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Cardiac Myosin Epitopes Recognized by Autoantibody in Acute and Convalescent Rheumatic Fever

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Abstract

Background—Acute rheumatic fever (ARF) is an autoimmune disorder associated with *Streptococcus pyogenes* infection. A prevailing hypothesis to account for this disease is that epitopes of self-antigens, such as cardiac myosin react to antibodies against *S. pyogenes*. The goal of our study was to confirm disease epitopes of cardiac myosin, identify immunodominant epitopes and to monitor the epitope response pattern in acute and convalescent rheumatic fever.

Methods—Enzyme-linked immunosorbent assays were used to determine epitopes immunodominant in acute disease and to track the immune response longitudinally to document any changes in the epitope pattern in convalescent sera. Multiplex fluorescence immunoassay was used to correlate anti-streptolysin O and anti-human cardiac myosin antibodies.

Results—Disease specific epitopes in rheumatic fever were identified as S2-1, 4 and 8. Epitopes S2-1, 4, 8, and 9 were found to be immunodominant in acute sera and S2-1, 8, 9, 29 and 30 in the convalescent sera. Frequency analysis showed that 50% of the ARF subjects responded to S2-8. S2-8 responders tended to maintain their epitope pattern throughout the convalescent period, while the S2-8 non-responders tended to spread their responses to other epitopes later in the immune response. There was a significant correlation between anti-cardiac myosin and ASO titers. In addition, S2-8 responders showed elevated ASO titers compared with S2-8 non-responders.

Conclusion—Our studies confirm the existence of S2-1, 4 and 8 as disease specific epitopes. We provide evidence that cardiac myosin S2-8 responders remain epitope stable in convalescence, while S2-8 non-responders shift to neoepitopes. Multiplex data indicated a correlation between elevated anti-streptolysin O and anti-human cardiac myosin antibody titers. Mapping of cardiac

myosin epitopes recognized in rheumatic fever sera may identify immunophenotypes of rheumatic fever.

Keywords

acute rheumatic fever; human cardiac myosin; autoimmunity; autoantibodies; group A streptococcus

INTRODUCTION

Understanding the immunologic targets of antibodies in acute rheumatic fever (ARF) may lead to greater insights into the autoimmune processes that control disease progression. In ARF, Group A Streptococcus (GAS) is believed to be the responsible triggering agent [1, 2].

The antibody response to GAS is protective against infection, but a few GAS components, such as M protein, also induce immune responses against human tissues [3-5]. One well-documented example is the cross reactivity of human cardiac myosin (HCM) with GAS [6, 7]. Cross-reactive epitopes in HCM have been localized using human and mouse monoclonal antibodies, and affinity purified antibodies from ARF sera [8]. These antibodies react with GAS and with epitopes in the rod S2 region of the HCM heavy chain.

Autoimmune susceptible individuals may tend to respond predominantly to pathogenic self-antigenic determinants. Ellis *et al.* [9] identified such disease-specific epitopes of HCM using sera from three globally disparate (US mainland, Hawaii and India) populations with rheumatic heart disease (RHD). They established that some epitopes were more prominent in ARF subjects compared with pharyngitis subjects and age matched controls. These disease-specific epitopes were localized to the S2 subfragment of HCM and were more prominent in the ARF group compared with controls.

The purpose of our study was to identify “immunodominant” epitopes in a cohort of ARF subjects from Hawaii and to determine if epitope shifting occurred in ARF by examining if new epitopes were uncovered by comparing serum antibody responses obtained from convalescent ARF subjects with sera from subjects at the acute stage of their disease. Epitope spreading has not been directly studied in ARF subjects. However, Ellis *et al.* [10] isolated T cell clones from peripheral blood and Fae *et al.* [11] found heart infiltrating T cell clones from RHD subjects undergoing surgery and identified T cells with the same T cell receptor that recognized different heart and streptococcal antigens. Fae *et al.* [11] proposed the degeneracy of the T cell receptor as a mechanism contributing to epitope spreading in ARF. Although both B and T cell epitopes of ARF have been investigated, no studies that follow the epitope recognition patterns of anti-human cardiac myosin antibodies in acute, convalescent and rheumatic heart disease have been done. Furthermore, anti-human cardiac myosin responses have not previously been correlated with elevation in ASO titers. In this study, we confirm the disease specificity of S2 subfragment epitopes, establish immunodominancy of the S2-8 epitope recognized by IgG antibody in both acute and convalescent sera, and analyze individual antibody responses for evidence of epitope shifting in rheumatic fever.

