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Identification of novel mimicry epitopes for cardiac myosin heavy chain-α that induce autoimmune myocarditis in A/J mice

Chandirasegaran Massilamany, Arunakumar Gangaplara, David Steffen, Jay Reddy *

School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, United States

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ABSTRACT

Myocarditis is one cause of sudden cardiac death in young adolescents, and individuals affected with myocarditis can develop dilated cardiomyopathy, a frequent reason for heart transplantation. Exposure to environmental microbes has been suspected in the initiation of heart autoimmunity, but the direct causal link is lacking. We report here identification of novel mimicry epitopes that bear sequences similar to those in cardiac myosin heavy chain (MYHC)-α 334–352. These epitopes represent Bacillus spp., *Magnetospirillum gryphiswaldense*, *Cryptococcus neoformans* and *Zea mays*. The mimicry peptides induced varying degrees of myocarditis in A/J mice reminiscent of the disease induced with MYHC-α 334–352. We demonstrate that the mimics induce cross-reactive T cell responses for MYHC-α 334–352 as verified by MHC class II IA^k/tetramer staining and Th-1 and Th-17 cytokines similar to those of MYHC-α 334–352. The data suggest that exposure to environmental microbes which are otherwise innocuous can predispose to heart autoimmunity by molecular mimicry.

1. Introduction

Myocarditis is one of the predominant causes of heart failure in young adolescents [1]; most affected individuals can remain asymptomatic and the disease is spontaneously resolved. However, chronically, 10–20% of those affected can develop dilated cardiomyopathy (DCM), and approximately half of the patients with DCM undergo heart transplantation due to the lack of effective treatments [2–4]. Myocarditis can occur as a result of exposure to noninfectious (drugs, metals, and chemicals) or infectious etiologies [5]. Microbes such as viruses, bacteria, rickettsia, and parasites have been implicated in the initiation of myocarditis, but the direct causal link remains tenuous [6–10]. It has been suggested that myocardial injuries due to infectious agents might involve the mediation of autoimmunity [11–13].

Microbes are believed to break self-tolerance by mechanisms that involve the release of cryptic or neoantigenic determinants, bystander activation of autoreactive cells, and molecular mimicry [3,14–18]. The heart is not an immunologically privileged organ, and immune cells and their products can freely access and interact with cardiac antigens. Cardiotropic pathogens inflict damage to the heart and cause varying degrees of inflammation, the sequel of which can be either death due to heart failure, recovery, or persistent inflammation. Autoimmune response is usually suspected in chronic inflammatory conditions of the heart in the absence of detectable infectious insult, and, in support of this theory, autoantibodies to various autoantigens have been detected in patients with cardiovascular diseases [19–21]. These autoantigens include cardiac myosin heavy chain (MYHC)-α, the adenine nucleotide translocator of the inner mitochondrial membrane, α-adrenergic receptors, extracellular matrix proteins, creatinine kinase, branched chain ketoacid dehydrogenase, and the muscarinic receptor [22–25]. Accumulated literature suggests that antibodies to MYHC-α have been used as indicators to mark the induction of autoimmunity in diseases of heart [26,27].

Although MYHC-α is an intracellular protein, the protein fragments can be complexed with MHC molecules on the surface of resident antigen presenting cells in heart tissue [28,29]. The pathogens that affect cardiac tissue can cause damage to cardiomyocytes, facilitating the release of MYHC-α to the extracellular milieu, the abundant availability of which can potentially activate self-reactive cells. Alternatively, structural homologies between microbial and self-antigens can trigger autoimmune responses by

Abbreviations: Ala, alanine; Asp, aspartic acid; BAC, Bacillus spp.; BAN, *Bacillus anthracis*; CAA, *N*-carbamoyl-L-amino acid amidohydrolase; CFA, complete Freund's adjuvant; CLIP, class II-associated invariant-chain peptide; cpm, counts per minute; CRN, *Cryptococcus neoformans*; DCM, dilated cardiomyopathy; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; FC, flow cytometry; HEL, hen egg white lysozyme; LN, lymph nodes; LNC, lymph node cells; MAG, *Magnetospirillum gryphiswaldense*; MYHC, cardiac myosin heavy chain; PE, phycoerythrin; PMA, phorbol 12-myristate-13 acetate; RNase, bovine ribonuclease; ROS, *Rhodobacter sphaeroides*; SA, streptavidin; tet^+, tetramer-positive; ZEM, *Zea mays*; 7-AAD, 7-aminoactinomycin D.

* Corresponding author. Address: Room 202, Bldg. VBS, School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, United States. Fax: +1 402 472 9690.

E-mail address: nreddy2@unl.edu (J. Reddy).

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cross-reactivity. Three examples of infectious agents exist, and autoimmunity can be an important component of their disease pathogenesis. These include β-hemolytic streptococci in rheumatic fever [30], coxsackievirus B3 in postinfectious myocarditis [13,21], and *Trypanosoma cruzi* in Chagas disease [31,32]. Emerging evidence, however, suggests that induction of organ-specific autoimmune diseases appears not to follow Koch's postulates; rather, exposure to multiple organisms might be a critical requirement to trigger autoimmunity [33]. In our studies using MYHC-α 334–352, we sought to identify the disease-inducing mimicry sequences in protein databases. This search resulted in the identification of three novel sequences from microbes and one sequence from maize; the mimicry peptides produce varying degrees of myocardial inflammation reminiscent of the disease induced with MYHC-α 334–352 in A/J mice through the induction of MYHC-α-reactive T cells capable of producing Th1 and Th17 cytokines.

2. Materials and methods

2.1. Mice

We used 6- to 8-week-old female A/J mice obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in accordance with the guidelines of the University of Nebraska-Lincoln.

