

Reactivity of Rheumatic Fever and Scarlet Fever Patients' Sera with Group A Streptococcal M Protein, Cardiac Myosin, and Cardiac Tropomyosin: a Retrospective Study

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Received 1 March 2000/Returned for modification 14 June 2000/Accepted 18 September 2000

Archived sera (collected in 1946) from acute rheumatic fever (ARF) and untreated scarlet fever and/or pharyngitis patients were reacted with streptococcal M protein, cardiac myosin, and cardiac tropomyosin. Except for very low levels to tropomyosin, antibodies to other antigens were not elevated in the sera of ARF patients relative to those of non-ARF patients, even though there was roughly equivalent exposure to group A streptococci. This suggests that antibodies to these molecules may not play a central role in the induction of ARF.

While a definitive link between group A streptococcal (GAS) pharyngitis and the pathogenesis of acute rheumatic fever (ARF) is still largely undetermined, early studies have pointed to serological cross-reactions between streptococcal antigens and cardiac tissue as a possible connection (3, 6, 24–28, 46, 48, 49). M protein, the primary virulence factor for GAS (32), has received the most scrutiny in this role, since the α -helical coiled-coil structure of M protein resembles that of mammalian myofibrillar proteins such as myosin and tropomyosin (39). A number of studies have involved cross-reactions between M protein and cardiac tissue components, generally focusing on the variable, amino-terminal region of the molecule (2, 7, 11, 12, 44). Recently, Mori et al. showed that ARF patient sera had higher reactivity to the conserved, carboxy-terminal region of the M protein than sera from patients with uncomplicated pharyngitis (38). In the Mori et al. study, the pharyngitis patients received prompt antibiotic treatment and therefore were likely exposed to GAS for only a few days (K. Mori, personal communication). Conversely, rheumatic fever patients, by definition, have not been treated prior to the onset of the disease and have been exposed to the organism for a much longer period of time, perhaps for weeks or longer. It is not unexpected, therefore, that in this context, rheumatic fever patients have higher titers of antibody to streptococcal antigens than do the pharyngitis patients. Due to the possible impact that this data might have on recent efforts to develop a cross-protective vaccine based on this part of the M molecule (4, 9, 19, 41), we examined sera from both ARF patients and patients with untreated, uncomplicated scarlet fever and/or pharyngitis (SF/P) for reactivity to M protein and cardiac myosin and tropomyosin. This study compares sera from patients who were not treated or who, due to the uncertain nature of antibiotic therapy at the time, had been exposed to streptococci for roughly equivalent periods of time.

Samples were selected from patients who had been seen during an outbreak of scarlet and rheumatic fever caused by

GAS at the Great Lakes Naval Training Station, Great Lakes, Illinois, in 1946 (1) (Table 1). A total of 27 serum samples from ARF patients were selected for this study. This included 8 samples from patients who had no antibiotic treatment and an additional 19 samples chosen randomly by matching available patient numbers to numbers produced by a random number generator. Sera from patients with uncomplicated SF/P who had not been treated with antibiotics ($n = 27$) were randomly chosen (as above). Serum samples were collected as close to 4 weeks postonset of scarlet fever as possible (as indicated by the study records), and all but one were collected within the 3- to 5-week period. The sera are part of the Rockefeller University Collection and were maintained under sterile conditions at 4°C. To confirm the reactivity of the antibodies in the serum after more than 50 years of storage, antistreptolysin titers were determined according to a microscale version of the manufacturer's instructions (Difco Laboratories, Detroit, Mich.). Eighteen of 22 (81.8%) ARF sera and 11 of 13 (84.6%) SF/P sera had levels of reactivity that were identical to or within a doubling dilution of published results (1), indicating that the antibodies in the sera had generally maintained their reactivity.

