Molecular Analysis of Human Cardiac Myosin-Cross-Reactive B- and T-Cell Epitopes of the Group A Streptococcal M5 Protein

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Received 25 November 1996/Returned for modification 6 March 1997/Accepted 20 June 1997

The group A streptococcal M protein is an important virulence determinant eliciting protective and auto-immune responses against the streptococcus and cardiac myosin, respectively. In this report, the major human cardiac myosin-cross-reactive T-cell epitopes of M5 protein are identified and localized to myosin-like repeats within the M5 molecule. BALB/c mice were immunized with human cardiac myosin, and the dominant myosin-cross-reactive T-cell epitopes of M5 protein were identified with a panel of 23 overlapping peptides spanning the A, B, and C repeat regions of M5 protein. Human cardiac myosin-cross-reactive T-cell epitopes of M5 protein were localized to several sequences in the M5 peptides NT4 (GLKTENEGLKTENEGLKTE), NT5 (KKEHEAENDKLKQQRDTL), B1B2 (VKDKIAKEQENKETIGTL), B2 (TIGTLKKILDETVDKIA), B3A (IGTLKKILDETVDKIA), and C3 (KGLRRDLASREAKKQ). The NT4 repeated sequence LKTEN was highly homologous with a site conserved in cardiac myosins, the B repeat region peptides were 47% homologous to human cardiac myosin amino acid sequence, and the C3 sequence RRDL was identical to a highly conserved site in skeletal and cardiac myosins. Immunization of BALB/c mice with each of the overlapping M5 peptides revealed myosin-cross-reactive B-cell epitopes throughout the A and C repeat regions and one major epitope in the B repeat region containing the previously reported Gln-Lys-Ser-Lys-Gln (QKSKQ) epitope. The data suggest that the M5 peptides elicited higher antibody titers to cardiac myosin than to skeletal myosin and that several sites in the A and B repeat regions of M5 protein induced myocardial inflammatory infiltrates.

Group A streptococcal M protein has long been recognized as the major virulence determinant of *Streptococcus pyogenes* (26), and it has been found to be structurally and immunologically similar to a number of host autoantigens, including myosin, tropomyosin, vimentin, keratin, and laminin (5–10, 13, 15, 20, 24, 25, 31, 44, 46). Although elevated antibody responses against host tissues may be important in the development of immune sequelae following streptococcal infection, T-cell responses within specific tissues such as the heart may be responsible for the permanent cardiac valve damage that results from rheumatic fever. T-cell responses to M protein have been investigated in a number of studies (11, 19, 34, 35, 40, 41, 48). Early work demonstrated that lymphocytes from patients with acute rheumatic fever (ARF) were highly responsive to streptococcal cell wall or membrane antigens (37–39). In further investigations, it was reported that cytotoxic lymphocytes were present in the blood of ARF patients (22) and that cytotoxic lymphocytes could be stimulated with M protein (11). More recent work has suggested that pepsin-extracted M protein, PepM protein, acts as a superantigen to stimulate human T cells expressing V β 2, V β 4, and V β 8 elements (47, 48, 50). However, researchers in other laboratories have reported that neither recombinant nor native forms of M proteins have superantigenic activity (12, 16, 42). Although there is disagreement over the superantigenicity of the M5 protein (12, 16, 42),

a superantigenic site of streptococcal M5 protein was reported to be localized to the amino acid sequence KEQENKETIGT LKKILDETVDKIAKEQKSKQNIQALKQEL (50). This sequence is located in M5 residues 157 to 197 as described in the sequence published by Manjula et al. (30) or in M5 residues 168 to 208 in the M5 gene sequence published by Miller et al. (32). The superantigenic site is the B3 repeat in the B repeat region of the M5 molecule (50). The M5 superantigenic sequence contains the QKSKQ site previously reported to react with antimyosin antibodies in ARF (7). The M5 superantigenic sequence was shown to have a significant amount of amino acid sequence homology with other superantigens (50).

The M5 serotype of *S. pyogenes* has been associated for many years with outbreaks of rheumatic fever (3) and is an important serotype to investigate for T-cell epitopes. T- and B-cell epitopes of streptococcal M5 protein defined by synthetic peptides in previous studies by Robinson and colleagues (40, 41), by Good and colleagues (34, 35), and in our own laboratory (unpublished data) are summarized in Table 1. M5 peptides reported to induce antimyosin antibodies and myosin-cross-reactive T cells are noted in Table 1. Kalil and colleagues recently reported that M5 protein-heart cross-reactive T-cell clones were isolated from rheumatic valves. The data imply that M protein-heart cross-reactive T cells are important in the pathogenesis of rheumatic heart disease (19).

