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Expression of Protective and Cardiac Tissue Cross-Reactive Epitopes of Type 5 Streptococcal M Protein in *Escherichia coli*

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The immunochemical properties of type 5 M protein antigens that were expressed in *Escherichia coli* K-12 by recombinant lambda bacteriophages isolated from a gene bank of serotype 5 *Streptococcus pyogenes* have been analyzed in detail. M proteins from partially purified bacteriophage lysates displayed precipitin lines of identity with a purified peptic extract of type 5 M protein (pep M5) in immunodiffusion assays. Immunoblot analyses of the M protein-positive lysates demonstrated that the cloned M protein component resided in five polypeptides with relative molecular weights of 57,900 (57.9K), 55.4K, 52.9K, 40.0K, and 32.6K. The hybrid λ phage (λM5)-produced M protein contained immunoprotective epitopes; λM5 protein inhibited opsonization of type 5 streptococci by pep M5 antibodies, and antiserum raised against λM5 lysates opsonized type 5 streptococci. Each of the five antigenic polypeptides of the recombinant phage M protein also shared epitopes with human heart tissue, as demonstrated by the reactivity of immunoblots of λM5 antigens separated on sodium dodecyl sulfate gels with anti-pep M5 antibodies absorbed to and eluted from human heart sarcolemmal membranes. Moreover, antiserum raised against the λM5 lysates reacted with sarcolemmal membrane proteins with relative molecular weights of 200K, 59K, 55K, 53K, and 27K as determined by immunoblot analyses. These results demonstrate that the structural gene coding for type 5 streptococcal M protein which was inserted into lambda DNA expresses immunoprotective epitopes, some of which are shared with human heart tissue.

A large body of evidence indicates that the surface fibrillar M protein is the major virulence factor in the pathogenesis of group A streptococcal infections (11, 15, 23). The M protein renders the organisms resistant to recognition and ingestion by phagocytic cells. Antibodies directed against exposed epitopes on the M protein fibrils opsonize the streptococci, which are then readily ingested by phagocytic cells in samples of fresh blood (11, 15). These observations have served as the basis for the development of M protein vaccines for protection against streptococcal pharyngitis and thus acute rheumatic fever. The production of a safe and effective M protein vaccine has been hampered by the finding that group A streptococci contain antigens which cross-react with host tissues (5, 12, 26, 28). Investigators have feared that M protein preparations may possess tissue cross-reactive moieties which may cause, rather than prevent, rheumatic carditis. Indeed, studies in our laboratory have shown that type 5 M protein contains, within its covalent structure, protective epitopes that share cross-reactivity with sarcolemmal membrane proteins of cardiac tissue (5).

Attempts to gain a thorough understanding of the structure as it relates to the function of the M protein molecule have been hindered by an inability to obtain M protein in an intact form and in sufficient quantities necessary for primary structural analyses of the entire M protein molecule (11). To overcome this problem, the techniques of molecular genetics have been applied to the expression of M protein determinants in a background that should enable isolation and purification of large quantities of intact M proteins. Scott and Fischetti (20) and Fischetti et al. (10) have recently cloned the gene that encodes for type 6 M protein, and Spanier et al. (22) cloned the type 12 M protein gene into *Escherichia coli*. We now have employed the molecular approach to study the protective and cardiac cross-reactive epitopes of type 5 M protein (13). A gene bank of type 5 *Streptococcus pyogenes* has been constructed with an *E. coli* K-12 host-lambda bacteriophage (L47.1) replacement vector system. The recombinant λ bacteriophages expressing the type 5 M protein antigen in *E. coli* have been identified and isolated from this gene bank (13). In this paper, we describe some of the immunochemical properties of the cloned type 5 M protein expressed by λM5 in *E. coli*. We show that antigenic determinants of the M protein are expressed in several molecular species. Moreover, the cloned M protein contains not only protective epitopes but also epitopes that are shared with membrane proteins of cardiac sarcolemma.