Recombinant, mature M6 protein (rM6) was isolated and purified from *Escherichia coli* strain C600NR carrying plasmid pJRS42.13 as previously described (20). PepM6, the amino-terminal half of the mature M6 protein, was purified from group A streptococcal type M6 strain D471 as previously described (34). Recombinant Δ M6 represents a fusion of amino acids 1 to 17 and 222 to 441 (the carboxy-terminal half) of the mature M6 protein with an 11-amino-acid multiple cloning site spacer between the two fusion sites (S. S. Whitehead, K. F. Jones, and V. A. Fischetti, unpublished data). PepM6 and Δ M6 fragments were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with amino- and carboxy-sequence-directed monoclonal antibodies (23). Peptides M6/240–260 and M6/256–277 represent amino acids 240 to 260 (SRKGLRRDLASREAKKNVEK) and 256 to 277 (KNVEKDLANLTAELDKVKEEKN), respectively, within the C-repeat region of the mature M6 protein. These peptides, as well as peptides MMY/930–950 and MMY/951–973 (below) were synthesized at the Rockefeller University Protein Sequencing Facility. Peptide M5/NT4 represents 19 amino acids (GLKTENEGLKTENEGLKTE) within the A-re-

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TABLE 1. ARF and SF/P patient serum samples

Patient diagnosis and case no.	Antibiotic treatment of patient prior to serum sample	M serotypes present in first four cultures
ARF		
GL2, GL50, GL120, GL128, GL129, GL144, GL173, GL234	None	M3, M17, M19, M30
GL13, GL30, GL45, GL65, GL88	Sulfadiazine or penicillin	M3, M17, M19, M30
GL42, GL52, GL61, GL164	Sulfa or sulfa powder	M3, M17, M30, NT ^a
GL16, GL23, GL44, GL51, GL92, GL175, GL181, GL217, GL247, GL251	Penicillin or penicillin spray	M1, M3, M17, M19, M30
SF/P		
GL3, GL8, GL10, GL11, GL14, GL17, GL31, GL36, GL49, GL55, GL78, GL80, GL97, GL99, GL110, GL112, GL123, GL137, GL158, GL174, GL192, GL222, GL235, GL243, GL279, GL304, GL310	None	M1, M3, M17, M19, M30, NT

^a NT, nontypeable.

peat region of the mature M5 protein (36) and was synthesized as previously described (11).

Human cardiac myosin (17) and human cardiac tropomyosin (37) were prepared as previously described. Peptides MMY/930–950 (DEEEMNAELTAKKRKLEDECS) and MMY/951–973 (ELKKDIDDLELTLAKVEKEKHAT) represent an area of mouse cardiac alpha-myosin, both of which have conformational homology with the streptococcal M6 conserved region at amino acids 256 to 277.

Serum reactivity of ARF and SF/P groups was determined by kinetic enzyme-linked immunosorbent assay (4). Microplates (Maxisorp plates; Nunc, Inc., Naperville, Ill.) were coated with 100 μ l of 1 μ g of rM6, pepM6, and Δ M6 per ml or 5 μ g of myosin, tropomyosin, and synthetic peptides per ml of antigen prior to processing with serum (dilutions are indicated in Fig. 1), alkaline phosphatase-conjugated anti-human immunoglobulin G, and substrate. Data (best absorbance per hour) was corrected to an internal human serum control (i.e., plates coated with a given antigen were adjusted according to the absorbance of a human serum control with known reactivity). Tests were performed twice in triplicate; significance was determined by Student's *t* test.

In general, there was no statistically significant difference between ARF and SF/P patient sera with respect to reactivity to M protein or fragments thereof (Fig. 1). The only M antigens for which this does not hold are rM6 (native molecule) and Δ M6 (carboxy-terminal half). However, in these cases, the reactivity of the SF/P sera was significantly higher than that of the ARF sera ($P = 0.0256$ [rM6] and $P = 0.0172$ [Δ M6]). In fact, except for peptide M6/240–260, the SF/P sera were more reactive than ARF sera with all of the M protein antigens. The degree of reactivity of both serum groups with rM6, Δ M6, and peptides M6/240–260 and M6/256–277 was not surprising in that there is a high degree of cross-reactivity between M6 and the M serotypes isolated during the 1946 outbreak (M1, M3, M17, M19, M30, and, possibly, the nontypeable strains) (Table 1) (23, 45). However, the degree of reactivity to pepM6 was not entirely expected (although certainly lower than that of Δ M6), since this part of the molecule, at least in those regions examined by epitope mapping, does not appear conserved between M6 and outbreak serotypes (23). A truer reflection of the relationship to the amino half of the M6 molecule by these sera is in the lower reactivity with M5/NT4 (at a 1:10 dilution), which is located in the A-repeat region of the M5 molecule and not conserved among these serotypes. This peptide is capable of inducing inflammatory heart disease in a mouse model (22) and for that reason was included in the study. Since M5/NT4-induced carditis is a cellular (not antibody-mediated) process, similar serum reactivity of the two groups for this peptide was not entirely unexpected.

