Preface

Many readers may recall a family member, friend, or acquaintance who died suddenly with no history of heart disease or who acutely developed heart failure of unknown cause. These are common clinical presentations of patients who have acute myocarditis. Myocarditis, often related to a viral infection, contributes substantially to dilated cardiomyopathy and the burden of heart failure worldwide. In the 8 years since the last monograph on myocarditis was published, knowledge of viral-induced myocardial injury, autoimmune pathways in the heart, and the clinical treatment of myocarditis has advanced significantly. New cardiotropic viruses have been described. The causal mechanisms between acute viral infection and dilated cardiomyopathy have been elucidated in great detail. It is timely to draw together in one volume the threads of research on viral myocarditis, autoimmune myocardial injury, and clinical advances in myocarditis for the benefit of the practicing physician and the specialized researcher. This book is written equally for the clinician confronted with suspected myocarditis and the specialized investigator. It seeks to give each a framework and greater context for study.

This is the first volume that attempts to cover the entire spectrum of myocarditis from basic research to bedside medicine. The first chapter provides an introduction to experimental myocarditis. Dr. Charles Gauntt is an authority in the field of virology who is well equipped to provide such an overview. The members of Dr. Steven Tracy's premier enteroviral research laboratory contributed the second chapter on viral life cycle and the earliest events in the molecular pathogenesis of experimental coxsackie and adenoviral myocarditis.

Chapters 3 through 6 address the immune reaction that results in postviral myocarditis. The multifaceted immune response is described by several closely related topics. Dr. Sally Huber begins the discussion with a chapter on the cellular immune response, followed by Dr. Bernhard Maisch's chapter on the humoral immune response. These chapters emphasize the central role of Th1 and Th2 lymphocytes in postviral autoimmune myocarditis. In Chapter 5, Dr. Akira Matsumori, discusses the beneficial and detrimental roles of cytokines in postviral myocarditis. The terrific expansion of knowledge of nitric oxide biology justified a separate chapter by Drs. Joshua Hare and Charles Lowenstein on the roles of nitric oxide in viral infection and postviral heart disease.

The next 4 chapters cover translational research and seek to bridge basic biologic investigations and the clinical disorders. Dr. Bruce McManus and his laboratory colleagues cover extensive experimental data on the role of programmed cell death, apoptosis, in viral myocarditis in Chapter 7. Dr. Makoto Kodama describes experimental autoimmune (versus postviral) giant cell and lymphocytic myocarditis in rat and mouse models in Chapter 8. The role of the adrenergic system in experimental and human dilated cardio-

myopathy and the implications for treatment of human disease are described next. This section concludes with a chapter on the latest available data on enteroviral proteases and cardiomyopathy from Dr. Kirk Knowlton's laboratory.

The diagnosis, prognosis, and treatment of nonspecific and specific myocarditides are covered in Chapters 11 through 24. The strengths and limitations of noninvasive tests, including serologic biomarkers, measures of apoptosis, nuclear imaging, and echocardiography, are covered in detail. The technique and interpretation of endomyocardial biopsy are placed at the center of this section. Separate chapters are devoted to major idiopathic clinical entities, including cardiac sarcoidosis, giant cell myocarditis, and the eosinophilic myocarditides. Specific infectious diseases that affect the heart include Chagas disease, rheumatic fever, and human immunodeficiency virus-related cardiomyopathy. The book concludes with state-of-the-art chapters on myocarditis in children and peripartum cardiomyopathy.

A second objective of this book is to foster exchange of new ideas between basic and clinical investigators through a collation of parallel, related research. Leaders in experimental myocarditis can read of their clinical colleagues' latest progress and opinions and vice versa. I hope this book forms a platform for new research collaborations to grow. I am heartened that students of the clinical and basic sciences can glimpse in these chapters the excitement of participation in a decades-long, worldwide, multidisciplinary effort and sense the satisfaction of many investigators.

I thank all of my colleagues who have generously contributed to this work, in particular, Dr. Kirk U. Knowlton for his counsel and encouragement early on, for without him this project would not have started; Dr. Joseph G. Murphy, whose experience I sought on many occasions and who heard my frustrations and shared in the pleasure of the final proof; Dr. Carol L. Kornblith (editor), who read every word at least four times and patiently heard and taught me; and the production staff, including Kathryn K. Shepel (art director), Roberta J. Schwartz (production editor), Virginia A. Dunt (editorial assistant), and John P. Hedlund (proofreader). I thank my dear wife, Jane, without whose support and encouragement (and ruthless editing) this project could not have happened.

Leslie T. Cooper, Jr., MD

CHAPTER

2

The Primary Viruses of Myocarditis

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INTRODUCTION

In any review of the viruses responsible for causing human myocarditis, one naturally focuses on the 6 serotypes of the group B coxsackieviruses (CVB1-6) (Table 2-1), human enteroviruses that have been well-established as primary causes of the disease since the mid-1950s. However, data from the mid-1990s suggested 2 other players whose roles seem also to be significant in the causation of this disease. Human adenovirus (Ad) DNA, specifically from adenovirus type 2 (Ad2) (Table 2-1), has been detected in a large proportion of human myocarditis cases. To date, there are no confirmed data suggesting that other Ad serotypes are involved, but it is not clear whether sufficient work has been done to screen for and so eliminate the possibility of other genotypes. Hepatitis C virus (HCV) (Table 2-1), a flavivirus, has been identified as a potential cause of human myocarditis, especially in studies of Japanese patients, although the evidence for a causal link elsewhere is less sound. Many viruses other than these 3 distinct viruses have been named as real or potential agents of viral inflammatory cardiomyopathy, but these 3—CVB, Ad2 and maybe other Ad, and HCV—seem, at present, to be the primary viral causes of the disease on the basis of isolation of virus from diseased tissue, serologic studies, or some type of

Table 2-1
Three Viruses Closely Linked as Agents of Human Myocarditis

Virus and classification	Virus description
Coxsackievirus, group B; genus *Enterovirus*, family Picornaviridae	Nonenveloped, icosahedral capsid, single-stranded RNA genome, positive sense, 7.4 kb (http://life.anu.edu.au/viruses/Ictv/fs_picor.htm#Genus1)
Adenovirus, genus *Mastadenovirus*, family Adenoviridae	Nonenveloped, icosahedral capsid, double-stranded linear DNA genome, 36-38 kbp (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_adeno.htm#Genus1)
Hepatitis C virus, genus *Hepacivirus*, family Flaviviridae	Enveloped capsid, linear, single-stranded RNA, positive sense, 9.4 kb (http://life.anu.edu.au/viruses/ICTVdB/26030001.htm)

Data from International Committee on the Taxonomy of Viruses, Virology Division, International Union of Microbiological Societies. http://www.ncbi.nlm.nih.gov/ICTV/ (February, 2000). Murphy FA, Fauquet CM, Bishop DK, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD. Virus taxonomy: the sixth report of the International Committee on Taxonomy of Viruses. Vienna: Springer-Verlag, 1995.
All the Virology on the World Wide Web at http://www.virology.net
To view images of these viruses online, see All the Virology on the World Wide Web, The Big Picture Book of Viruses, http://www.virology.net/Big_Virology/BVHomePage.html
Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE, eds. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven, 1996.

molecular detection method such as reverse transcription polymerase chain reaction (RT-PCR) or nucleic acid hybridization. Although this review deals with these 3 groups of viruses specifically, the reader should understand that these are not the sole agents of viral myocarditis but those for which strong cases have been or can be made for a primary etiologic role. This review examines papers published mainly since 1996; we direct the reader to earlier[1-4] and more recent[5-11] reviews for other references.

