2013

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Published in *Journal of Cardiovascular Translational Research,* doi: 10.1007/s12265-013-9519-3

Submitted August 15, 2013; accepted November 11, 2013; published online November 22, 2013.

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Abstract
Heart disease, the leading cause of death in humans, is estimated to affect one in four American adults in some form. One predominant cause of heart failure in young adults is myocarditis, which can lead to the development of dilated cardiomyopathy, a major indication for heart transplantation. Environmental microbes, including viruses, bacteria, and fungi that are otherwise innocuous, have the potential to induce inflammatory heart disease. As the list is growing, it is critical to determine the mechanisms by which microbes can trigger heart autoimmunity and, importantly, to identify their target antigens. This is especially true as microbes showing structural similarities with the cardiac antigens can predispose to heart autoimmunity by generating cross-reactive immune responses. In this review, we discuss the relevance of molecular mimicry in the mediation of infectious myocarditis.

Keywords: heart, infectious myocarditis, autoimmunity, molecular mimicry, coxsackievirus, microbial mimics, mimicry epitopes

Introduction

Heart disease is the leading cause of death in humans [1]. An estimated 80 million American adults (one in four) have some form of cardiovascular disease [1, 2], with a projected incidence of 40.5 % by 2030 [3]. Myocarditis is a predominant cause of heart failure in young adults [4, 5]. Most individuals affected by myocarditis remain asymptomatic, and the disease spontaneously resolves frequently; however, 10–20 % of the patients with myocarditis—an estimated 20,000 to 40,000 patients per year in the USA—can develop chronic disease leading to dilated cardiomyopathy (DCM), with an incidence of 3.5 to 8.5 cases per 100,000 population, or 9,000 to 20,000 new cases annually [6, 7]. Approximately half of DCM patients undergo heart transplantation due to lack of available, effective therapeutic options. The estimated cost of caring for DCM patients is $7 billion annually in the USA alone [8, 9]. Two lines of evidence exist to suggest that inflammatory infiltrates can be present in the hearts of healthy individuals to a low degree: (1) Myocarditis is suspected to be the cause in 5–22 % of sudden deaths in athletes less than 35 years old [4, 10–12] and (2) a necropsy study involving more than 12,000 accidental deaths revealed the presence of inflammatory infiltrates in the hearts of 1.06 % of individuals [13]. But what triggers myocarditis in these apparently healthy individuals is a fundamental question to be addressed in cardiology research. Accumulated literature suggests that the genesis of myocarditis requires the mediation of a variety of immune cells of both the innate (natural killer T cells, γδ T cells, mast cells) and adaptive immune systems (CD4 T cells, CD8 T cells, and B cells) involving multiple mechanisms or pathways [2, 14]. In this review, we discuss how environmental microbes can cause myocarditis by molecular mimicry.

Environmental Microbes in the Causation of Myocarditis

Generally, it is believed that microbial infections can trigger autoimmune responses in genetically susceptible individuals. However, unlike other diseases caused by known specific etiologies, it is practically difficult to prove all of the Koch's postulates for autoimmune diseases [15]. Furthermore, a theme is now emerging to suggest that exposure to multiple microbes, rather than a single microbe, may be critical to initiate autoimmune diseases [15]. Mechanistically, microbes that primarily infect the target organ can lead to secondary generation of autoimmune responses by facilitating the release of intracellular self-antigens, which are then recognized by the immune system as foreign antigens. A variety of pathogens like viruses, bacteria, rickettsia, and parasites have been implicated in the causation of myocarditis (reviewed in reference [2]), but their direct causal links remain tenuous clinically, with the challenge being able to prove the cause-and-effect relationship.