MATERIALS AND METHODS

Peptides and enzyme-linked immunosorbent assay (ELISA)

Sera were obtained from 20 ARF subjects in Hawai'i over a 5-year period. Of the 20 ARF subjects, 15 were diagnosed with carditis and arthritis, while 5 had arthritis without detectable carditis at the time of diagnosis.

Among these subjects, 12 had multiple samples taken after the initial visit. Only 10 subjects had samples both in the acute phase (<40 days after diagnosis) and convalescent phase (>40 days post diagnosis). ARF subjects fulfilled the revised Jones criteria diagnosis of ARF [12, 13].

Control sera (n=68) were obtained from subjects ASO titers <150 Todd units. All participants gave written informed consent. ARF subjects were all treated with anti-inflammatory drugs and given intramuscular penicillin prophylaxis on a monthly basis and fully recovered. The research protocol was approved by the Institutional Review Board of the University of Hawaii, John A. Burns School of Medicine and were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Twenty-five S2 fragment peptides that comprise human cardiac myosin heavy chain used in this study have been described previously [9].

Multiplex Fluorescence Immunoassay (MFIA)

Sera from 13 ARF subjects were tested using the Luminex technology (Luminex Corp., Austin, TX). Streptolysin O from *Streptococcus pyogenes* (Sigma, St. Louis, MO) and purified whole intact human cardiac myosin were used for coupling to microsphere beads as previously described by Martins *et al.* [14, 15]. Data are represented as median fluorescence intensity (MFI).

Statistics

Mann Whitney U test were used to compare optical density (O.D.) values of each S2 peptide obtained for the ARF (n=14), convalescent (n=16), and control (n=68) to identify disease specific epitopes. Differences between samples were deemed significant if *p*-values <0.05.

Immunodominant epitopes were identified as those that exceeded the cutoff of the mean of the median OD value for all peptides plus two standard deviations. The frequency of response to each S2 peptide was determined by evaluating how many ARF subjects (n=14) with acute sera showed a positive reactivity (O.D. >0.300) to each S2 peptide and determining the percentage of the total.

Spearman's rank correlation coefficient was used to assess the correlation between anti-streptolysin O and anti-human cardiac myosin antibodies in ARF subjects (n=13) using the multiplex fluorescence immunoassay.

RESULTS

Disease specific epitopes recognized by antibodies from acute and convalescent subjects

The purpose of this study was to determine the epitopes recognized early in the disease (acute) and compare epitopes recognized later (convalescent) in the disease process. Acute sera from ARF subjects less than 40 days after diagnosis (n=14) and convalescent sera greater than 40 days after diagnosis (n=16) were analyzed. Sera from ARF and from controls (n=68) were reacted with peptides spanning the HCM S2 rod region (peptides S2-1 through 32). Median reactivities were compared among the three groups using Mann Whitney U test. All samples were plotted on a dot graph with the interquartile range indicated. Only those peptides that showed a significant difference between any two groups are shown (Figure 1). The peptide S2-1 had a higher median O.D. of 0.237 in the acute subjects compared to controls at 0.110 ($p<0.0001$).