GROUP B COXSACKIEVIRUSES: INTRODUCTION

The CVB are human enteroviruses (family Picornaviridae), a genus that includes the well-studied and closely related polioviruses.[12] There are 6 CVB serotypes (CVB1-6); by definition, immunity against 1 serotype does not confer immunity against any of the other 5 serotypes. The CVB are typical enteroviruses and, after the polioviruses, are the best studied enterovirus group.[1,7]

The CVB genome is a single strand of positive (message) sense RNA, 7,400 nucleotides in length.[13] The 5′ end of the genome is not capped but is linked covalently to the viral protein, VPg. The single open reading frame is preceded by the 5′ nontranslated region (5′ NTR), which at 741 nucleotides represents 10% of the viral genome. As in all picornaviruses, the enterovirus 5′ NTR is a highly structured RNA sequence[14] whose functions include both viral protein translation and viral genome replication.[15-19] Although the primary structure (nucleotide sequence) of enteroviral or different CVB serotype 5′ NTRs differs significantly, the overall higher-order RNA structures that are predicted to form are sufficiently similar to permit artificially engineered viruses, in which the 5′ NTR from one enterovirus is replaced by that from another, to replicate and function in cell culture at near wild-type levels.[20,21] The CVB ORF encodes 11 proteins within a polyprotein of 2,185 amino acids. Two viral proteases process the CVB polyprotein cotranslationally so that, as in polioviruses and other picornaviruses, no full-length viral polyprotein is observed. The structure of the CVB3 capsid has been solved[22] and it, like other picornaviruses, is an icosahedron, made up of 60 copies each of the 4 viral capsid proteins. The virus receptor, human coxsackievirus and adenovirus receptor (CAR), a protein of the immunoglobulin superfamily,[23-25] most likely interacts with the virus capsid in the depressions (called canyons) that surround the 5-fold axes of symmetry. Interestingly, CAR is also a primary receptor for the Ad (see below).

EVIDENCE FOR CVB INVOLVEMENT IN MYOCARDITIS

Strong evidence supports an etiologic role for the CVB in myocarditis and dilated cardiomyopathy. From the mid-1950s onward, coxsackieviruses had been repeatedly isolated

from myocarditic hearts;[26-31] furthermore, models of the disease were being established that indicated the virus could cause myocarditis in mice. The first demonstration that enteroviral RNA could be detected in myocarditic hearts was published by Bowles and colleagues;[32]these workers used slot blot detection of human heart RNA that was probed with radioactively labeled complementary DNA (cDNA) transcribed from CVB3 RNA. Much interest was generated by the findings that claimed as positive for viral RNA more than 50% of the myocarditic heart samples assayed. The claim that the viral RNA detected was specifically coxsackieviral represented a misunderstanding of the technique, which as designed was sufficiently generic to enable the detection of RNAs from many different enteroviruses. Numerous subsequent studies used filter hybridization, in situ hybridization, and RT-PCR to probe for the presence of enterovirus RNA in human heart tissues (reviewed in references 2 and 5). While some studies failed to detect any samples that were positive for enteroviral RNA, most detected enteroviral RNA in approximately 20% to 25% of hearts examined. Unfortunately, no multicenter study—to avoid possible biases or errors specific to a single laboratory and to screen samples from around the world—has yet rigorously defined by sequence the enteroviral genotypes detected in numerous endomyocardial biopsy or explanted heart muscle samples.

As representatives of all the CVB genomes have been sequenced and as there is a deep database of enteroviral genomic sequences, the design of the appropriate primers for the identification and characterization of the primary enteroviruses by sequence analysis is straightforward. The diagnostic sequence to target should be the region encompassing the junction of the capsid protein 1D with the viral protease 2Apro.[33-35] The strong molecular evidence of enteroviral involvement with myocarditis combined with the earlier findings of various CVB serotypes in diseased hearts and the excellent models of murine myocarditis that recapitulate many of the human disease symptoms make it highly likely that the CVB are the enterovirus most often responsible for causing human myocarditis.

THE COXSACKIEVIRUS-ADENOVIRUS RECEPTOR

Evidence that several of the coxsackieviruses and Ad use a common cell surface receptor was provided initially by experiments that tested the abilities of different viruses (CVB1, CVB3, and Ad2) to interfere with one another for binding to and infecting target cells.[36,37] These early results have now been confirmed in detail. The shared receptor is commonly referred to as CAR (HCAR and MCAR are used to denote CAR of human or mouse origin, respectively). The CAR has been shown to function as the cellular receptor for Ad subgroups A, C, D, E, and F as well as the CVB.[38]

Three laboratories concurrently reported the isolation and identification of CAR as a unique new membrane protein in 1997.[23-25] All 3 of these groups used reagents or methods developed earlier in Crowell's laboratory.[39,40] The CAR amino acid sequence has been

determined both by partial amino-terminal sequencing and by sequence analysis of the cloned cDNAs. The CAR is a member of the immunoglobulin superfamily of proteins, containing an amino-terminal V-like domain (frequently referred to as D1), followed by a C2-like domain (D2), a single membrane-spanning sequence, and a 107-residue cytoplasmic domain (Fig. 2-1; see color plate 1). The amino acid sequence provides a calculated molecular weight for the polypeptide chain of 37,969 after removal of the signal sequence. The mature protein that is expressed in cultured cells is glycosylated; there are 2 AsnXSer/Thr sites, 1 in each extracellular domain, which potentially contain N-linked carbohydrate. By sodium dodecyl sulfate-polyacyl-amide gel electrophoresis, the mature protein has an apparent mass near 46 kDa. Neither the cytoplasmic domain nor the membrane-spanning domain are required for CAR to function as a receptor for Ad or CVB: viruses of both groups can infect cells which express only the extracellular domain anchored to the membrane by either the native membrane-spanning sequence or glycophosphatidylinositol[43,44] (Carson S, Chapman N, unpublished data).

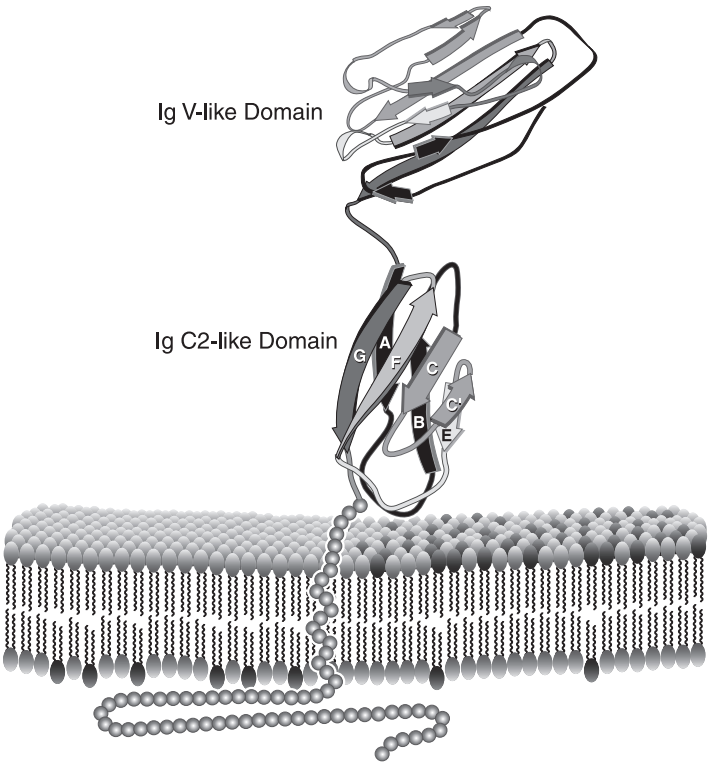

Fig. 2-1. Human coxsackievirus and adenovirus receptor. See color plate 1.