One mechanism by which microbes can break self-tolerance is by inducing cross-reactive immune responses through molecular mimicry because of their antigenic similarities with host proteins (reviewed in references [6, 16]). Cross-reactivity can occur for antibodies and/or T cells [6, 16, 17]. For antibodies, cross-reactivity has been noted for linear sequences containing amino acids identical to the self-antigens, and also for homologous, but non-identical sequences, including for epitopes, derived from unrelated molecules such as proteins and carbohydrates [18]. For T cells however, cross-reactivity occurs as a result of degeneracy in the recognition of peptides displayed by major histocompatibility complex (MHC) molecules (Fig. 1). T cell degeneracy can occur for microbial peptides sharing stretches of amino acids significantly identical to self-antigens or even for those peptides bearing the minimal sequence identities, so long the critical MHC- and T cell receptor (TCR)-contact residues are preserved [19]. While, the autoantibodies produced by B cells can activate complement cascade leading to the induction of type II and type III hypersensitivity reactions, the CD4 helper T cells and cytotoxic T lymphocytes (CTLs) mediate their effects through the production of cytokines and cytolysis, respectively [20].

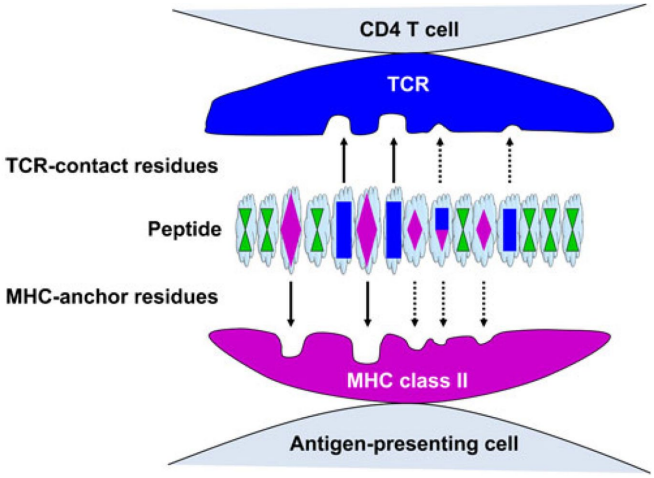

Figure 1. Mechanistic basis for TCR degeneracy. αβ TCR-expressing CD4 T cells recognize peptide fragments presented in the context of class II MHC molecules by antigen-presenting cells. The side chains of some amino acids in the peptide sequence are extended. Such residues preferentially anchor MHC molecules, which are commonly referred as MHC-anchor residues (**bottom arrows:** solid, primary; dotted, secondary). Similarly, side chains of solvent-exposed residues also can interact with TCRs, and they are called TCR contact residues (**top arrows:** solid, primary; dotted, secondary). Substitutions, if any, in the primary positions can lead to complete ablation of the T cell response, but similar substitutions are tolerable for secondary TCR contact residues [90]. Such precise recognition has been demonstrated in the case of the hemoglobin (Hb) molecule, where a single amino acid substitution at the primary TCR contact site (glutamic acid 73) in Hb 64–76 resulted in 1,000-fold weaker recognition by the T cells [90]. Such an event can occur for two entirely different ligands, if the primary T cell contact residue alone is preserved in both. For example, in the transgenic mouse model of spontaneous rheumatoid arthritis, a TCR specific for bovine ribonuclease 42–56 can recognize another irrelevant ligand, glucose-6-phosphate isomerase 281–293, which is a self-peptide [90]. Thus, enormous potential exists for microbes that bear the sequences of self-antigens to induce cross-reactive T cell responses, as long as the critical MHC- and TCR-interacting residues are preserved.

Association of Autoimmunity in Diseases Caused by Common Infectious Pathogens of Cardiovascular System

Evidence from Human Studies

In at least four examples, autoimmunity can be an important component of cardiovascular diseases caused by infectious agents: coxsackievirus B (CVB)3 in post-infectious myocarditis [21], *Trypanosoma cruzi* in Chagas heart disease [22], *Chlamydia* in myocarditis and coronary artery disease [23, 24], and β-hemolytic streptococci in rheumatic heart disease (RHD) [25–28].

Coxsackieviruses are generally implicated as causes of myocarditis in North America [29]. In the USA, approximately five million enterovirus infections are attributed to CVB1–5, and 12 % of those may have myocardial involvement, in which CVB1, CVB3, and CVB5 serotypes are commonly implicated [30, 31]. Serologically, CVB3-reactive antibodies are found in about 50 % of DCM patients, while enterovirus genomic material can be detected in up to 70 % [32–34], suggesting CVB3-mediated autoimmunity can play a role in myocarditis/DCM pathogenesis. Isolation of infectious CVB particles is not commonly reported in patients with myocarditis/DCM as the myocardial biopsies are taken mostly in rare cases [35]. But, when isolated for example at necropsy, the viral isolates were found to be pathogenic in mice [36].