The median O.D. value was found in >50% of the acute subjects where as 7 of 68 control subjects had O.D values at or above 0.237. Peptide S2-4 had a higher median O.D. of 0.220 in the acute subjects compared to convalescence at 0.104 ($p<0.0005$) and controls at 0.095 ($p<0.05$). Peptide S2-8 was the strongest reacting peptide epitope and had the highest median O.D. in acute and convalescent subjects, respectively, at 0.443 and 0.292 when compared to controls at 0.080 with significance of $p<0.0001$ and $p<0.005$ respectively. The peptides that had significant reactivity in acute rheumatic fever compared to controls were S2-1, 4 and 8 (Figure 1A-C). Peptide S2-4 was the only peptide that had significantly higher reactivity in acute when compared to convalescent and controls (Figure 1B). In testing controls, as noted previously [9] a single peptide, S2-10, was statistically higher in controls (median O.D. = 0.505) in comparison to acute (median O.D. = 0.084) and convalescent (median O.D. = 0.035) sera with a significance of $p<0.05$ and $p<0.005$ respectively. Therefore in acute sera S2-1, 4 and 8 were significantly elevated, while in convalescent sera only S2-8 remained significantly elevated.

Identification of immunodominant epitopes

Immunodominant epitopes were identified by plotting the median of each group of samples (acute, convalescent and controls) for each peptide. A peptide value that was statistically higher than the mean of the median of all other peptides was designated as an immunodominant epitope. The immunodominant epitopes found in the acute samples (n=14) taken after diagnoses were S2-1 ($p<0.0001$), S2-4 ($p<0.005$), S2-8 ($p<0.05$) and S2-9 ($p<0.05$) (Figure 2A). The convalescent sera samples (n=16) taken 40 days after onset identified S2-1 ($p<0.01$), S2-8 ($p<0.0001$), S2-9 ($p<0.05$) and, S2-29 ($p<0.0005$) and S2-30 ($p<0.005$) as immunodominant (Figure 2B). Healthy control sera showed S2-10 ($p<0.0001$) as an immunodominant epitope (Figure 2C). Peptides S2-1, 8 and 9 were identified as immunodominant in both acute and convalescent sera. Only Peptides S2-29 and S2-30 were immunodominant in convalescent and not in other groups and may indicate neoepitopes found later in the immune response. We examined clinical data [erythrocyte sedimentary rate (ESR), C-reactive protein (CRP) and anti-streptolysin O (ASO) titers] between S2-8 responders and S2-8 non-responders. Among the S2-8 responders (n=5), median ASO titers were 952 and S2-8 non-responders (n=7), median ASO titers were 337 with a significant

difference of $p=0.030$ (Table 1). These data suggest a strong link between the S2-8 responses during group A streptococcal infection. There were no significant differences in ESR and CRP between S2-8 responders and S2-8 Non-responders.

It is expected that the number of ARF subjects would respond to immunodominant epitopes more frequently than to other epitopes. Using a cutoff of an optical density 0.300, S2-8 and 9 were recognized by 50% of the 14 acute ARF subjects. In contrast, S2-1 and 4 were each recognized by 36% of subjects.

Assessing responses of neoepitopes in convalescent rheumatic heart disease: potential for intramolecular epitope shifting

Ten longitudinal ARF subjects were available to investigate changes in the epitope pattern since these had samples taken before and after 40 days. Of these 10 longitudinal subjects with matching acute and convalescent sera available as shown in Figure 3, five displayed immunodominant responses to S2-8 in acute sera (subjects 1-5), which for convenience, will be termed “S2-8 responders” and five “S2-8 non-responders,” respectively (subjects 6-10) did not respond to S-8. Interestingly, S2-8 remained one of the immunodominant epitopes in both acute and convalescent samples. In contrast, four of the five S2-8 non-responders gave responses to epitopes S2-9, 22, 23, 26 that were potentially neoepitopes in the later convalescent samples than in the acute stage (see Figure, SDC 1). Only subject 9 did not show a substantial increase in any of the epitopes between the acute and convalescent sera, but the time difference between the acute and convalescent samples was 46 days.

Correlation of anti-cardiac myosin titers with anti-streptolysin O titers

Our study found that elevated anti-cardiac myosin antibody titers strongly correlated ($r = 0.79$; $p < 0.001$) with elevated ASO titers (Figure 3). In addition, we found that S2-8 responders demonstrated significantly ($P = 0.03$) higher ASO titers (Table 1) while the S2-8 non-responders did not correlate with elevated ASO titers.