Adenoviruses bind the V-like domain of CAR. The structure of the Ad12 fiber knob complexed with the V-like domain, produced in *Escherichia coli*, has been solved.[41] Three CAR V-like domains bind a single fiber knob. The structure of the C2-like domain and the overall CAR structure remain to be determined, but similarity to C2 domains of other members of the immunoglobulin superfamily is anticipated. Although several groups have expressed the individual V-like and C2-like domains, the binding site for CVB has not been reported. The silence on this issue suggests that CVB binding may require contributions from both domains or that carbohydrate moieties, present on the native protein but absent from protein that is artificially expressed in *E coli*, play a role in CVB association with the CAR. The potential importance of glycosylation on CVB binding was suggested by experiments that predate the isolation and characterization of CAR.[45] Although virus overlay blots use radiolabeled CVB to detect CAR isolated directly from cultured cells, such approaches fail to demonstrate CVB3 binding to the glycosylation-deficient extracellular domain of CAR expressed in *E coli* (Carson S, Chapman N, unpublished data).

Interestingly, the CAR has a high degree of sequence homology to A33, a protein known for its prevalence on colon carcinoma cells, and CTX, a *Xenopus laevis* protein expressed on thymocytes; the chicken and human equivalents of CTX have also been cloned and are quite similar.[46] This group of closely related proteins has an unusual second disulfide bond in the C2-like domain that is not present in other immunoglobulin superfamily proteins, indicating that they represent a subgroup that has been conserved during evolution. The degree of evolutionary conservation from amphibians to humans portends an important but unknown physiologic function for CAR.

The localization of the CAR gene to chromosome 21 was inferred by matching the CAR sequence data with sequences in the Expressed Sequence Tag database that had been assigned to chromosome 21.[23-25] Subsequently, mapping studies localized the CAR gene to 21q11.2 and identified 3 pseudogenes located on chromosomes 15, 18, and 21. The CAR gene has been characterized further in the context of genomic organization and shown to be divided into 7 exons.[47] Regulatory elements that control CAR transcription and translation remain to be identified, and evidence suggesting that CAR expression is highly regulated (discussed below) indicates that such elements will be interesting and important.

With rare exceptions (see below), CVB infect only cultured cells which express CAR (eg, HeLa cells but not RD cells). It may, therefore, be anticipated that CAR should be expressed in tissues and organs that are infected by CVB. Similar expectations may hold for Ad infection, but established roles for coreceptor proteins and for secondary receptors indicate that simple interpretations of infection-receptor expression association may not be generally appropriate with Ad (eg, alpha V integrins[48] and glycosaminoglycans[49]). The CVB are appreciated for their ability to infect brain, pancreas, and heart. Northern blot analyses readily detected CAR mRNA in human pancreas, heart, prostate, testis, intestine,

and brain.[25,50] The CAR mRNA was also present in murine kidney, liver, and lung. Although northern blot analyses failed to detect CAR mRNA from human lung, human airway epithelial cells in culture have been shown to express CAR on the basolateral aspects of the cell surface.[51]

Consistent with decreasing susceptibility of both mice and humans after the early neonatal period, recent studies have found decreased expression of CAR in muscle cells of adult rats and mice.[52,53] Similar results were obtained in vivo in mice.[54] In rats, immunohistochemical analysis revealed broad neural and epithelial distribution of CAR during development, with expression more pronounced in epithelial cells of adult animals.[55] Significantly, CAR was reexpressed in adult hearts during myocarditis and could also be induced in cultured cardiomyocytes treated with supernatants from phytohemagglutinin-stimulated leukocytes.[52] We have shown that human umbilical vein endothelial cells increased the expression of CAR per cell as the density of cultured cells increased, in sharp contrast to HeLa cells in which CAR expression per cell was constant at all densities.[56] Expression of CAR by HeLa cells is down-regulated by an unknown mechanism when the cells are transfected for expression of plasminogen activator inhibitor 2.[57] These findings, considered in context of the epidemiology of CVB disease, suggest that susceptibility to CVB diseases and the pathology of the viral infection will be closely tied to the regulated expression of CAR. Because males are more susceptible to viral myocarditis than females, it will be interesting to determine whether androgenic hormones increase the expression of CAR, especially in tissues associated with CVB pathologies, as was suggested by Lyden et al.[58]

Much of the work predating the discovery and characterization of CAR was reviewed in 1997 by Kuhn.[59] In the course of efforts to identify and isolate CAR, other proteins that bind CVB have been identified, including decay accelerating factor (DAF, CD55), a complement regulatory protein on the cell surface, and nucleolin, an intracellular protein that had not previously been observed on the cell surface.[60,61] The roles of DAF and nucleolin with regard to receptor-associated functions during CVB infection remain unclear. Findings published since the initial reports that these proteins bind CVB have largely served to increase our confidence that CAR is the principal receptor for CVB (and Ad). Although nucleolin appears to bind CVB on virus overlay blots,[61] it does not support infection of Chinese hamster ovary cells,[62] cells that are refractory to CVB infection owing to lack of a suitable receptor.

In contrast to the sparse data on nucleolin, several reports provide compelling evidence that DAF binds not only strains of CVB that have been selected for growth on RD cells (which lack CAR and are generally refractory to CVB replication) but that DAF also binds CVB in general.[63,64] Although DAF-binding strains of CVB3 require CAR to productively infect and lyse RD cells,[65] demonstrating that binding DAF alone is insufficient for CVB to enter RD cells, a combination of antibodies against DAF and CAR is required to block

infection of HeLa cells by strains of CVB3, which differ in their DAF-binding affinity.[66] Moreover, CVB which bind human DAF fail to bind DAF from rat and mouse,[67] but CVB do infect mouse 3T3 cells, which express human CAR[25] (Carson S, Chapman N, unpublished data). Overall, the data clearly show that CVB bind DAF, and probably nucleolin, but that neither of these proteins is necessary and sufficient for infection. Current data, however, do indicate that CAR is both necessary and sufficient for CVB infection of cells on which it is expressed, either naturally or after transfection. While it is tempting to accept CAR as the singular receptor for CVB, other data show that antibodies to DAF or removal of DAF from cells with phospholipase diminishes CVB infectivity.[63,66] Consequently, we speculate that DAF and CAR may be closely associated on the cell surface, that DAF may serve to provide a cell-associated reservoir of virus thereby increasing access to CAR, and that the optimally functional CVB receptor may be a complex of proteins in which CAR is the principal and key component. Resolution of this issue awaits detailed structural analysis of CVB bound to CAR, to DAF, and to the putative DAF-CAR complex and molecular description of the participation of these proteins in virus binding and cell entry.