2.2. Identification of mimicry epitopes for MYHC-α 334–352

MYHC-α 334–352 (DSAFDVLSFTAEEKAGVYK) is an immunodominant epitope in which putative MHC class II and TCR binding sites have been identified (Fig. 1) [28]. Using this sequence, we performed pattern searches in the non-redundant database using the prosite scan of the Bioinformatics Toolkit (http://www.toolkit.tuebingen.mpg.de/patsearch, Max-Planck Institute for Developmental Biology, Tuebingen, Germany). The pattern we used was XXXX**D**XXXXXAXXXAXXXX (underlined: TCR-contact residues; bold: MHC-anchor residue; X: non-critical residues). Peptides that showed greatest identity with MYHC-α were selected for further study (Fig. 1).

2.3. Peptide synthesis

All the peptides were synthesized on 9-fluorenylmethyloxycarbonyl chemistry (Neopeptide, Cambridge, MA), and more than 90% purity was confirmed by HPLC and mass spectroscopy. The peptides include: MYHC-α 334–352; MYHC-α 335–350 (SAFDVLSFTAEEKAGV); bovine ribonuclease (RNase) 43–56 (VNTFVHESLADVQA); Bacillus sp. (BAC) 25–40 (EGFTRLSFTAEEKAAH); *Rhodobacter sphaeroides* (ROS) 192–207 (EAFDSLSAIAEEKAAI); *Magnetospirillum gryphiswaldense* (MAG) 726–741 (GKMVAPSFTAEEKAQK); *Cryptococcus neoformans* (CRN) 93–108 (RNIPPPMFTAEEKAEA); *Zea mays* (ZEM) 159–174 (LGSDLRPTFTAEEKALL); *Bacillus anthracis* (BAN) 301–316 (IATDHAPHTAEEKAQG); and N-terminal biotinylated hen egg white lysozyme (HEL) 46–61 (YNTDGSTDYGILQINSR). The peptides were dissolved in 1 × PBS, aliquoted, and stored at −20 °C.

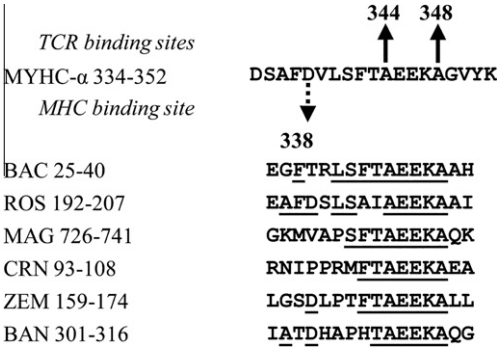

Fig. 1. Comparison of mimicry epitopes with MYHC-α 334–352. The peptide sequence of MYHC-α 334–352 was compared with peptide sequences of mimicry epitopes; identical residues are underlined. Top arrows: TCR-contact residues. Bottom arrow: MHC-anchor residue. BAC, Bacillus spp.; ROS, *R. sphaeroides*; MAG, *M. gryphiswaldense; CRN, C. neoformans;* ZEM, *Z. mays*; BAN, *B. anthracis*.

2.4. Immunization procedures

Immunizations involved peptides emulsified in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* H37RA extract (Difco Laboratories, Detroit, MI) to a final concentration of 5 mg/ml [28,34]. To measure recall responses, 100 μg of each peptide was administered s.c. in multiple sites: sternum, inguinal regions, and foot pads. For disease induction, peptide emulsions (50, 100 or 200 μg/animal in 200 μl volume) prepared as above were administered s.c. in inguinal regions and sternum twice with an interval of seven days. In addition, each animal received pertussis toxin i.p. on days 0 and 2 after the first immunization (List Biological Laboratories, Campbell, CA; 100 ng/mouse).

2.5. Histopathology

Mice were euthanized 21 days after immunization with peptides, and hearts were collected at necropsy and fixed by immersion in 10% phosphate buffered formalin. Three cross-sectional levels of each heart were sectioned in 5 μm thickness and stained with hematoxylin and eosin (H and E) [35]. Hearts were evaluated for inflammation and scored for severity based on the numbers of inflammatory foci added across all sections. Inflammation was primarily classified as suppurative, lymphocytic, or mixed, representing, respectively, neutrophils, lymphocytes, and a mixture of both. The degree of inflammation was scored as normal (0), mild multifocal (1–5 foci), moderate multifocal to coalescing (6–25 foci), and diffuse (26 or more foci) [28,35,36]. In addition, the percent involvement of cross-sectional areas of myocardium infiltrated with cells was scored as 0, normal; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%.

2.6. Proliferation assay

Ten days after immunizations, groups of mice were killed and the draining lymph nodes (LN) (maxillary, mandibular, axillary, inguinal, and popliteal) were harvested. Single cell suspensions were prepared by gently pressing the LN using sterile 3-ml syringe pistons in cell strainers (70 μm) (BD Biosciences, San Diego, CA) and cell pellets were obtained by centrifugation which were then resuspended in 1 × ammonium chloride potassium buffer to lyse erythrocytes (Lonza, Walkersville, MD). After washing, cell pellets were finally resuspended in growth medium containing RPMI supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1× each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin–streptomycin (Lonza) and cell viability was verified to be more than 95% by trypan blue staining. Lymph node cells (LNC) were stimulated at a density of 0.5 to 1×10^7 cells per ml in triplicates with the indicated peptides (0–100 μg/ml) for 2 days in growth medium. In this setup, the cells cultured with no peptides served as medium controls. Proliferative responses were measured as counts per minute (cpm) after pulsing with tritiated ^{3}[H] thymidine (1 μCi per well; MP Biomedicals, Santa Ana, CA) for 16 h