Chagas heart disease caused by T. cruzi is the leading cause of infectious myocarditis in the world [37]. Autoimmunity is suspected in the disease pathology, and autore-

activity appears to reside in the CD4 T cell compartment, although CD8 T cells outnumber CD4 T cells in heart infiltrates (3:1) [38, 39]. By deriving T cell clones and antibodies from patients with Chagas disease, reactivities were observed for various cardiac proteins including cardiac myosin, and also for antigenic epitopes from *T. cruzi* proteins such as B13, cysteine protease cruzipain, and ribosomal proteins P1 and P2. But their disease-inducing potential remains to be tested experimentally [38, 40, 41].

Chlamydial myocarditis characterized by subclinical or asymptomatic phenotype has been noted in 5–15 % of the individuals' positive for *Chlamydia psittaci* infection. Likewise, two other chlamydial species are also implicated in the causation of myocarditis, namely *Chlamydia pneumoniae* and *Chlamydia trachomatis*, where *C. pneumoniae* appears to induce more severe disease than *C. trachomatis* [42, 43]. The underlying autoimmune mechanisms in chlamydial infections, if any, are unknown.

In group A streptococcal infections, the concept of molecular mimicry has been implicated as a major mechanism of disease pathogenesis. Antibodies and T cells specific to streptococcal M protein have been shown to cross-react with host protein, cardiac myosin, and M protein [26, 27]. A cytotoxic human monoclonal antibody against the group A carbohydrate epitope *N*-acetyl-β-D-glucosamine generated from rheumatic carditis patient has delineated the sharing of epitopes between the group A streptococcus, cardiac myosin, and the valve endothelium, basement membrane, and laminin [44]. The basis of this mimicry is the alpha helical structures of the shared molecules [45]. Also, peptide epitopes of human cardiac myosin have been identified in RHD [46]. Antibodies in rheumatic carditis are believed to damage the valve endothelium in human disease leading to edema of the valve and activation of the endocardium and T cell infiltration in the valve [47]. Thus, mimicry is believed to play a role in attacking the heart valve through both antibodies and T cells in human rheumatic carditis. Furthermore, T cell clones derived from intralesional fragments from patients with RHD react with three different regions within the streptococcal protein M5, as well as cardiac proteins derived from valvular tissue, and the immunodominant epitope of M5 protein has been localized to a region spanning the amino acids 81–103 [48–50]. Similarly, T cell clones recognizing other peptide regions of M5, namely B2 and B3A, can react with S2 and light meromyosin regions of human cardiac myosin, suggesting that the immune pathogenesis of RHD involves the mediation of cross-reactive T cell responses potentially for a broad spectrum of cardiac antigens [18, 27, 48, 50].

Interestingly, molecular mimicry has not been limited to cross-reactivity between host and pathogen, but has been extended to include antibodies generated against one host antigen (cardiac myosin or its peptide fragment S2-16 from the S2 hinge region) which can cross-react with another host antigen, β-adrenergic receptor-1 (BAR), possibly leading to apoptosis of cardiomyocytes via the cyclic adenosine monophosphate-dependent protein kinase A signaling pathway [51, 52]. Such an effect by antibodies which signal receptors on cardiomyocytes could be blocked by human cardiac myosin and β-adrenergic blocker [51]. Likewise, cardiac adenine nucleotide translocator 1 (ANT) and anti-CVB3 antibodies can cross-react with each other, suggesting a role for ANT autoantibodies in CVB3/DCM pathogenesis [53], possibly by altering the functional activities of ANT (e.g., energy metabolism) [54, 55]. Thus, antibodies which affect the physiology and function of the heart may be important in leading to altered heart function and arrhythmias in cardiomyopathy.