DISCUSSION

The purpose of our study was to examine the disease specific epitopes to HCM recognized by ARF subjects, to identify the immunodominant epitopes and to track the changes of epitope recognition over time after the onset of ARF. We found that acute ARF subjects had antibodies that reacted significantly higher to peptides S2-1, 4, and 8 when compared to controls. These epitopes were similar to disease specific epitopes identified by Ellis *et al.* [9] who used a larger number of subjects from three geographically distinct populations, including Hawaii. We expanded on the sample set previously analyzed by Ellis *et al.* [9] that included 3 subjects without detectable carditis by clinical means and excluding these samples did not alter the results or p-values. ARF/RHD subjects from Australia [16] had higher reactivity to S2 peptides 1 and 2 of human cardiac myosin compared with controls when all of the peptides in S2 were analyzed.

S2-10 was found to be a prominent epitope recognized by antibodies from controls with no evidence of current GAS infection as determined by having ASO titers within the control range. Ellis *et al.* [9] also found S2-10 was higher in controls compared with ARF subjects

in Hawaii but controls from US mainland or India did not react prominently to S2-10. It is tempting to speculate that S2-10 may be a protective epitope, however, heightened reactivity was not found in other populations, including pharyngitis subjects. Overall, our studies clearly show that in ARF disease-specific HCM epitopes are found in the S2 hinge region of cardiac myosin, but the identity of specific epitopes may vary among subjects depending potentially on the GAS serotype responsible for triggering the response and the genetic variation in immune response among different populations.

Martins *et al.* [17] compared antibody responses to cross-reactive tissue proteins in ARF and age matched control groups over a one-year period and found that subjects had significantly higher levels of antibody to porcine myosin compared with controls early after diagnosis. Gorton *et al.* [16] compared antibody responses from acute ARF/RHD subjects in Australia with those on prophylaxis 1 year after diagnosis. They found that in the prophylactic group, the reactivity response after a year to peptides S2-1 and S2-2 were the same as controls and their data suggested that S2 response could be used as a marker for disease activity and effectiveness of treatment regimen. In our study, reactivity to S2-8 remained high in convalescent subjects, 40 days after diagnosis. S2-30 was significantly higher in the majority of convalescent sera indicating that it may be a neoepitope.

Immunodominant epitopes in subjects from Hawaii were identified by comparing the median reactivity of each peptide to the mean of the median of all other peptides. In acute sera, peptides S2-1, 4, 8 and 9 were the immunodominant. In convalescent sera, S2-1, 8, 9, 29 and 30 were immunodominant and in control sera, S2-10 was found to be immunodominant. Peptide S2-8 showed the highest reactivity in acute (median O.D. = 0.444) and convalescent (median O.D = 0.292) sera in the immunodominant analysis and was also identified as disease specific. Peptides S2-29 (median O.D = 0.160) and 30 (median O.D = 0.419) were only identified in the convalescent sera and not in the acute sera, and not all convalescent sera recognized S2-30. Frequency analysis assumes that immunodominant epitopes were more readily recognized by the host. Indeed, of all the peptides, only S2-8 was recognized by at least 50% of the subjects. Frequency analyses on other purported autoimmune disease have reported a much lower frequency of antibodies to a single epitope from a self-protein. For example, Docheva *et al.* [18] reported that no more than 20% of uveitis subjects responded to a single 25-mer peptide of alpha-crystallin. Only when the responses to five peptides were totaled, did the frequency of autoantibodies approach 50%. Thus, S2-8, in the Hawaii population, appears to be a particularly potent immunodominant epitope and the most consistent in that it was found to be immunodominant in both acute and convalescent sera in addition to being recognized by 50% of the ARF subjects.

