enzyme.

4. CONCLUSIONS

The CVB3-murine models of acute and/or chronic myocarditis have convincingly established that major virus-induced pathologic mechanisms are murine strain-specific and have a strong gender (male) bias. Recent data suggest that the initial interactions of CVB3 virions entering the blood with cells of the innate defenses can affect subsequent adaptive immune responses and determine severity of disease induced. Information obtained from comparative studies of responses induced by viral infections in susceptible males versus those found in relatively resistant females have defined and identified roles of the immune systems in defense versus cardiopathologic processes. Contributions of heart-specific gamma/delta T cell subsets to these processes are being determined. The roles of various cytokines in development of the disease are under intense investigation. Autoimmune humoral and cell-mediated immune responses, including molecular mimicry, with potential roles in the disease process are well documented. The correlation between RT-PCR detection of persistent viral RNA synthesis, but not infectious virus production, in heart tissues of CVB3-challenged mice with chronic disease is quite strong. This finding appears to have prognostic value for patients with the disease. Molecular studies show that cardiovirulence in the CVB3 genome generally maps to a region near the 5'-terminus that does not encode a protein, however, the genomic region associated with persistent viral RNA synthesis is not known. Also, we still don't understand at a molecular level what is different between cells of the heart or in immune responses of specific murine strains that develop chronic disease which permit continued residency and persistent synthesis of viral RNA versus similar parameters of most murine strains that block virion production and persistent viral RNA synthesis in heart tissues which undoubtedly contribute to resolution of the acute disease. Because CVB3-induced cardiopathogenic responses are murine strain-dependent, experimental immunosuppression treatments are efficacious only in certain strains, in parallel agreement with the anecdotal range of outcomes of immunosuppressive treatment of humans with myocarditis. Thus, the complexity of myocarditis found in CVB3-murine models underscores the need for further studies to identify major host immune responses that would be amenable to treatment, a small step toward providing some future hope to patients suffering from myocarditis due to one major group of etiologic agents, the coxsackieviruses of group B.

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