Evidence from Animal Models

Animal models are useful tools to delineate the autoimmune events in various diseases caused by infectious pathogens. One such model is experimental autoimmune myocarditis (EAM), and the EAM models are valuable because their histological features bear resemblance to post-infectious myocarditis induced with pathogens like CVB3 [56, 57]. The disease is induced in susceptible strains of rodents by immunizing the animals with cardiac proteins, such as cardiac myosin heavy chain-α (Myhc-α), and cardiac troponin I or their peptide fragments [58–60]. The immune pathogenesis of EAM is typically described as delayed-type hypersensitivity reaction that involves the mediation of T cells and macrophages. Although Myhc-α is located intracellularly, its peptide fragments have been found to be displayed by antigen-presenting cells in several mouse strains (C.B-17, SCID, B10.D2, DBA/2J, and A/J) leading to the recognition of myosin peptides by T cells [58, 61]. Using Myhc-α 334–352 as a putative antigen, we recently attempted to identify the microbes that have the potential to trigger myocardial injuries by molecular mimicry. We identified three novel microbial agents—*Cryptococcus neoformans*, *Bacillus* spp. (BAC), and *Magnetospirillum gryphiswaldense*—that carry the mimicry sequence for Myhc-α 334–352 [62]. The mimicry epitopes induce lymphocytic myocarditis in A/J mice reminiscent of the disease induced with Myhc-α 334–352, indicating that exposure to these microbes might predispose to heart autoimmunity [62].

CVB3 is one of the bona fide pathogens of cardiovascular system being used in the myocarditis research. Infection studies with CVB3 in mice have shown that the disease course exhibit two distinct stages that occur in continuum similar to the biphasic nature of the disease in humans [63, 64]: the acute phase (14–18 days postinfection), in which infectious virus is present causing damage to cardiac myocytes, and the chronic phase (beyond 18 days postinfection), in which inflammation reappears, although the extent of virus replication is much reduced due to selection of a defective virus [56, 65].

Previously, it was shown that the severity of CVB3-induced myocarditis was relatively high in mice deficient for CD8, but the disease was attenuated in mice deficient for CD4. Conversely, the mice deficient for both CD8 and CD4 were better protected, suggesting that both cell types modulate the disease outcome [66]. Evidence of autoimmunity in CVB3 infection was first provided based on the observation that the CTLs generated in Balb/C mice infected with CVB3 were lytic to cardiomyocytes, and the CTLs were capable of inducing the disease in naïve mice, but their antigen-specificity was unknown [67, 68]. Using MHC class II/IA^k dextramers for Myhc-α 334–352, we recently showed that the post-infectious myocarditis induced with CVB3 in A/J mice is accompanied by the generation of pathogenic cardiac myosin-specific CD4 T cells that can transfer disease into naïve recipients [69]. We are currently investigating whether CVB3 infection also can lead to the generation of T cells that react with other cardiac antigens, and if so, whether these are pathogenic. Such a possibility exists because CVB3 infection is accompanied by the generation of antibodies for several cardiac antigens [70, 71]. Whether molecular mimicry hypothesis is relevant to CVB3 pathogenesis continues to be uncertain as there are no significant sequence identities between CVB3 proteome and a well-characterized cardiac antigen, Myhc-α. But, the recent data suggest that CVB3 infection can possibly lead to the generation of cross-reactive antibodies for actin because certain degree of sequence homology exists between the two [72]. Likewise, whether the virus that can persist in defective form [65], reactivates, and aggravates disease as a result of viral damage of cardiac tissue is unknown.

In experimental Chagas heart disease, immunization of mice with *T. cruzi* proteins led to the induction of cross-reactive immune responses to cardiac myosin [73]. Likewise, the animals immunized with cardiac myosin also showed parasite-reactive immune response [38, 74]. Consistent with these observations, tolerance studies also suggest that cardiac myosin might be the primary autoantigen recognized in Chagas heart disease [73, 74]. Recent data, however, indicate that other antigens such as BAR and muscarinic cholinergic (MC) receptors also may be targeted in the progression of Chagas heart disease as autoantibodies generated against BAR and MC receptors reacted with *T. cruzi* ribosomal proteins [75, 76].