Cardiac myosin has been shown to produce myocarditis in immunized animals (23, 33, 45, 51), and development of disease has been shown to be dependent upon the presence of CD4⁺ T cells (45). A myocarditis-producing peptide from A/J mouse myosin heavy-chain α isoform (residues 334 to 352) was I-A^k restricted and bound strongly to the I-A^k molecule (14). These findings suggested that autoantigenic peptides can bind

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TABLE 1. Summary of T-cell epitopes of streptococcal M5 protein

Epitope position (peptide)	Sequence ^a	Mouse strain or human origin of T-cell clone (no. of clones or response)
27–44	LKTKNEGLKTENEGLKTE	BALB/c (<i>H-2^d</i>) rM5 ^b
59–76 (NT5)	<u>KKEHEAENDKLKQORDTL</u> ^c	BALB/c (<i>H-2^d</i>) PepM5 ^b
72–89 (NT6)	QRDTLSTOKETLEREVQN	BALB/c (<i>H-2^d</i>) rM5 ^b
85–102 (NT7)	REVQNTQYNNETLKIKNG	BALB/c (<i>H-2^d</i>) rM5 ^b
98–115 (NT8)	KIKNGDLTKELNKTROEL	BALB/c (<i>H-2^d</i>) PepM5 ^b
111–129 (B1A)	<u>TROELANKQOESKENEKAL</u>	BALB/c (<i>H-2^d</i>) rM5 ^b
150–167 (B2)	<u>TIGTLKKILDETVKDKIA</u> ^c	BALB/c (<i>H-2^d</i>) PepM5 ^b
176–193 (B3A)	<u>IGTLKKILDETVKDKLAK</u> ^c	BALB/c (<i>H-2^d</i>) PepM5 ^b
1–35	AVTRGTINDPQRAKEALDKYELENHDLKTKNEGLK	BALB/c (<i>H-2^d</i>) (14 clones) ^d C57BL/6 (<i>H-2^b</i>) (1 clone) ^d
28–54	<u>KTKNEGLKTENEGLKTENEGLKTENEG</u>	BALB/c (<i>H-2^d</i>) (2 clones) ^d CBA/Ca (<i>H-2^k</i>) (1 clone) ^d
55–70	LKTEKKEHEAENDKLK	BALB/c (<i>H-2^d</i>) (1 clone) ^d CBA/Ca (<i>H-2^k</i>) (1 clone) ^d
103–132	DLTKELNKTROELANKQOESKENEKAINEL	BALB/c (<i>H-2^d</i>) (2 clones) ^d
133–162	<u>LEKTVKDKIAKEQENKETIGTLKKILDETV</u>	BALB/c (<i>H-2^d</i>) (2 clones) ^d
209–223	<u>TIGTLKKILDETVKDK</u>	CBA/Ca (<i>H-2^k</i>) (1 clone) ^d
217–237	<u>ISDASRKGLRRDLASREAKK</u>	C57BL/6 (<i>H-2^b</i>) (1 clone) ^d
300–319	<u>DASREAKKQVEKAIEEANSK</u>	BALB/c (<i>H-2^d</i>) (6 clones) ^d CBA/Ca (<i>H-2^k</i>) (5 clones) ^d
312–331	ALEEANSKLALEKLNKELE	C57BL/6 (<i>H-2^b</i>) (1 clone) ^d
329–359	ELEESKKLTEKEKAELQAKLEAEAKOLKEQL	BALB/c (<i>H-2^d</i>) (3 clones) ^d
359–388	AKQAEELAKLRAGKASDSQTPDTKPGNKAV	C57BL/6 (<i>H-2^b</i>) (3 clones) ^d
389–425	VPGKGQAPQAGTKPNQNKAPMKETKRQLPSTGETANP	C57BL/6 (1 clone) ^d
295–313	<u>LRRDLASREAKKQVEKAJ</u> ^{c,e}	B10.BR (<i>H-2^k</i>) (SI = 8.04) ^f
305–324	<u>AKKQVEKALEEANSKLAAL</u> ^{c,e}	B10.BR (<i>H-2^k</i>) (SI = 2.06) ^f
335–354	<u>KLTEKEKAELQAKLEAEAK</u> ^c	B10.D2 (<i>H-2^d</i>) (SI = 5.52) ^f
345–364	<u>QAKLEAEAKALKEQLAKQAE</u> ^c	B10 (<i>H-2^b</i>) (SI = 3.61) ^f
355–374	<u>LKEQLAKQAEELAKLRAGKA</u> ^{c,e}	Humans
1–25	TVTRGTISDPQRAKEALDKYELENH ^c	Humans with ARF ^g
81–96	DKLKQORDTLSTOKETLEREVQNI ^c	Humans with ARF ^g
163–177	ETIGTLKKILDETVK ^c	Humans with ARF ^g

^a Amino acid sequence taken from the sequence of *S. pyogenes* serotype 5 M protein gene as published by Miller et al. (32). Underlined sequences are sites common to the M5 peptides tested in these studies.

^b Stimulation of lymphocytes from lymph nodes of BALB/c mice immunized with rM5 protein or PepM5 protein as indicated. This datum was obtained from Cunningham and Dale (9a).

^c Myosin-cross-reactive T-cell epitope in mice or humans.

^d Stimulation of T-cell clones from rM5-immunized BALB/c mice. This datum was obtained from Robinson et al. (40).

^e Induced antimyosin antibodies reactive with heart tissue extracts or porcine myosin.

^f SI response to HCL M5 protein extract obtained from Pruksakorn et al. (35). Sequence data were obtained from Pruksakorn et al. (34).