The reactivity of the two groups of sera with myosin and its two synthetic peptides (MMY/930–950 and MMY/951–973) was virtually identical (Fig. 1). In contrast, reactivity with tropomyosin, although low, was significantly higher with ARF sera than with SF/P sera ($P < 0.001$). Since the levels of reactivity were so low against this antigen, it is difficult to speculate whether it might have any significant impact on initiating or exacerbating ARF. A recent study identified the cardiac tissue-reactive component recognized by ARF sera as cardiac tropomyosin (29). Although tropomyosin shares structural homology with M protein, based on its coiled-coil fibrous structure (35) and serological cross-reactivity (18), the immunochemical data presented (high reactivity to M protein, low reactivity to tropomyosin) affords little evidence to suggest that M protein is responsible for the differences seen between ARF and SF/P sera with respect to tropomyosin reactivity. In addition, Cummins et al. showed that patients with myocardial infarction had elevated levels of tropomyosin in their sera relative to those of normal donors (10). It is very possible that the modest, though significant, difference seen between the two serum sample groups is due to autoantibodies raised in response to the release of tropomyosin after the onset of ARF. Such antibodies were also detected by Latif et al. in cardiomyopathy patients who had elevated serum antibodies to cardiac antigens, including tropomyosin (33).

Reactivity of ARF and SF/P sera was also compared for those individuals who had four consecutive positive throat cultures (i.e., who were presumably exposed to equal amounts of GAS antigens; ARF, $n = 10$; SF/P, $n = 17$). No change from the above results was seen (data not shown). In addition, to determine if antibiotic treatment might have had any effect on the number of GAS organisms present and, hence, exposure to GAS antigens, sera from ARF or SF/P patients who received no treatment (ARF, $n = 8$; SF/P, $n = 27$) were compared. Again, there was no discernible change from the original results (data not shown). It should be stressed that despite the treatment of many of the ARF patients with penicillin and/or sulfa drugs, this treatment was clearly ineffective, since most of these patients were still culture positive after treatment (Table 1).

It is well established that antibodies in ARF patient sera will bind to M protein (5, 6, 12, 38) and, conversely, that M protein can induce heart-reactive antibodies in experimental animals (2, 7, 8, 13–16, 31, 40, 44). Within the context of the present study, initiated in part by Mori's data on the reactivity of ARF sera with the conserved, C-repeat region of M protein (38), this region has only recently been investigated with respect to the issue of heart reactivity. Vashishtha and Fischetti demonstrated that C-repeat peptides could induce myosin-reactive