Proteins other than CAR can bind Ad2 and Ad5, including integrins, MHC-1, and glycosaminoglycans.[48,49,68] The role of glycosaminoglycans remains to be studied, and the involvement of major histocompatibility complex-1 in Ad infection is controversial following reports that CAR, and not major histocompatibility complex-1, is required for Ad infection.[69,70] In contrast, it appears that CAR functions as a primary receptor for Ad binding to cells, but alpha (v) integrins are required for viral uptake and infection.[68] Viral tropism, however, depends on other factors in addition to the expression of CAR and integrins.[71]

The different protein-virus interactions required for infection indicate that the mechanisms of cell entry, or at least the determinants for cell entry, differ for CVB and Ad, and that CAR does not provide equivalent access to the cell for these different viruses. Although mice are readily infectable by CVB, human Ad do not replicate in mice. If the primary component in the CVB infection of murine tissues is the presence of MCAR, a protein closely similar to HCAR, then MCAR alone must be insufficient for human Ad replication. This may be due to differences in the abilities of equivalent coreceptor proteins from mouse and human to support viral entry into cells, or it may be the result of subtle differences between the HCAR and MCAR structures important for Ad binding. Because mutation of L54→A can abrogate Ad binding to HCAR, it is possible that apparently minor differences between HCAR and MCAR are sufficient to render MCAR incapable of binding human Ad. The Ad12 fiber knob contacts HCAR in the C, C′, C″, and F strands of the V-like domain.[41] Although key contact residues are conserved in MCAR, 3 of the 9 amino acid differences found in this domain occur in the C′ and C″ strands (A93→S, K97→I, and D63→N).

From studies published since 1997, one can conclude that the predicted human receptor shared by Ad and CVB has been isolated and partially characterized. Although the Ad12 binding site on HCAR has been defined at near atomic detail, these details have not illuminated the molecular mechanisms of Ad infection of cells, especially in view of requirements for secondary receptor (or coreceptor) proteins. Details of the molecular interaction between CAR and Ad may provide new avenues for pharmacologic interventions in infection and approaches for targeting Ad-based gene therapy vectors to target tissues that do not express CAR. At this writing, the fine details of CVB binding to CAR are not yet known. It has been suggested that the CAR-CVB association may require glycosylation of the receptor or features contributed from both of the extracellular protein subdomains. The few reports of CAR expression in different tissues support the idea that CVB tropism may be directed by receptor expression. This interpretation may be simplistic because prostate, testis, kidney, and lung have been reported to express CAR, yet the pathologic feature of CVB-associated disease is associated primarily with pancreas, heart, and brain. The findings that CAR is expressed in cultured endothelial cells, which can harbor chronic CVB infections, and that CAR can be regulated by inflammatory mediators indicate that understanding the epidemiology and pathology of CVB and Ad infections depends on elucidating the cell biology and physiology of CAR expression and function.

THE GENETICS OF CVB CARDIOVIRULENCE

The genetics of viral virulence phenotypes have not been worked out for any of the picornaviruses, with the exception of the CVB, in which work has just begun. We distinguish here between the genetics of *artificially altered* virulence phenotypes (as, for example, by mutation or animal passage) and the genetics of *naturally occurring* virulence phenotypes, such as are found in clinical viral isolates. In the former case, the genetics and the mechanism of artificially induced attenuation have been worked out for the Sabin poliovirus vaccine strains,[12,72-77] but the natural genetic determinants of the poliovirus neurovirulence phenotype were never mapped. With poliovirus approaching worldwide eradication as a cause of disease,[78-81] it is doubtful that this mapping will occur for poliovirus.

ARTIFICIAL ATTENUATION VERSUS NATURALLY OCCURRING VIRULENCE PHENOTYPES

The CVB are, however, not under a cloud of eradication like the related polioviruses and are an excellent model system with which to study the genetics that underlie naturally occurring virulence phenotypes and approaches to artificial attenuation. A distinct advantage to the study of CVB virulence phenotypes is the ability of these viruses to replicate well and to high titers in mice and the ability to induce diseases, such as acute myocarditis and pancreatitis, that are close counterparts to the human disease.[4,82-84] Another key aspect is

the availability of numerous clinical isolates of the 6 CVB serotypes that permit sorting by virulence phenotype in mice. Finally, the genomes of all CVB serotypes have been cloned as infectious cDNA copies, permitting ready mapping of viral genetics by comparative sequence analysis and by chimeric genome construction.

Until recently, the studies that have dealt with the topic of cardiovirulence have focused on the genetics of artificially induced attenuation; the exception has been a series of studies mapping sites of CVB4 pancreovirulence. In 1995, we[85] reported the mapping and identification of the single nucleotide mutation that was responsible for the attenuation of the cardiovirulent phenotype of a well-characterized noncardiovirulent CVB3 strain called CVB3/0.[86] The U234C mutation in the CVB3 5′ NTR attenuated CVB3/0 for replication in murine heart cells in culture and in the mouse and was responsible for attenuating the myocarditic phenotype in mice. This work also showed that replication of CVB3/0 in severe combined immunodeficient mice that lack functional T- and B-cell immune responses resulted in the rapid acquisition of a cardiovirulent phenotype and that this was linked to a C to U reversion at nucleotide (nt) 234.

Our findings have been extended by inducing transversion mutations within the 5mer.[87] Transversion mutations (Py/Pu or Pu/Py) more deleteriously affect the replicative vigor of the resulting progeny viral strains in cell culture; the strains are effectively unable to replicate successfully in mice. As opposed to the key attenuating 5′ NTR mutations of the Sabin poliovirus strains that slowed viral translation in cells of neural origin,[88] we found that the 5mer mutations altered the positive-to-negative viral RNA strand ratio in infected cells, a finding that suggests a lesion in positive strand viral RNA synthesis. Subsequent investigation demonstrated that the nt234C mutation was unique to CVB3/0 and that nt234 existed always as U in a completely conserved 5mer defined by nt232-236 (5′-CGUUA) in all enteroviruses known. Therefore, the 234C was most likely an artificial mutation and not a naturally occurring determinant of a noncardiovirulent phenotype.[89]

Much attention was drawn by a report that CVB3/0 rapidly became virulent on replication in selenium-deficient mice,[90] data that suggested a correlation between selenium deficiency and the possibility of a normally benign CVB3 infection causing Keshan disease,[91] with greater implications for nutrition and viral diseases. The data were generated by using the artificially attenuated CVB3/0 strain, a strain that reverts even in well-fed mice. These findings have not been tested, however, with a series of avirulent CVB strains to determine whether a naturally occurring avirulent strain will become virulent during replication under conditions of selenium deficiency. Recently, CVB-induced murine myocarditis has been shown to be linked to murine pancreatitis, and myocarditis does not apparently exist in the absence of pancreatitis.[84] The noncardiovirulent CVB3/0 is nonetheless partially pancreovirulent in mice, inducing pancreatitis (acinar tissue destruction). Replication of CVB in the murine pancreas occurs soon after inoculation and reaches

titers higher than those observed in sera during viremia. This work has also shown that CVB strains exist that replicate well in mice, yet cause no detectable disease in murine pancreas or heart. Such naturally occurring avirulent strains are potentially valuable candidates for naturally stable vaccine strains. Knowlton and colleagues[92] identified a mutation in the capsid protein 1B (also termed "VP2") that attenuated a highly cardiovirulent CVB3 strain; however, like the mutation characterized by Tu et al.,[85] the capsid protein mutation has not been found in any other CVB strains and must be considered artificial.