^g This datum was obtained from Guilherme et al. (19).

strongly to major histocompatibility complex (MHC) molecules and behave similarly to epitopes from foreign antigens such as pathogens (14). The evidence for specific cardiac myosin epitopes in the development of experimental autoimmune myocarditis is compelling. Researchers in several laboratories have identified specific sites in cardiac myosins which produce inflammatory heart disease (14, 28, 36, 51). Myocarditic regions of cardiac myosins have been localized to the S1 (14, 36), S2 (28, 36), and light meromyosin (LMM) (51) fragments of the molecule. Neu and colleagues found that the myosin peptide (residues 334 to 352) which produced myocarditis in A/J (I-A^k) mice was not myocarditic in the BALB/c (class II MHC I-A^d) strain of mice, illustrating the importance of host susceptibility.

A region within the streptococcal M5 protein which pro-

duces myocarditis in MRL/++ mice was found to be a sequence unique to cardiac myosin and I-A^k restricted and dependent on the presence of CD4⁺ T cells (21). In our report herein, we investigate M5 protein for myocarditogenic sites in BALB/c mice and find that there are several sites capable of inducing inflammation in the heart. Myosin-cross-reactive T-cell epitopes within the M proteins may break immune tolerance to epitopes unique to cardiac myosin and target the immune response to the heart in streptococcal rheumatic carditis.

In our experimental approach to define the myosin-cross-reactive T-cell epitopes of M5 protein, we immunized mice with human cardiac myosin in order to identify murine anti-myosin T-cell cross-reactivities with 23 overlapping M5 peptides. This approach differed from approaches used in previous studies in which M5 peptide-specific T cells were investigated

TABLE 2. Overlapping synthetic peptides of streptococcal M5 protein^a

Peptide residue	Sequence	Amino acid positions
NT1	AVTRGTINDPQRAKEALD	1-18
NT2	KEALDKYELENHDLKTKN	14-31
NT3	LKTKNEGLKTENEGLKTE	27-44
NT4	GLKTENEGLKTENEGLKTE	40-58
NT5	KKEHEAENDKLKQQRDTL	59-76
NT6	QRDTLSTQKETLEREVQN	72-89
NT7	REVQNTQYNNETLKIKNG	85-102
NT8	KIKNGDLTKELNKTRQEL	98-115
B1A	TRQELANKQQESKENEKAL	111-129
B1B	ENEKALNELEKTVKDKI	124-141
B1B2	VKDKIAKEQENKETIGTL	137-154
B2	TIGTLKKILDETVDKIA	150-167
B2B3A	KDKIAKEQENKETIGTLK	163-180
B3A	IGTLKKILDETVDKILAK	176-193
B2B3B	DKLAKEQKSKQNIQALKQ	189-206
B3B	GALKQELAKKDEANKISD	202-219
C1A	NKISDASRKGLRRDLAS	215-232
C1B	DLASREAKKQLEAEHQK	228-245
C1C2	AEHQKLEEQNKISEASRK	241-258
C2A	EASRKGLRRDLASREAK	254-271
C2B	SREAKKQLEAEQQKLEEQ	267-284
C2C3	KLEEQNKISEASRKGLRR	280-297
C3	KGLRRDLASREAKKQ	293-308

^a Peptides are in numerical order from the start of the mature protein (32).

for cross-reactivity with myosin (35). Our experimental design enabled us to define dominant myosin-cross-reactive epitopes located in the A, B, and C repeat regions of M5 protein. In streptococcal sequelae, pathogenic cardiac myosin-specific T cells may be triggered by rheumatogenic M proteins. For these reasons, we chose to investigate the M5 peptide cross-reactivity of myosin-specific T lymphocytes taken from animals after they were immunized with human cardiac myosin. In another group of experiments, we investigated the 23 M5 peptides for the induction of antimyosin antibody responses against human cardiac myosin. Our studies describe previously unidentified murine B- and T-cell epitopes of streptococcal M5 protein which

cross-react with human cardiac myosin. The identification of these epitopes should be important for the understanding of potentially pathogenic epitopes and the evaluation of potential group A streptococcal M protein subunit vaccines.

MATERIALS AND METHODS

Antigens and immunoassays. Purified human cardiac myosin was prepared in our laboratory by previously described procedures (13). The human cardiac myosin contained a single protein band at 200 kDa and did not contain actin or other contaminants. Purity of antigens was determined as homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (13). Myosin was identified in Western blots with a myosin-specific monoclonal antibody (MAb), CCM-52 (a gift from William Clark, Cardiovascular Research Institute, Michael Reese Hospital and Medical Center, Chicago, Ill.). MAb CCM-52 recognizes a site in human cardiac myosin LMM near the HMM-LMM chymotrypsin cleavage site (13).

The synthetic peptides of M5 protein shown in Table 2 were prepared by Ken Jackson, Molecular Biology Resource Facility, W. K. Warren Medical Research Institute, by using the 9-fluorenylmethoxycarbonyl strategy and subsequent high-pressure liquid chromatography purification (4). With the exception of peptide NT5, the synthetic peptides were composed of 18 amino acids with a 5-amino-acid residue overlap at each end. A diagram of the M protein shows the positions of the 23 peptides (Fig. 1). The NT peptides span the A repeat region, the B peptides span the B repeat region, and the C peptides span the C repeat region. Figure 2 displays the complete amino acid sequence of the M5 protein as reported by Miller et al. (32), with the A, B, and C repeats marked. Peptides shown in Table 2 do not include the 142 C-terminal residues downstream of the C repeat region.

The enzyme-linked immunosorbent assay (ELISA), SDS-polyacrylamide gel electrophoresis, and Western blot analysis were performed according to routine procedures as described previously (7-9, 15, 25).