As to the chlamydial infections, peptides from cysteine-rich outer membrane protein—*C. trachomatis* serovar E (ChTR1) 25–40; *C. trachomatis* serovar C (ChTR2) 25–40; *C. trachomatis* serovars L1, L2, and L3 (ChTR3) 25–40; *C. pneumoniae* (ChPN) 25–40; and *C. psittaci* (ChPS) 25–40—were found to share sequence identities with Myhc-α [23]. By active immunization, the mimicry epitopes induced comparable myocarditis in mice accompanied with the generation of cross-reactive T cells and antibodies [23, 77]. However, in infection studies, although animals infected with *Chlamydia* showed myocarditis and heart-specific antibodies [23, 78], it was not clear whether the antibodies were produced secondarily as a result of recognition of cardiac antigens released following bacterial damage to the heart tissue or as a consequence of molecular mimicry. Regardless of their mechanisms however, the disease-inducing abilities of cardiac antibodies produced in the infected animals has not been tested in the adoptive transfer protocols. Likewise, whether the animals infected with chlamydia show the generation of pathogenic cardiac-reactive T cells also has not been investigated. This is a fundamental question to be addressed because T cell help is critical for production of antibodies. Thus, whether autoimmune response contributes to the pathogenesis of chlamydial myocarditis continues to be debated.

The relevance of molecular mimicry in the pathogenesis of RHD induced by streptococci has been well-studied in various rodent models. Antibodies against the group A streptococcal carbohydrate epitope, *N*-acetyl-β-D-glucosamine, have been shown to recognize alpha helical sequences [45, 79, 80] and represent mimicry between two dissimilar structures. Anti-group A streptococcal antibodies can also cross-react with both CVB3 and cardiac myosin [81, 82]. These cross-reactive immune responses were shown to be pathogenic in the mouse model of CVB3 infection [83]. Mouse monoclonal antibodies that recognize the motif QKSKQ within the streptococcal proteins M5 and M6 can react predominantly with Myhc-α [84], and other proteins, such as tropomyosin, laminin, vimentin, and keratin, may play a role in group A streptococcal cross-reactivities with heart and other tissues [44, 45, 79, 81, 85–87]. Studies in mice suggested that T cells against streptococcal M proteins are pathogenic and have been shown to react with the myocarditogenic peptide NT4 from streptococcal M5 protein which can cross-react with cardiac myosin and CVB3 [83], and the severity of viral myocarditis is reduced in mice tolerized with NT4 peptide [25, 83]. Similarly, streptococcal M protein and/or certain M protein peptides were shown to induce valvulitis in rats [88] and myocarditis in mice [83, 89]. Molecular mimicry although is thought of as a disease process can also be part of the normal immune response to protect against pathogens, as the mechanism dictates "survival of the fittest." For example, an antibody recognizing both host and multiple microbial ligands can be thought of as "killing two birds with one stone" as the host protects itself using an antibody which neutralizes multiple pathogens. Studies have demonstrated this principle when mouse monoclonal antibodies generated against the group A streptococcus also neutralized CVB3 and were cytotoxic for heart cells [81].

Conclusion

The general belief is that environmental microbes, mostly viruses, can trigger autoimmune diseases in ge-

netically susceptible individuals; the challenge is being able to establish their cause and effect relationship. More recent data suggest that exposure to environmental microbes of non-viral origin carrying the sequences identical to Myhc-α, which are otherwise innocuous like BAC, has the potential to trigger heart autoimmunity through molecular mimicry. But a formal proof is needed to verify whether such a phenomenon can occur in humans. Likewise, whether exposure to multiple microbes is required to trigger heart autoimmunity also is not known. This can be addressed in animal models by exposing the myocarditis-susceptible animals to different microbes sequentially like CVB3 followed by BAC or vice versa. To this end, we are setting up an infection model with BAC. Our preliminary data suggest that A/J mice infected with BAC show the generation of Myhc-α-reactive T cells (unpublished observations). Although translating the information generated in animal models to humans is challenging, the growing list of microbes of non-viral origin as potential candidates for heart autoimmunity may create opportunities to target them therapeutically.

Acknowledgments — This work was supported by the American Heart Association and the Children's Cardiomyopathy Foundation (SDG2462390204001, JR), and the National Institutes of Health (HL114669, JR; HL108371, SAH; HL56267, MWC). CM is a recipient of a postdoctoral research fellowship grant awarded by the Myocarditis Foundation, NJ. Associate Editor DeLisa Fairweather oversaw the review of this article for *Journal of Cardiovascular Translational Research*.

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