Ramsingh and co-workers[83,93-97] have focused on CVB4 involvement as an agent of pancreatitis. They used an avirulent strain of CVB4 and a derivative of the same strain passed in mice that acquired a pancreovirulent and cardiovirulent phenotype to map 2 primary sites that determine the pancreovirulent phenotype to the capsid proteins. Although in this case the phenotype that was acquired was a virulent rather than an attenuated one, experiments to show that the mutations are not murine adaptations (for example, by mapping pancreovirulence of clinical CVB4 isolates) have yet to be performed. Iizuka and colleagues[98] deleted approximately 100 nt of the CVB1 5′ NTR from just upstream of the translational start site and demonstrated that the resultant virus was less virulent in mice. All of these artificially altered genomes, especially the chimeric and deletion strains, also represent potentially useful models for the study of viral quasispecies and viral evolution in cell cultures and in mice.

A key set of experiments mapped cardiovirulence of clinical isolates of CVB3 to the 5′ NTR. Using several different CVB3 strains that naturally varied in their cardiovirulence phenotypes and the CVB3/20 infectious clone (a virus that is both pancreovirulent and cardiovirulent in mice[13]) as the background, Dunn[99] and others constructed several genomic chimera and tested their virulence phenotype in mice. The capsid protein coding region or the 3′ half of different CVB3 genomes (encoding the nonstructural proteins) in the CVB3/20 background caused no change in the cardiovirulent phenotype, regardless of whether the donated sequences were obtained from cardiovirulent or noncardiovirulent strains. However, when the 5′ NTR of the noncardiovirulent strain CVB3/CO was used to replace its homolog in CVB3/20, the resultant virus was no longer cardiovirulent. Replacement of the CVB3/CO 5′ NTR with that from the parental CVB3/20 or from another, different cardiovirulent strain (CVB3/AS) restored the cardiovirulent phenotype. These data are strong evidence that the CVB3 5′ NTRs naturally encode cardiovirulence. The site that controls the cardiovirulent phenotype has been more finely mapped to the stem-loop II region of the 5′ NTR. Although these exciting results are unique and for the first time indicate where in the genome cardiovirulence is likely encoded, many questions remain. Are these results applicable to other CVB3 strains and other CVB serotypes? Does this same site control pancreovirulence and avirulence? Although the pancreovirulent and cardiovirulent phenotypes appear to be linked,[84] similar chimeric cDNA constructs are

needed using pancreovirulent and avirulent strains to determine the answer. How does stem-loop II interact with the infected host cell to set the chain of events in motion that culminates in clinically describable disease?

The importance attached to understanding the natural genetics of CVB virulence is understandable. Different attenuating approaches may be taken to reduce the replicative fitness of CVB strains and thus generate a potentially useful vaccine strain. However, as evident from Sabin poliovirus vaccine strains (reviewed in reference 77) and an attenuating mutation in CVB3,[85] the attenuated phenotype can rapidly revert during replication in the host, resulting in reacquisition of a virulent phenotype. It can be argued that, were the Sabin poliovirus strains developed today to fight poliomyelitis, this rapidity of reversion would not be commercially acceptable in a vaccine. A vaccine against the 6 CVB types would be useful to reduce the incidence of viral myocarditis and virus-induced dilated cardiomyopathy but, given the relatively small market (about 10% of that for poliovirus during the epidemic years of the 1940s and 1950s), it is unlikely that any company will spend the funds necessary to bring such a vaccine to market.

It is possible that CVB will be used in the future as chimeric vaccine and virus expression vectors as well as therapeutic vectors. Much evidence, obtained using polioviruses as vectors, has shown that enteroviruses could succeed as chimeric vectors against other diseases of viral and bacterial origin and perhaps even cancer.[100-107] Höfling and colleagues[108] demonstrated that an attenuated strain of CVB3, expressing an antigenic polypeptide from adenovirus type 2 (Ad2) hexon protein, induced both anti-Ad2 and anti-CVB3 immunity in mice. These workers demonstrated that the chimeric virus induced anti-Ad2 immunity even in mice that had been immunized previously with CVB3 to mimic preexisting immunity in humans, suggesting the potential for reuse of the viral vector and expression of multiple epitopes. In another demonstration of the potential clinical utility of chimeric CVB vectors, Chapman et al.[109] showed that an attenuated strain of CVB3 can express biologically active murine interleukin-4 and that its replication in mice can induce CVB3-binding IgG1 antibodies. These results cumulatively raise many interesting questions. For example, can a different cytokine, interleukin-2, when linked to virus-expressed antigens, potentiate the host immune response to the foreign vector and to the foreign antigen, as has been shown for nonviral systems?[110] Can CVB be used to express polyvalent epitope arrays, similar to those that have been studied for use as immunogens against *Plasmodium falciparum* (malaria) infection?[111]

PROGRESS TOWARD UNDERSTANDING THE MOLECULAR MECHANISMS OF CVB-INDUCED INFLAMMATORY HEART DISEASE

The molecular and immunologic mechanisms that determine CVB inflammatory heart

disease are being studied. The first step in pathogenesis once the virus has entered the body (ie, entry into the cell) has been resolved through the discovery and characterization of the receptor that the virus uses to gain entry into cells (see above).

Two findings have made important strides in the overall understanding of intracellular molecular mechanisms of viral heart disease. The src kinase p56lck plays a role in CVB3 heart disease in knock-out mice: mice lacking p56lck did not develop CVB3-induced disease.[112] Although the mechanism of this finding has not been derived, the findings suggest that p56lck might be targeted to intervene in CVB-caused disease. Importantly, these findings extended to other organs besides the heart, making the possibility of an anti-p56lck compound of significant interest.

How CVB3 replication interacts with components of the infected cell is the subject of 2 further papers. Lloyd's[113] and Knowlton's[114] groups showed that 1 of the 2 CVB proteases, termed "2Apro," cleaves poly A binding protein; Knowlton showed it also cleaves dystrophin. Dystrophin and the dystrophin-associated glycoproteins alpha-sarcoglycan and beta-dystroglycan were morphologically disrupted in infected myocytes. They also determined that there was a closer relationship between the kinetics of cleavage of poly A binding protein and HeLa cell host cell capped mRNA translational shutoff and viral protein synthesis than for the cleavage of eIF4G1, traditionally named as the key intracellular protein inhibited by 2Apro during enterovirus cleavage (primarily from poliovirus studies).