Immunization of mice with human cardiac myosin. For the T-cell studies, 4-week-old, BALB/c (*H-2^d*) sex-matched mice obtained from the colony at the Oklahoma Medical Research Foundation were immunized in the footpad, inguinal region, axillary region, and dorsum between the scapulae with 100 µg of purified human cardiac myosin in complete Freund's adjuvant (CFA). The immunization and peptide mapping procedure as described above was reported earlier for the mapping of dominant T-cell epitopes of multideterminant protein antigens (1, 17).

Isolation of lymphocytes and tritiated thymidine uptake assay. Ten days postimmunization, the peripheral popliteal, axillary, and inguinal lymph nodes were removed from immunized test and control animals (three animals/group). The cells were harvested into a single cell suspension in 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Life Technologies Inc., Grand Island, N.Y.). The cells were washed three times in medium and counted with a hemocytometer. The single cell suspension was placed in a 96-well tissue culture plate (Nunc, Inc., Naperville, Ill.) in the amount of 5×10^5 cells per well in 100 µl of IMDM

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FIG. 1. Diagram of the M protein showing 23 overlapping synthetic peptides representing the N-terminal region (NT), the B repeat region (B), and the C repeat region (C). Table 2 shows the 23 M5 peptide sequences, and Fig. 2 displays the complete amino acid sequence marked. The synthesized peptides do not include the downstream 142 C-terminal residues.

1 A V T R G T I N D P Q R A K E A L D K Y E L E A1 N H D L K T K 30
 31 A2 N E G L K T E A3 N E G L K T E A4 N E G L K T E A5 N E G L K T E K K 60
 61 E H E A E N D K L K Q Q R D T L S T Q K E T L E R E V Q N T 90
 91 Q Y N A6 N E T L K I K I N G D L T K E L N K T R Q E L A N K B1 K Q Q 120
 121 E S K E N E K A L N E L L E K T V K D K I A B2 K E Q E N K E T 150
 151 I G T L K K I L D E T V K D K I A B3 K E Q E N K E T I G T L K 180
 181 K I L D E T V K D K L A B4 K E Q K S K Q N I G A L K Q E L A K 210
 211 K D E A N K I S D A S R K G L R R D L D A S R E A K K Q L E 240
 241 A E H Q K L E E Q C2 N K I S E A S R K G L R R D L D A S R E A 270
 271 K K Q L E A E Q Q K L E E Q C3 N K I S E A S R K G L R R D L D 300
 301 A S R E A K K Q V E K A L E E A N S K L A A L E K L N K E L 330
 331 E E S K K L T E K E K A E L Q A K L E A E A K A L K E Q L A 360
 361 K Q A E E L A K L R A G K A S D S Q T P D T K P G N K A V P 390
 391 G K G Q A P Q A G T K P N Q N K A P M K E T K R Q L P S T G 420
 421 E T A N P F F T A A A L T V M A T A G V A A V V K R K E E N 450

FIG. 2. Complete amino acid sequence of group A streptococcal M5 protein beginning with the amino acid at the start of the mature protein as reported by Miller et al. (32) and used to synthesize the overlapping peptides listed in Table 2. The peptides shown in Table 2 do not include the 142 C-terminal residues downstream of the C repeat region. The A, B, and C repeat regions are marked.

containing 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, and 0.001 M sodium pyruvate.

Lymphocytes were incubated at 37°C in 5% CO₂ for 5 days with intact protein antigen (2 to 200 µg/ml) or with peptide (0.1, 1.0, or 10 µg/ml) before the addition of [³H]thymidine (1 µCi/well) (NET-207, NEN Research Products Dupont, Boston, Mass.). The cells were ³H-labeled for 18 h prior to being harvested onto glass scintillation filters with a cell harvester (Brandel Biomedical Research and Development Laboratories, Inc., Gaithersburg, Md.). The filter was rinsed in trichloroacetic acid followed by methanol, placed into 4 ml of Readyase scintillation fluid (Beckman Instruments, Inc., Fullerton, Calif.), and counted on the L1218 Rackbeta beta counter (LKB, Wallac Oy, Finland). Each sample was counted for 2 min. The samples were tested in triplicate, and results were recorded in counts per minute. Negative controls consisted of lymphocytes alone, and positive controls were lymphocytes stimulated with 10 µg of concanavalin A per ml. Lymphocytes from control mice immunized only with CFA were tested simultaneously to determine the effects of adjuvant on the proliferation ability of the lymphocytes. In all experiments, there were three animals per group. The stimulation index (SI) for each sample was calculated by using the following formula: SI = sample counts per minute/negative control counts per minute. Test samples with an SI over 3.0 were considered positive. Standard error was calculated for each set of triplicate tests. Five separate experiments were performed to determine the human cardiac myosin-cross-reactive epitopes of the M5 protein. The averaged data from four proliferation experiments at peptide antigen concentrations of 1 and 10 µg/ml and the results of a separate proliferation assay at the peptide antigen concentration of 0.1 µg/ml are shown (see Fig. 3). In all experiments the standard error was calculated.