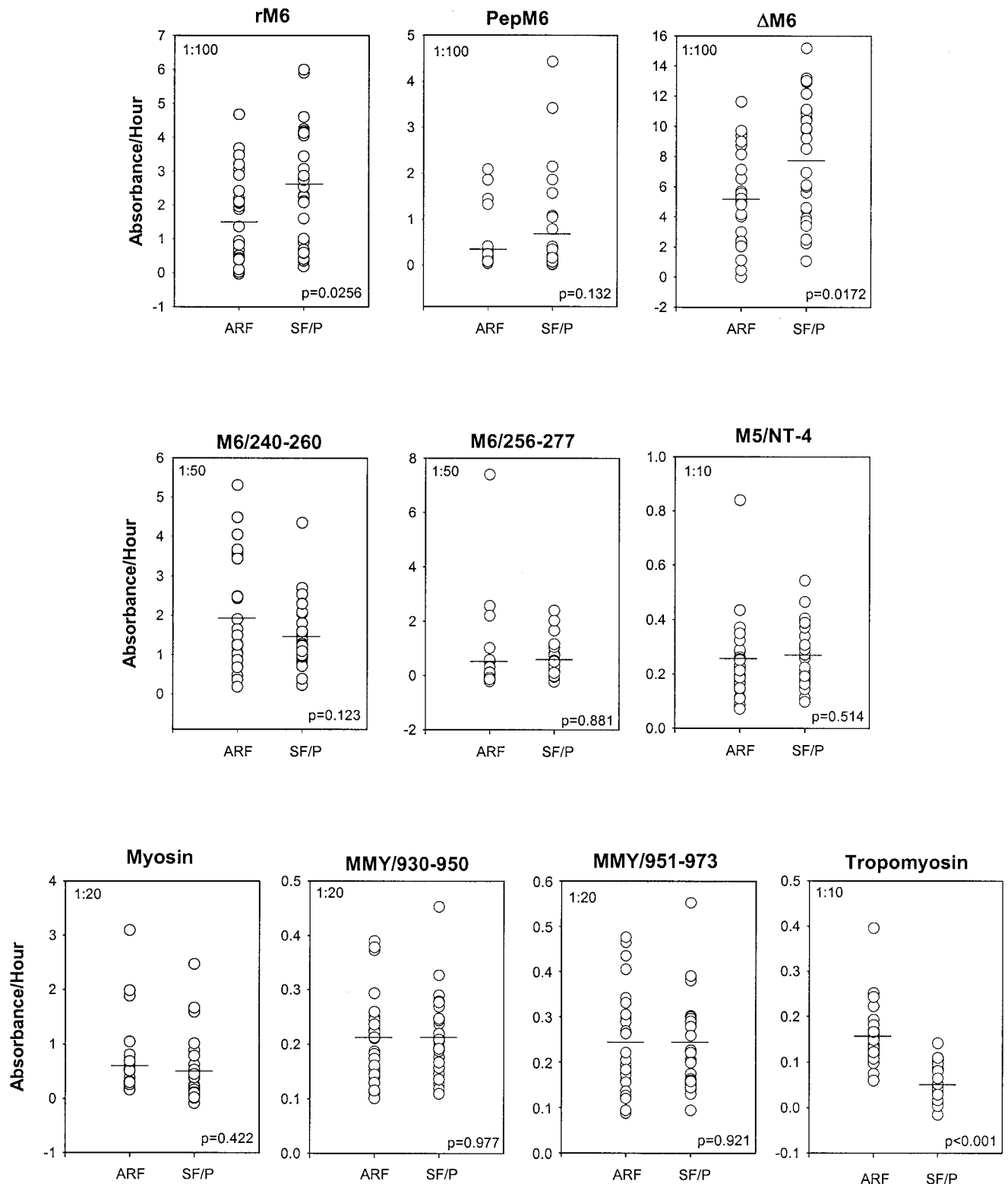


FIG. 1. Kinetic enzyme-linked immunosorbent assay data showing the reactivity of ARF and uncomplicated SF/P patient sera for various streptococcal M protein and heart antigens. Each data point represents the mean of six readings. *P* values were determined by Student's *t* test.

antibodies in rabbits, but only with denatured forms of myosin (47). In addition, Cunningham et al. found that the immunization of mice with human cardiac myosin revealed activated T-cells directed to epitopes within the amino terminal half of

the M5 molecule, but also to a single epitope in the conserved, C-repeat region (11). Interestingly, while many peptides from other parts of the M5 molecule induced lymphocytic infiltrates in immunized mice, those from the C-repeat region did not.

The present study, therefore, confirms that ARF patients have serum antibodies to streptococcal M protein. However, in contrast to Mori et al. (38), the current data indicate that these ARF antibody levels are not significantly higher and, in fact, are lower than those in SF/P patient sera. These data were recently corroborated by Quinn et al. (43). What the present study could not answer, due to low serum availability is whether there are higher levels of anti-heart antibodies within the anti-M protein population of ARF sera than in SF/P sera. Nor can it address the possible role that M-reactive lymphocytes may play in ARF pathogenesis (11, 21, 42). However, we do find that those individuals with SF/P who have significantly higher levels of anti-M protein antibody do not progress to ARF, suggesting that other factors, including potential genetic predisposition (30), may play a significant role in ARF pathogenesis.

This work was supported by a grant from SIGA Pharmaceuticals and USPHS grant AI11822 to V.A.F. and USPHS grant HL35280 to M.W.C.

We gratefully acknowledge the capable technical assistance of Virginia Summerville, Matthew Hollander, and Mary Windels. In addition, we thank John Zabriskie of The Rockefeller University for helpful discussions regarding the Great Lakes Study and the sera therefrom and Mark Hemric for purification of human cardiac myosin.

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Editor: A. D. O'Brien