Detecting epitope shifting by examining individual patterns was challenging because each had variations in epitope patterns and our sample size was limited. However, we noticed that those individuals who did not react to S2-8 as the immunodominant epitope in their acute sera tended to shift to neoepitopes whereas those individuals who responded to S2-8 in the acute sera did not show an alteration in their pattern. It will be interesting to test the hypothesis that those individuals who did not initially react to the immunodominant epitope, spread to other epitopes whereas those who reacted to the immunodominant epitope did not spread to other epitopes. Confirmation of this finding will require more subjects, longer and

more consistent sampling, and a more detailed evaluation of the clinical conditions during the entire course of the study. The term “epitope spreading” has been defined by T cell responses, not by antibody reactivities. The concept of immunodominant determinants was first introduced in T cell responses [19] when it was recognized that the immune response to foreign protein antigens is limited usually to a small number of determinants and whether similarities exist between B- and T-cell epitope spreading will require additional study. Previous studies have shown that B-cell epitope spreading exist in both rabbits and mice immunized with peptides [20, 21].

Our findings strengthen the proposal that autoantibodies against cardiac myosin are involved in the pathogenesis of ARF reviewed by Cunningham [4] and that such cross-reactive antibodies may persist for over a month after diagnosis. As discussed in detail recently [5], GAS triggers the inflammatory events and is the key first step. Antibodies induced as a result of GAS infection in RHD subjects are cross-reactive not only to host proteins such as myosin and laminin but to carbohydrate entities, especially N-acetyl glucosamine which allows deposition of antibodies to the valve surface endothelium. These antibodies may serve as the “first hit” on the susceptible heart. Developing a monoclonal antibody against S2-8 would reveal if this epitope crossreacts with other tissue antigens or to carbohydrate moieties. Antibodies and T cells lead to further damage on the heart and the release of more self-proteins, especially collagen resulting in induction of other autoantibodies. In fact, Martins *et al.* [15] showed that antibodies to collagen and porcine myosin were higher in subjects with carditis than those without carditis. In a recent review, collagen was discussed as a possible autoimmune strategy and pathogenesis in ARF/RHD [22].

Our preliminary results indicate that individuals who respond to S2-8 originally tend to maintain that response later and do not show shifts to new epitopes whereas those that do not respond to S2-8 shift to other epitopes. Evidence exists that shows that repeated GAS infections are required to trigger ARF reviewed by Carapetis [23]. The only clinical difference between S2-8 responders and S2-8 non-responders were clearly higher ASO titers in the responders. Perhaps this indicates that the more vigorous S2-8 response can be reflected in a higher ASO titer indicating group A streptococcal infection. Indeed we have noted that antibody titers to human cardiac myosin correlates with the ASO titers ($r = 0.79$, $p = 0.001$; Figure 4). It is well known that sequences of HCM cross-react with group A streptococcal antigens [24, 25]. If reactions to dominant epitopes do not occur, a more heterogeneous response may ensue to many different epitopes. In both situations a high titer of anti-cardiac myosin antibody may occur. It would be interesting to examine epitope patterns of S2-8 responders and non-responders during another episode of rheumatic fever. The development of critical memory cells and epitope spreading may contribute to the manifestations of the disease spectrum as discussed originally by Lehman *et al.* [26]. In rheumatic heart disease, epitope shifting may indicate the release of more self-proteins later in the immune response against cardiac myosin such as in the S2-8 non-responders. As discussed by Martin *et al.* [27], these pathogenic mechanisms may play a role in other streptococcal diseases including pediatric autoimmune syndromes associated with streptococcal infections but more importantly related to anti-phospholipid syndromes that includes Libman-Sacks endocarditis [27, 28].

Limitations of our study include the small cohort size and a lack of correlation of immunophenotype with outcomes of heart disease or severity due to treatment of the patients with steroid therapies and penicillin prophylaxis early in disease.