Immunization of mice with synthetic peptides. Synthetic peptides were prepared individually in IMDM and were mixed with incomplete Freund's adjuvant, and 500 µg was injected intraperitoneally into groups of three BALB/c mice. Injections were administered at 0, 2, and 4 weeks, and a final injection was administered at 6 weeks in phosphate-buffered saline (PBS). A group of three mice was euthanized and bled prior to the beginning of the experiment, and control animals were given injections of adjuvant alone for the entire 8 weeks of the experiment. Two weeks after the final injection, the animals were euthanized, serum was collected, and the animals' hearts were fixed in buffered formalin.

Histological examination of tissues. Hearts were fixed in buffered formalin and blocked in paraffin, and sections were cut and stained with hematoxylin and eosin. Stained tissue sections were examined microscopically for cellular infiltrates or damage. Lesions were graded according to the number of cells and the size of the area of infiltration. Mild infiltrates were defined as cellular infiltrates containing mononuclear leukocytes or lymphocytes. The scoring protocol for the Dallas criteria, for the pathological diagnosis of human myocarditis in heart

biopsies, was used to characterize the lesions in the hearts of the mice immunized with M5 peptides. The scoring protocol is as follows: 0, no inflammatory foci; 0 to 1+, fewer than three foci involving <10 myocytes in the cellular infiltration; 1+, more than three foci involving <10 myocytes in the cellular infiltration; 2+, 1 to 2 foci of myocarditis involving >10 myocytes; 3+, more than two foci of myocarditis with confluent foci observed; 4+, multifocal or confluent myocarditis throughout many regions of the myocardium. Each heart was scored, and animals with a score of 0.5 to 1+ or greater were considered positive. Examples of positive lesions are shown in the Results section.

RESULTS

Myosin-cross-reactive T-cell epitopes of streptococcal M5 protein. Lymphocytes isolated from the lymph nodes of myosin-immunized mice were incubated with human cardiac myosin at concentrations from 1 to 200 µg/ml and with M5 protein peptides at concentrations of 10, 1, and 0.1 µg/ml. The optimum proliferation of sensitized lymphocytes (20,000 cpm) was observed at 50 µg of purified human cardiac myosin per ml.

Myosin-cross-reactive epitopes of M5 protein were identified in four separate experiments. The M5 peptides were tested at 10 µg/ml with the myosin-sensitized lymphocytes, and the average of these four proliferation experiments is shown in Fig. 3A. The data revealed that the cardiac myosin-cross-reactive M5 peptides were NT5 (M5 residues 59 to 76; KKEHEAEND KLKQQRDTL [SI = 7.0]), B1B2 (M5 residues 137 to 154; VKDKIAKEQENKETIGTL [SI = 8.1]), B3A (M5 residues 176 to 193; IGTLKKILDETVKDKLAK [SI = 6.3]), and C3 (M5 residues 293 to 308; KGLRRDLASREAKKQ [SI = 6.3]). Less dominant but positive sites (having SIs of ≥3.0) were peptides B2 (M5 residues 150 to 167; TIGTLKKILDETVKD KIA) and C2A (M5 residues 254 to 271; EASRKGLRRDLDA SREAK). These experiments were compared to four assays in which the M5 peptides were tested with the myosin-sensitized lymphocytes at a concentration of 1 µg/ml. The average of these four experiments is shown in Fig. 3B, which reveals that the human cardiac myosin-cross-reactive epitopes of M5 protein are located in B1B2 (M5 residues 137 to 154; VKDKIAKEQENKETIGTL [SI = 14.5]) and B2 (M5 residues 150 to 167; TIGTLKKILDETVKDKIA [SI = 11.1]). Other less dominant but positive sites were peptides NT5 (M5 residues 59 to 76; KKEHEAENDKLKQQRDTL) and NT8 (M5 residues 98 to 115; KIKNGDLTKELNKTRQEL). The medium or no-antigen control (1,773 cpm) was used to calculate the SIs for the experiments described.

In an additional experiment (Fig. 3C), M5 peptides (0.1 µg/ml) were reacted with lymphocytes from the human cardiac myosin-immunized mice; peptide NT4 (M5 residues 40 to 58; GLKTENEGLKTENEGLKTE) gave an SI of 3.4, and B1B2 (M5 residues 137 to 154; VKDKIAKEQENKETIGTL) gave an SI of 2.8. In this experiment, the background counts per minute for sensitized lymphocytes were 3,720 in medium alone or no-antigen control and 1,180 in mice immunized with CFA alone. M5 peptide B1B2 clearly stimulated myosin-sensitized lymphocytes at all of the concentrations tested. However, NT4 did not stimulate lymphocytes from myosin-immunized animals at the higher concentrations and was not an epitope commonly recognized by the immune lymphocytes. Nevertheless, we believe that NT4 is important to mention, because it was positive in one of our lymphocyte proliferation assays and was demonstrated to produce myocarditic lesions in the hearts of immunized mice as shown below.

Collectively, the data identify the dominant human cardiac myosin-cross-reactive epitopes of M5 protein in the NT4/NT5 and the B1B2/B2/B3A regions and in the C3 sequence in the C repeat region. The averaged data revealed several subdominant myosin-cross-reactive epitopes which include NT8 and

FIG. 3. Stimulation of lymphocytes from mice immunized with human cardiac myosin with each of 23 overlapping M5 peptides at concentrations of 10 (A), 1 (B), and 0.1 (C) $\mu\text{g/ml}$ in a tritiated thymidine incorporation assay. (A and B) Data from four separate experiments were averaged; the no-antigen control was 1,773 cpm. (C) Data from one experiment in which myosin-immune lymphocytes were stimulated are shown; the no-antigen control was 3,720 cpm. Error bars indicate standard deviations. Standard error was calculated for the average of four assays shown, and SIs are shown for the dominant peptides. The four individual assays demonstrated similar results. The most dominant myosin-cross-reactive site was B1B2/B2, which appears in all three panels. Other dominant peptides included NT5, B3A, and C3.