In other work, using mice with the inducible nitric oxide synthase gene knocked out, Zaragoza and colleagues[115] have demonstrated that CVB3-induced heart disease is worse than in normal mouse controls; similar results hold for pancreatitis,[116] a likely precursor to CVB-induced myocarditis.[84] The inflammatory response defines myocarditis; the inducible nitric oxide synthase gene can be induced by Th1-type cytokines such as interferon-γ and tumor necrosis factor-α, cytokines associated with CVB infection. The relationship of the extent of inducible nitric oxide synthase induction and the phenotype of the CVB strain used to inoculate mice needs, however, to be investigated; to date, the viruses used are all cardiovirulent and such strains are a minority of those that circulate naturally.[84] Is inducible nitric oxide synthase induced even during infections by avirulent strains of CVB? Heim and others[117] showed that proinflammatory cytokines interleukin-6 and interleukin-8 are transiently up-regulated in myocardial fibroblasts immediately after exposure to CVB3, consistent with others' findings that proinflammatory cytokines can also be expressed in the murine model of CVB3-induced myocarditis.[118] Huber and colleagues[119,120] demonstrated that $\gamma\delta$+ T cells modulate the major histocompatibility complex class II susceptibility to CVB3-induced heart disease, and that these cells primarily induce Fas-mediated killing and are more effective in inducing cardiomyocyte apoptosis than $\alpha\beta$+ T cells.

ADENOVIRUSES: INTRODUCTION

Human Ad have been highlighted as probable causes of human myocarditis. The Ad are double-stranded DNA viruses with linear genomes of approximately 40 kbp.[121,122] Like picornaviruses, their capsids consist of proteins arranged in an icosahedral design.[123] The discovery of the receptor protein, CAR, used by the CVB to enter human cells (see above) and subsequent work has shown that CAR is also the primary receptor used by Ad. Because the ability of a virus to enter cells represents a primary first step toward causing a disease in a given tissue, the ability of both CVB and Ad to use CAR as their receptor and their linkage to human inflammatory heart disease are perhaps not surprising. Nonetheless, viral pathogenesis requires more than mere cell access; many viruses can infect various tissues but cause no disease. Thus, much remains to be done to understand the propensity for Ad to replicate and cause disease in myocardial cells.

THE ADENOVIRUS GENOME AND REPLICATION

The human Ad are members of the genus *Mastadenovirus* (family Adenoviridae), which contains the nonavian Ad (reviewed in reference 122). Forty-nine serotypes of human Ad are organized into 6 subgroups by red blood cell agglutination. Human Ad2 and 5 of subgenus C are the best studied because of their ease of replication in HeLa or KB cell culture. The human Ad have a 30- to 38-kb linear double-stranded DNA genome with inverted terminal repeats of about 100 to 140 bp and are packaged in an icosahedral protein capsid containing 240 hexon and 12 penton proteins enclosing the core of the genome complexed with 4 viral proteins.

Ad of all but the B subgenus[38] have been shown to use either CAR[23-25] with high affinity or the α2 domain of major histocompatibility complex class I[124] with low affinity as a human cell receptor via binding of the fiber protein of the capsid. However, Ad37 of the D subgenus has been shown to use sialic acid, not CAR, as a receptor.[125] Adenovirus entry into cells appears to be greatly stimulated by internalization receptors, the vitronectin-binding integrins $\alpha V\beta 3$ and $\alpha V\beta 5$, which are bound by RGD (arg-gly-asp) motifs of the penton base protein of the capsid.[48] This binding results in signal transduction required for endocytosis of the virus.[126] On entry into the cell, the virus escapes the endocytic vacuoles[127] and uncoats, and the core reaches the nucleus[128] in which the viral DNA serves as a transcriptional template for viral RNAs and is replicated.

Three temporal classes of RNAs encoding proteins are transcribed from Ad DNA: early (including E1A, E2, and E3 among others), delayed early, and late (mostly proteins of the virion) (reviewed in reference 122). Transcribed RNAs are spliced to generate mRNAs of multiple viral proteins.[129] The E1A proteins activate viral RNA transcription, affect cellular gene expression, induce entry of infected cells into the S phase (optimal for viral DNA replication), and prevent viral-induced apoptosis of infected cells. E1A and

E3 proteins have effects that decrease major histocompatibility complex class I expression on infected cells, thus decreasing the ability of cytotoxic T lymphocytes to eliminate infected cells (reviewed in reference 122). In addition, E3 and E1B proteins have antagonistic effects toward the antiviral activities of tumor necrosis factor-α.

The E2 proteins are involved in Ad DNA replication and include the precursor termination protein, the DNA polymerase, and DNA binding protein. These proteins and cellular transcription factors (Oct-1 and NFII) are necessary for viral DNA replication (reviewed in reference 130), a process that occurs by binding a replication complex to the terminal 5′ end of the repeated region of the genome, initiating transcription at nucleotide 4-6, jumping back to the initial 3 nucleotides (which have the identical sequence) and elongating the nascent strand of DNA. The precursor termination protein remains covalently attached to the 5′ end of the nascent DNA. The displaced strand of DNA can form base pairs at the terminal inverted repeats, generating a "panhandle" with a suitable 5′ end for initiation of DNA replication of a second strand. Alternatively, 2 replication forks from opposite ends of a replicating genome can meet, allowing the 2 parental strands to separate and nascent strands to be completed on them.

Replication of the viral genome results in increased activation of the major late promoter for expression of late RNAs (many different transcripts are produced by splicing and differential use of poly [A] sites), a process that involves binding of multiple cellular factors and adenovirus protein IVa2. Production of these late proteins depends on controlled elongation of the viral RNAs. Host cell protein synthesis is curtailed in Ad-infected cells: cellular RNAs are blocked from transport to the cytoplasm[131] and an initiation factor that is required for translation of most cellular RNAs is inactivated.[132] Adenovirus late RNAs use a different cap-dependent form of translational initiation, ribosome shunting,[133] which is functional with the late RNA tripartite leader. In addition to these processes, activation of the cellular protein kinase R by interferon or double-stranded RNA (produced during Ad infection), which stops translation by phosphorylation of eIF-2 to block initiation of translation, is prevented by the production of small viral RNAs, VAI RNA and VAII RNA (reviewed in reference 122). As late proteins accumulate, penton-hexon complexes are transported to the nucleus where Ad DNA is encapsidated in a process dependent on a packaging signal in the genome. Activity of the L3-encoded viral protease results in maturation of viral proteins in the virion, generating an infectious particle. Virus particles are released by lysis of the cell.