In summary, our data suggest that in ARF there are immunodominant antibody epitopes to human cardiac myosin, some of which are disease-specific. This study of human cardiac myosin peptides resulted in the identification of different epitopes in S2-8 responder and S2-8 non-responder immunophenotypes. Furthermore, our study identified higher ASO titers in S2-8 responders and the correlation of elevated anti-streptolysin O and anti-cardiac myosin antibody titers. Further studies are needed to understand the role of immune responses to immunodominant epitopes in cardiac myosin during the disease process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison analysis of S2 peptide reactivities among serum samples from acute, convalescent and healthy control subjects

The median ELISA O.D. reactivities of each S2 peptide in acute (●; <40days; n=14), convalescent (■; >40 days; n=16) and healthy control (▲ n=68) samples were plotted. Median reactivities were compared among the three groups using Mann Whitney U test. All samples were plotted on a dot graph with the interquartile range indicated. Only those peptides that showed a significant difference between any two groups were shown in Figure 1. (A) The median of S2-1 reactivities were significantly higher in acute subjects than controls with a significance of $p < 0.0001$. (B) S2-4 peptide reactivities were significantly higher in acute sera when compared to convalescent sera with a significance of $p < 0.05$ and acute sera compared to control sera had a significance of $p < 0.0005$. (C) S2-8 peptide reactivities were significantly higher in acute and convalescent sera when compared to control sera with a significance of $p < 0.0001$ and $p < 0.005$ respectively. (D) S2-10 peptide was significantly elevated in control sera compared to acute and convalescent sera with a significance of $p < 0.05$ and $p < 0.005$ respectively. Acute (●), Convalescent (■) and Controls (▲)

Figure 2. Immunodominant epitopes

The median ELISA O.D. reactivities of each S2 peptide in (A) Acute (<40days; n=14), (B) Convalescent, (>40 days; n=16) and (C) Control (n=68) samples were graphed. The median values of each peptide were compared to the mean all other peptides using the Mann Whitney U test. The dotted line represents the mean of the median of all peptides. Peptides shown above the dotted line were identified as immunodominant and were analyzed for significance. (A) Acute sera reactivities identified peptides S2-1 ($p < 0.0001$), S2-4 ($p < 0.001$), S2-8 ($p < 0.05$), and S2-9 ($p < 0.05$) as immunodominantly significant. (B) Convalescent sera reactivities identified peptides S2-1 ($p < 0.01$), S2-8, ($p < 0.0001$), S2-9 ($p < 0.05$), S2-29 ($p < 0.001$) and S2-30 ($p < 0.01$) as immunodominantly significant. (C) Control sera reactivities identified peptide S2-10 ($p < 0.0001$) as immunodominantly significant. All Subjects (■)

Figure 3. Epitope pattern of individual subjects “S2-8 Responders” and “S2-8 Non-responders”
 Sera obtained from the acute (□) and convalescent (■) visits of ten longitudinal ARF (Subjects 1-10) were tested using ELISA against S2 myosin peptides. Subjects 1-5 (left column) are shown as ‘S2-8 Responders’ and subjects 6-10 (right column) are ‘S2-8 Non-responders’. The S2-8 Responder tended to show similar or lower reactivities in the convalescent than acute sera. In contrast the S2-8 non-responders tended to shift to neoepitopes in the convalescent phase except for subject 9.
 Acute (□) and Convalescent (■)

Figure 4. Correlation of Anti-Streptolysin O and Anti-Human Cardiac Myosin Antibodies
Sera obtained from 13 ARF subjects were examined using the multiplex fluorescence immunoassay against streptolysin O and human cardiac myosin proteins. Anti-cardiac myosin antibody strongly correlated with anti-streptolysin O antibodies ($r = 0.79$; $p < 0.001$). ARF subjects (●)

Table 1

ASO Titers are Significantly Higher in S2-8 Responders: Comparison of ASO Titer, CRP, and ESR in S2-8 Responders versus Non-Responders

	S2-8 Responders	S2-8 Non Responders	p-values
ESR (mm/h)	119.5 (n=6)	115 (n=7)	0.47
CRP (mg/L)	7.4 (n=5)	16.2 (n=3)	0.79
ASOT (IU/mL)	952 (n=5)	337 (n=7)	0.03

ESR, erythrocyte sedimentation rate; CRP, c-reactive protein; anti-streptolysin O (ASO) titer, p-value obtained by Mann Whitney test for statistical analysis

n = number of samples in acute phase (< 40 days post diagnosis)