C2A. Peptides C3 and C2A have a large amount of sequence identity.

Anti-M5 peptide sera reacted with cardiac and skeletal myosins. To determine which peptides could induce anti-myosin antibody, BALB/c mice (groups of three) were immunized with each of the 23 overlapping synthetic M5 peptides. Antipeptide sera were titrated in the ELISA against human cardiac and rabbit skeletal myosin coated onto Immulon 4 microtiter plates at 10 $\mu\text{g/ml}$. Titers were calculated at a 0.1 optical density titration endpoint. Figure 4 shows the titers of the 23 antipeptide serum samples compared with the adjuvant-immunized control. Titers against purified human cardiac myosin ranged from 25,600 to >204,800 in the ELISA for antipeptides NT4, NT6, NT7, NT8, B1A, B1B, B2B3B, C2B, and C3 (sequences are in Table 2). The titer for the control serum was 400. When skeletal myosin was used as the antigen, titers for anti-NT4, -NT6, and -B2B3B ranged from 1,600 to 6,400 in the ELISA, and the titer for the control serum was 400. These data suggest that the M5 sequences induce stronger responses to cardiac myosin than to skeletal myosin.

In the Western immunoblot assay, the antipeptide sera were reacted with human cardiac myosin to verify the ELISA results. Sera from each group of three immunized mice were pooled. Figure 5 shows the reactivity of each one of the antipeptide sera with cardiac myosin in the blot. The strongest reacting sera were anti-NT3, -NT4, -NT5, -NT6, -NT7, -B2B3B, -C1A, -C1B, -C2C3, and -C3 (sequences are in Table 2). A PBS and adjuvant control serum pool was shown to

produce no reaction with the myosin. All sera were tested at a 1:1,000 dilution.

Histological examination of heart tissues from M5 peptide-immunized mice. Hearts from mice immunized with the M5 peptides were checked for lesions. Formalin-fixed heart tissues were stained with hematoxylin and eosin and examined microscopically for cellular infiltration. Some of the BALB/c mice immunized with peptides NT4 (M5 residues 40 to 58; GLKTE NEGLKTENEGLKTE), NT5 (M5 residues 59 to 76; KKEHE AENDKLKQQRDTL), NT6 (M5 residues 72 to 89; QRDTL STQKETLEREVQN), B1A (M5 residues 111 to 129; TRQE LANKQESKENEKAL), and B3B (M5 residues 202 to 219; GALKQELAKKDEANKISD) developed mild lymphocytic infiltrates as described in Table 3. The infiltrates were mild (0.5 to 1.5+) lesions found in $\geq 10\%$ of the total myocardium in a tissue section of the entire heart and scored by the Dallas criteria for myocarditis as described in Materials and Methods. Figure 6A shows the histology of a heart lesion from an NT4-immunized mouse, and Fig. 6C shows a heart section from a B1A-immunized animal. The lesions in animals given peptides NT5, NT6, and B3B were similar. Figure 6B shows tissue from a control animal which had no lesions. Hearts from animals given incomplete adjuvant were normal upon histologic examination. The M5 peptides NT1, NT2, NT3, NT7, NT8, B1B, B1B2, B2, B2B3A, B3A, B2B3B, C1A, C1B, C1C2, C2A, C2B, C2C3, and C3 (sequences are in Table 2) did not produce any heart lesions following immunization.

DISCUSSION

Since the streptococcal M protein is structurally and immunologically similar to myosin, the purpose of our study was to identify human cardiac myosin-cross-reactive B- and T-cell epitopes of the group A streptococcal M5 protein. Although a strong antibody response against the M protein is required for protection against homologous-type streptococcal infection, T-

FIG. 4. Reaction of anti-M5 peptide sera with human cardiac myosin in the ELISA. Sera were diluted twofold and reacted with 10 μ g of human cardiac or rabbit skeletal myosin per ml in the ELISA. Endpoints were calculated at optical densities of 0.1 and 0.2. The endpoints at 0.1 are shown and were slightly higher but not significantly different from those at 0.2. The relative differences between cardiac and skeletal myosins observed at both endpoints and in different assays were the same. Titers of the anti-peptide sera for human cardiac myosin (A) and rabbit skeletal myosin (B). Titers of the anti-peptide sera against cardiac myosin indicated a more than fourfold increase in reactivity.

cell responses are important in B-helper T-cell function and in the development of inflammation in streptococcal sequelae. T-cell epitopes of streptococcal M5 protein have been reported previously (34, 35, 40, 41). Table 1 summarizes our current knowledge of T-cell epitopes of M5 protein. Among animals expressing different MHC haplotypes, the processed peptides presented to T cells by MHC class II molecules differ for each haplotype. Sercarz and colleagues review the structural constraints in the binding of a peptide to MHC class II molecules (18, 43). These researchers report that a small number of determinants of a given antigen are presented in an immuno-

dominant manner, with the rest of the peptides remaining silent or cryptic. Sercarz and colleagues report that the selection process for presentation of a determinant is dependent on a number of factors, including enzymatic processing sites in the protein antigen, competition with other determinants on the protein itself, T-cell suppression against a particular determinant, the type of antigen-presenting cell, and the affinity of the peptide fragment for the MHC molecule (18, 43). Studies suggest that there is a strong correlation between the affinity of binding and the ability of a peptide to be presented by a particular MHC molecule.