EVIDENCE FOR ADENOVIRUS INVOLVEMENT IN MYOCARDITIS

Relatively little evidence links human Ad to myocarditis, and it is primarily molecular in nature. Towbin's laboratory has promoted Ad as potential causes of human heart disease, beginning with the detection of Ad DNA by PCR in 15 of 38 pediatric heart samples.[134]

Concurrently, the detection of Ad DNA in the hearts of 2 patients with clinically unsuspected but histologically proven myocarditis was described by another group,[135] and Ad DNA was also detected by using PCR in the myocardium of an infant with myocarditis and pericarditis.[136] Matsumori[137] detected enteroviral RNA in 1 patient of 36 with cardiomyopathy and myocarditis; no other virus, including Ad, of those assayed was detected. Human heart biopsy samples from patients with dilated cardiomyopathy were probed with PCR; Ad DNA was detected in 17% of samples with inflammation and in 15% of samples without inflammation.[138]

Only in 1999 was sequence analysis applied to answer the question of which Ad serotype was being detected in human heart tissues.[139] With PCR and sequence analysis of the amplified fragments, 94 heart samples were examined from patients with left ventricular dysfunction; 13% were positive for Ad, and these were consistent with Ad serotype 2 (Ad2). To date, no other report has confirmed another serotype in the heart, but this is itself interesting, for 3 Ad serotypes are closely related: 1, 2, and 5 (previously called type C Ad). Is Ad2 the only cardiotropic and cardiovirulent Ad? If so, why? It is clear that a concerted effort is needed to resolve this question. Also in 1999, another group probed 31 samples from patients with myocarditis (n = 15) and dilated cardiomyopathy (n = 16) for enteroviral and Ad sequences.[140] These workers failed to find evidence for Ad DNA in their samples, although enteroviral RNA was detected in 27% of the patients. Towbin's group published another study on the use of tracheal aspirates as an approach to the detection of viruses in pediatric myocarditis and pneumonia.[141] Comparison of findings with the PCR analysis of endomyocardial biopsies showed 3 children with Ad and the confirmation of Ad2 in another patient who had both myocarditis and pneumonia.

Cumulatively, these reports suggest that similar problems exist for the detection of Ad DNA as for enteroviral RNA. Primarily, these are believed to revolve about the sensitivity of any specific group's enzymatic amplification assay and the as yet unresolved issue of prevalence of a specific virus at any time in a specific population. The results are highly inferential, however, that human Ad are involved as etiologic agents of human heart disease. At present, there is no small animal model with which to study Ad involvement in heart disease. Although there are murine adenoviruses (Mav) that replicate in their natural host and these viruses have been reasonably well characterized (reviewed in references 142 and 143), there is no Mav-induced mouse myocarditis model, although reports exist regarding myocarditis and endocarditis in mice induced by Mav type 1.[144,145] Given the apparent etiologic connection in humans, the molecular understanding of much of the Ad and Mav genomes, and the ready availability of inbred mouse strains, refinement of these early results into a working model of Mav-induced heart disease has merit.

HEPATITIS C VIRUSES: INTRODUCTION

Infection by HCV (family Flaviviridae, genus *Hepacivirus*), the cause of most cases of non-A, non-B viral hepatitis, is a major cause of chronic hepatitis, resulting in liver cirrhosis and hepatocellular carcinoma worldwide[146] and infects 175 million people globally. More than 80% of infected patients develop chronic disease while remaining essentially asymptomatic.[147] In the United States, an estimated 2 to 3 million people are currently infected and more than 150,000 new cases of HCV infection occur per year. The sequelae of HCV-induced and serious chronic liver disease result in 8,000 to 10,000 deaths annually.[147] Since the first report of viral genomic sequences from HCV in 1989, a greater understanding of the HCV infection has been achieved.

HCV infection usually develops after direct blood-borne percutaneous exposure. High risk of HCV infection has been reported often after blood transfusion.[148] Epidemiologic evidence exists for the transmission of HCV to renal dialysis patients, among intravenous drug users, during heterosexual sex, from mother to baby, and even by needle-stick victims.[149] With anti-HCV screening tests for blood donors, advanced assay techniques (eg, RT-PCR and in situ hybridization) have made it possible to detect early infection before the progression to acute disease.

THE HCV GENOME AND REPLICATION

Hepatitis C does not easily replicate to usable titers in cell culture, a fact that has hindered the study of the molecular biology of this virus. The viral genome is a positive-sense, single-stranded, 9.6-kb RNA molecule with highly conserved 5′ and 3′ NTR, approximately 341 and 500 nt, respectively, and is encapsidated with a core protein, C, that is enclosed in a membrane containing at least 2 envelope proteins, E1 and E2.[150] The 5′ NTR is a complex secondary structure, indicating the function of an internal ribosomal entry site,[151] whereas the 3′ NTR contains a highly conserved 98-nt sequence with a secondary structure which in combination with other sequences and the viral polymerase, NS5B, is necessary for the replication of the minus strand viral RNA.[152,153] The genome's single large open reading frame produces a polyprotein that is cotranslationally and post-translationally processed by the combination of host signal peptidases and viral proteinases, resulting in all 11 known viral proteins, including the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (reviewed in reference 154). Viral proteases include NS2 acting in complex with NS3 and NS3 in complex with NS4A. NS3 also has RNA-stimulated NTPase and RNA helicase activities and has the potential to transform cells in culture. The functions of the protein p7, which is derived from processing of the structural region by signal peptidases, NS4B and NS5A, is currently unknown.

A host signal peptidase associated with transport into the endoplasmic reticulum is responsible for maturation of the structural proteins located in the N-terminal one-third of the polyprotein, whereas viral proteinases process the mature nonstructural proteins, NS2 to NS5B.[155] Thus far, 4 enzymatic activities encoded by the nonstructural proteins have been reported. The NS2-NS3 region codes for an autoproteinase responsible for cleavage at the 2/3 site.[156] Cleavage at the NS2/NS3 junction is accomplished by a metal-dependent, autocatalytic proteinase encoded within NS2 and the N-terminus of NS3. The remaining cleavages downstream from this site are effected by a serine proteinase also contained within the N-terminal region of NS3 (reviewed in reference 157). The N-terminal one-third of NS3 functions as the catalytic subunit of a serine proteinase, which cleaves at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites; NS4A is a membrane protein that acts as a cofactor of the proteinase.[155] NS3 in a heterodimeric complex with NS4A also encodes an RNA-stimulated NTPase/RNA helicase domain at its C-terminus.[158] To date, no functions have been reported for NS4B or NS5A in RNA replication; however, NS5A has been implicated in modulating the sensitivity of HCV to interferon.[159]

EVIDENCE FOR HCV INVOLVEMENT IN MYOCARDITIS

The literature on HCV involvement in heart disease is not vast and can be divided by results from Japanese and European groups. Matsumori and colleagues[160] initially suggested that HCV may play an etiologic role in the development of heart disease. Thirty-six patients with dilated cardiomyopathy were screened for anti-HCV antibodies in serum; 16.7% of the patients and 2.5% of controls (n = 40) were positive. HCV type II RNA was detectable by RT-PCR analysis in 4 of the 6 patients with antibodies against HCV, and RNA was detected in 3 of the positive patients' heart muscle. Matsumori et al.[161] then reported finding serologic evidence of HCV infection in 6 of 35 patients with hypertrophic cardiomyopathy versus 2% to 3% of patients with ischemic heart disease; HCV RNA was detected in the heart RNA from 3 of the 6 positive patients who had hypertrophic cardiomyopathy. This was followed by another group's findings that HCV RNA could be detected in the hearts and livers of 3 patients with chronic active myocarditis.[162] A multicenter study in Japan published in 1998 had examined 697 patients with hypertrophic cardiomyopathy and 663 patients with dilated cardiomyopathy for the presence of anti-HCV antibodies in sera:[163] 10% to 11% of the former and 6% of the latter were positive. In normal blood donors 2% to 3% were positive. In another Japanese study, 9 of 65 patients with hypertrophic cardiomyopathy tested positive for anti-HCV antibodies (13%-14%) versus 2% to 3% of the control population;[164] HCV RNA was also detected in 5 of the HCV positive patients.