Our immunization protocol was designed to map dominant T-cell epitopes by a method described by Sercarz and colleagues (17). This method is not one of hyperimmunization; on the contrary, it is defined by a single immunization so that only immunodominant regions of a protein will be identified in proliferation assays in which peptides are used as antigens. The immunodominant sites of a protein are the regions that are recognized by a large number of T cells (1, 17). Therefore, host proteins which are homologous to immunodominant regions of a strong bacterial antigen might be expected to have an increased chance of producing cross-reactive T-cell responses. Our study is unique because we immunized mice with cardiac

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FIG. 5. Reactivity of the anti-M5 peptide sera in the Western immunoblot assay of human cardiac myosin. The 200-kDa protein band of the purified human cardiac myosin, shown in the stained (S) portion of the Western blot, reacted most strongly with anti-peptide sera from mice immunized with M5 peptides NT3-7 and B2B3B and C1A, C1B, C2C3, and C3. Sera were tested at a 1:1,000 dilution. A control anti-myosin MAb, CCM-52 (a gift from William Clark, Cardiovascular Research Institute, Michael Reese Hospital and Medical Center), reacted with the 200-kDa band present in our purified preparation of human cardiac myosin heavy chain. Purification of the human cardiac myosin heavy chain to homogeneity has been previously described by Dell et al. (13). The Western blot confirms the data in the ELISA with human cardiac myosin.

TABLE 3. Histological examination of cellular infiltrates in hearts from BALB/c mice immunized with M5 peptides

M5 peptide	No. positive ^a /no. negative (Dallas score)
NT4.....	2/3 (1-1.5+)
NT5.....	1/3 (0.5+)
NT6.....	2/3 (0.5-1+)
B1A.....	1/3 (1.5+)
B3B.....	1/3 (0.5+)
All other M5 peptides ^b	0/3 (0)
None (PBS control).....	0/3 (0)

^a Positive was defined as cellular infiltrate present in $\geq 10\%$ of the total heart section and lesions graded mild to moderate (0.5 to 1.5+) according to the Dallas criteria and myocarditis scoring protocol described in Materials and Methods.

^b NT1, NT2, NT3, NT7, NT8, B1B, B1B2, B2, B2B3A, B3A, B2B3B, C1A, C1B, C1C2, C2A, C2C3, and C3.

FIG. 6. (A and C) Histological examination of formalin-fixed, hematoxylin-and-eosin-stained heart tissue sections from NT4- and B1A-immunized mice, respectively, revealed mild cellular infiltrates. Similar mild infiltrates were also seen in mice given peptides NT5, NT6, and B3B (Table 3). (B) A hematoxylin and eosin stain of a section of heart tissue representative of that from control mice given incomplete Freund's adjuvant. Magnification, $\times 400$ (A and C) and $\times 200$ (B).

TABLE 4. Summary of myosin- or heart-cross-reactive T-cell epitopes of streptococcal M5 protein

Epitope position (peptide)	Sequence ^a	Origin of T-cell clone or response (reference)
1-25	TVTRGTISDPQRAKEALDKYELENH ^b	Valve from human with ARF (19)
81-96	<u>DKLKQQRDTLSTOKETLREVNQ</u> ^c	Valve (19)
163-177	<u>ETIGTLKKILDETVK</u> ^c	Valve (19)
337-356	LRRDLASREAKQVEKAL	Normal peripheral blood lymphocytes (34)
347-366	AKKQVEKALEEANSKLAAL	Mice (35)/normal peripheral blood lymphocytes (34)
397-416	LKEQLAKQAEELAKLRAGKA	Peripheral blood lymphocytes from humans with ARF (34)
40-58 (NT4)	GLKTENEGLKTENEGLKTE	Lymph node of BALB/c mice ^d
59-76 (NT5)	KKEHEAENDKLKQQRDTL	
137-154 (B1B2)	VKDIAKEQENKETIGTL	
150-167 (B2)	TIGTLKKILDETVKDIA	
254-271 (C2A)	EASRKGLRRDLASREAK	
293-308 (C3)	KGLRRDLASREAKQ	

^a Underlined sequences are common to the epitopes and sequences compared in Table 4.

^b The amino-terminal TVTRGTIS sequence was taken from the M5 amino acid sequence published by Manjula et al. (30) and deviates from the M5 sequence published by Miller et al. (32) at positions 1 and 8.

^c Sequences 81-96 and 163-177 were taken from the PepM5 sequence as reported by Manjula et al. (30). These sequences appear in the PepM5 sequence as 67 to 89 and 174 to 188, respectively, as reported by Miller et al. (32). All other sequences shown are from the M5 gene sequence reported by Miller et al. (32).

^d BALB/c mice immunized with purified human cardiac myosin and the recovered lymph node lymphocytes were stimulated with each of the peptides in tritiated thymidine uptake assays.

shown to contain human cardiac myosin-cross-reactive T- and B-cell epitopes, the C repeat peptides did not produce cellular infiltrates in the hearts of immunized BALB/c mice. Previous studies have reported that a C repeat region peptide could generate antibodies reactive with denatured myosin (49). Additional studies have shown the C repeat region to contain T-cell epitopes cross-reactive with selected human cardiac myosin peptides (34). In our study, M5 protein sequences demonstrated to contain potential human cardiac myosin-cross-reactive epitopes were within highly repeated regions of the M5 molecule. Repeated regions of M protein which are homologs of cardiac myosin may break tolerance to this potent autoantigen.

The immunological and sequence similarity of cardiac myosin to M5 protein may be an important observation. The comparison of anti-M5 peptide titers against cardiac and skeletal myosin revealed higher titers to cardiac myosin. Other rheumatogenic serotypes will need to be investigated to determine if this is a characteristic of rheumatogenic M protein serotypes. Potential human cardiac myosin-cross-reactive B-cell epitopes of M5 protein were identified by immunization with the 23 different peptides and then reaction of the antipeptide sera with cardiac and skeletal myosin. Streptococcal M5 peptides NT3 to NT7 in the A repeat region, peptide B2B3B in the B repeat region, and peptides C1A to C3 in the C repeat region were capable of inducing B-cell responses and antibody against human cardiac myosin. In the past (7), we identified in M5 protein the Gln-Lys-Ser-Lys-Gln (QKSKQ) site recognized by affinity-purified antimyosin antibody from sera of patients with ARF. The B2B3B peptide stands out vividly as a strong inducer of antimyosin antibody in BALB/c mice, against both skeletal and cardiac myosin. Although the B2B3B peptide contains a strong myosin-cross-reactive epitope recognized by humans and mice, it was not found to contain a T-cell epitope.

Antipeptide ELISA titers generally correlated with the Western blot analysis results. However, in a few cases, such as that of the anti-NT3 sera, the ELISA titer to myosin was low, while the anti-NT3 sera were positive in the blot assay. This might be explained by conformational changes between the myosin protein on the ELISA plate and SDS treatment in the Western blot analysis. Thus, the differences between the results

of the Western blot assay and the ELISA can most likely be explained by the difference in the conformational states of the protein. Although the role of antimyosin antibodies in rheumatic heart disease is not clear, antimyosin antibody was shown elsewhere to deposit in the hearts of susceptible animals and produce myocarditis (29).

Table 4 summarizes the myosin-cross-reactive T-cell epitopes of M5 protein that we demonstrated in BALB/c mice and compares our results with those of Kalil and colleagues, who defined the M5 sequences recognized by T-cell clones from rheumatic heart valves (19). Table 4 also shows studies by Good and colleagues, who demonstrated that human peripheral blood cross-reactive T-cell clones responded to M5 peptides and myosin peptides (34). A most important correlation shown in Table 4 is that M5 peptides NT5/NT6 and B1B2/B2, dominant myosin-cross-reactive epitopes in BALB/c mice, contain sequences that overlap with the M5 peptides recognized by T cells from rheumatic heart valves (19). T-cell clones from rheumatic heart valves were reactive with the peptides of M5 protein and responsive to heart proteins in purified heart extracts. The collective evidence strongly suggests that certain regions of the M5 protein are involved in inflammatory heart disease in animals and in ARF.

Due to MHC restriction of T-cell responses, epitopes would not be expected to be identical in the human and the BALB/c mouse; however, we know very little about the binding of the cross-reactive peptides to MHC molecules or the T-cell receptor. It is interesting that viral, bacterial, and self peptides structurally similar but lacking sequence similarity contained important contact residues for HLA class II molecule and the T-cell receptor and were capable of stimulating an individual T-cell clone known to be responsive to the self peptide (52). Therefore, peptides with diverse sequences could be recognized by a single T-cell receptor. These data suggest that the peptide surface contacted by the HLA class II and T-cell receptor molecules is important in the recognition and stimulation of the T cell. It is then understandable why immunologically cross-reactive sequences may not be required to have a large amount of sequence homology for the activation of specific T cells. In the present study there is sequence homology

between myosin and the M protein, molecules which appear to contribute to disease.

Since cardiac myosin has a well-known ability to produce myocarditis, while skeletal myosin does not (23, 33), amino acid sequences that are unique to cardiac myosins may be important for their cross-reactivity to M proteins and may target T-cell responses to the heart in rheumatic fever. The identification of the M protein and/or myosin epitopes presented by MHC class II molecules in the susceptible host is important for understanding how rheumatic carditis evolves. Data in our paper suggest that immune responses against M protein are directed more at cardiac myosin and that sequences such as NT4 and B1B2/B2/B3A are more homologous to cardiac myosins than to skeletal myosins. The hypothesis that M proteins may break immune tolerance to unique and pathogenic epitopes of human cardiac myosin may be important for defining the immune mechanisms of rheumatic carditis.

ACKNOWLEDGMENTS

We thank Carol Crossley for excellent technical assistance and Ken Jackson, Director, Molecular Biology Resource Facility, W. K. Warren Research Institute at the University of Oklahoma Health Sciences Center, for the synthesis and purification of M protein peptides.

This work was supported by NIH grants no. HL35280 and HL35289 to M.W.C. from the National Heart, Lung and Blood Institute.

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Editor: V. A. Fischetti