In contrast to these findings of about 10% to 15% HCV positivity in patients with heart disease in Japan, Figulla and colleagues in Germany found a much lower percentage

of dilated cardiomyopathy or myocarditis patients (n = 73) with anti-HCV sera: 1%-2% as opposed to 6% in the control group.[165] These workers concluded there was little obvious correlation between HCV exposure and heart disease. Another European study found no correlation in the sera or by PCR analysis between HCV or other microorganisms and end-stage idiopathic dilated cardiomyopathy (n = 37); 39 patients with end-stage dilated cardiomyopathy of known etiology were used as controls.[166] Prati and colleagues[167] studied HCV involvement in 752 patients with dilated cardiomyopathy and other heart diseases (along with 443 control samples) and found a lower percentage of anti-HCV antibodies in sera from heart patients (3% to 4%) than in the control group (6% to 7%), concluding that HCV exposure did not correlate with myocarditis or dilated cardiomyopathy. A study in Greece also found no evidence for linkage between HCV infection and dilated cardiomyopathy.[168] Patients with chronic HCV infection (n = 102) and dilated cardiomyopathy (n = 55) were assayed for exposure to HCV serologically and by RT-PCR. No chronic HCV patients had evidence of dilated cardiomyopathy, and no dilated cardiomyopathy patients showed serologic or molecular evidence of HCV infection.

The possible etiologic role of HCV infection in the development of myocarditis is still debatable: the reasons for the disparity in results between the Japanese and European studies are not evident. A difference in viral strains that circulate locally may represent a key factor.[169] HCV is a long-term persistent infection, permitting quasispecies variations to occur[170,171] and be selected within the unique environment of any human; such viral quasispecies might not be dominant in a general viral population.[172] This has been well-documented in HIV-infected patients. Individual variations or those endemic in a population as a locally dominant quasispecies can be postulated to influence disease outcome in those infected.[173] Therefore, it might be illuminating to determine whether significant differences in HCV sequences exist between those strains isolated from diseased heart tissues in Japan and those from HCV strains circulating generally in Japan and in Europe.

The problematic correlation of HCV infection with heart disease might also be said to exist for enteroviral infections and myocarditis, although the weight of the cumulative evidence makes for an extremely strong inferential argument for enterovirus causation of human myocarditis despite some reports in which enteroviral RNA has not been detected in diseased hearts. However, a similar weight of evidence does not exist at present supporting the role of HCV as a key agent of myocarditis. One primary aspect of the argument for enteroviral, specifically CVB, involvement in heart disease derives from correlative animal studies; it is clear that human CVB can cause acute myocarditis in mice and that this is both virus strain (eg, reference 84) and mouse strain[174] dependent. As for Ad involvement in heart disease, there is no current murine model as easily used for HCV heart disease as that available for the CVB, but this may be changing. A report offers a murine

model of flavivirus infection,[175] thus raising the possibility of eventually deriving a murine model for flavivirus-induced myocarditis. Use of a transgenic mouse model[176] to model anti-HCV protection using vaccinia virus-HCV chimeric vaccines has also been reported. GB virus-B is a member of the flavivirus family and is closely related to HCV. Bukh and colleagues[177] have shown that GBV-B can replicate in tamarins and induce hepatitis, demonstrating a potential small primate model for HCV infection that might be adapted to heart disease.

REMAINING QUESTIONS

Although a review of the literature reveals known, suspected, or potential viral causes of human myocarditis, only the 6 serotypes of CVB and Ad2 appear at present to be the primary etiologic agents. The involvement of HCV may be important, especially in Japan and perhaps elsewhere on the western Pacific rim, but this is not yet clear. Other viruses come and go in reports, but none with the regularity of detection of enteroviruses and Ad. With enzymatic amplification and genetic arrays now commonplace, permitting rapid and sensitive detection of specific nucleic acids, a well-designed multicenter study is needed to determine which viruses (and other microorganisms) might be detected in myocarditis. Such studies would be expected to confirm and extend current knowledge, providing a stronger rationale for the targeting of key infectious agents as subjects of prophylactic, and possibly therapeutic, vaccine development. Clearly, a side but significant benefit of such vaccines would be the reduction in other diseases associated with these agents.

Much of what is understood about enteroviral involvement in human myocarditis stems from the availability and exploitation of excellent murine models of CVB-induced myocarditis. It makes sense to pursue such a model for adenovirus, using murine Ad because human Ad do not replicate in mice. This effort should also be linked with an effort to understand the role of CAR in the development of viral disease in mice and humans. Clearly, entry to cells is a prerequisite of viral disease but, conversely, the mere presence of the viral receptor does not imply that a specific tissue promotes productive viral infection that leads to disease. What is the natural purpose and ligand of CAR? Interventional therapy such as blocking CAR might be considered in severe cases of CVB disease such as in neonatal and pediatric patients.

For none of the 3 virus groups examined in this review is there complete consensus on the involvement of the specific virus in myocarditis. Viral antibodies have been found in sera or viral nucleic acid sequences in cells from diseased heart tissue; this contrasts with other studies that have failed to find significant linkage between viral exposure and heart

disease. The efficacy and diligence of specific detection methods can always be argued as one cause of this variance, but, in general, serology and molecular detection techniques are now so well developed and so widely used that their credibility can no longer be raised as a fundamental issue. (Of course, how such data are interpreted will always remain problematic for some.) All viruses, far from being single entities, are better understood as populational swarms of related yet distinguishable strains termed "quasispecies."[178,179] With this concept firmly in mind, one can more easily cope with the idea that a specific "virus" in one part of the world may differ signficantly from its counterpart elsewhere or from one person to another in the same community. Studies of virus involvement in human disease, such as those discussed in this review and elsewhere, must also consider which viruses are in common circulation in the community in which the patients live. Is the failure to find virus nucleic acid in diseased hearts due to a limited circulation of virus for a specific time in a specific community? Serologic data are more telling in this respect: antibodies persist for long periods and represent a longer view. The 2 techniques—serology and molecular analysis—are complementary in defining the relationship of humans, viruses, and the diseases they cause.

Evidence suggests that vaccines can be created that can immunize humans against the primary viral agents of myocarditis—CVB and Ad2. The initial step in that direction has already been taken with the demonstration that an antigenic fragment of Ad2 hexon protein can be expressed by CVB3 and the replication of this chimeric virus in mice induces anti-Ad2 and anti-CVB3 neutralizing antibodies, even in the face of preexisting mouse anti-CVB3 immunity.[108] Unlike the Ad, which are also potential chimeric vaccine vectors, we understand much more about attenuating CVB for disease, primarily because of the advantage of a relevant disease system in mice.[20,85-87,180] Also, the CVB are closely related to the polioviruses against which excellent vaccines have been made. So successful have these vaccines been that poliovirus should soon be an extinct pathologic agent. Work in progress shows that multiantigenic chimeric CVB strains can induce protective immunity in mice against virulent challenge by CVB and Ad (the latter can be modeled by using Mav-1).[143] Coxsackievirus constructs can express cytokines and redirect the host immune response,[109] a finding that suggests therapeutic and immunomodulatory possibilities similar to what has been demonstrated by viral and subunit vaccines.[110,181,182]

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