MATERIALS AND METHODS

Bacteria, bacteriophage strains, and culture conditions. Cultures of serotype 5 *S. pyogenes* were grown as previously described (4). The construction of recombinant λ bacteriophages expressing the type 5 M protein (λM5) is described elsewhere (13). Individual λM5 phages are described in the text. The λS5.2 is a recombinant phage, isolated from the type 5 *S. pyogenes* gene bank, which did not express M5 antigen. This phage was selected at random to be used as a negative control for M5-producing recombinant phages. Media, growth conditions, and buffers used in phage propagation were carried out as described by Maniatis et al. (16).

Preparation of λM5 lysates and pep M5. Crude lysates were prepared by infecting *E. coli* LE392 with sufficient λM5 to give confluent bacterial lysis on λ agar plates. Phage-de-

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rived material was eluted from agar surfaces with 6 ml of λ buffer containing protease inhibitors (0.01% each of phenylmethyl sulfonyl fluoride and benzamidine) and 0.25 ml of chloroform. Particulate material was removed from the eluate by centrifugation at 100,000 × *g* for 90 min, and the supernatant was dialyzed against distilled water and lyophilized. The lysates contained ca. 100 μg of protein per mg (dry weight) of material. Pepsin-extracted type 5 M protein (pep M5) was prepared as previously described (1, 21).

Immunodiffusion analysis of λM5 lysates. Agar gel immunodiffusion analyses were carried out as described by Ouchterlony (18). Undiluted rabbit antiserum against pep M5 was reacted with 3-mg/ml samples of lyophilized λM5 and λS5 lysates or with 10-μg/ml samples of pep M5 dissolved in 0.02 M phosphate buffer–0.15 M saline (PBS; pH 7.2).

ELISA inhibition assay. An enzyme-linked immunosorbent assay (ELISA; 8) was used to determine the presence of M protein in λM5 lysates by the inhibition of the interaction of antibody with pep M5 as previously described (7). Briefly, lysates (20 mg [dry weight]) were diluted twofold in 0.25 ml of PBS with 0.05% Tween-20 (PBS-Tween) and then incubated with a constant dilution (1:3,200) of rabbit antiserum to pep M5 for 2 h at 37°C. The pep M5 (0.25 ml of a 5-μg/ml solution in 0.1 M carbonate buffer [pH 9.6]) was immobilized on the walls of polystyrene cuvettes by incubating for 2 h at 37°C. After washing with normal saline with 0.05% Tween-20 (saline-Tween), the pep M5-coated wells were incubated with the lysate-antibody mixture. The cuvettes were again washed with saline-Tween, and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) was used to quantitate any remaining (uninhibited) rabbit anti-pep M5 antibodies by standard procedures (7).

Opsonization inhibition. Opsonization inhibition tests were used to demonstrate the presence of protective type 5 M protein epitopes in λM5 lysates (3). Samples of opsonic antiserum, which were diluted with PBS to an ELISA titer of 3,200 (to produce ca. 50% opsonization in the final mixture), were incubated with serial twofold dilutions of λM5 lysate. Immunoprecipitates were removed by centrifugation, and the absorbed and unabsorbed antisera were used to preopsonize homologous streptococci (3). The streptococci were then added to fresh human blood to determine the percent inhibition of opsonization (3).

Immunoelectrophoretic assay of λM5 protein. The λM5 and λS5 lysates and *E. coli* LE392 (2.5 mg [dry weight] per well) were boiled for 3 min in 2% sodium dodecyl sulfate (SDS) and then electrophoresed on 11% or 8 to 18% (continuous gradient) polyacrylamide gels containing 0.1% SDS (14). After electrophoresis, the proteins were transferred to nitrocellulose paper (0.2 μm; 15 by 15 cm; Schleicher and Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (24). After the transfer, the nitrocellulose paper was soaked in 0.01 M Tris–0.9% NaCl–0.05% Tween-20–3% bovine serum albumin (pH 7.6; TS-BSA). The membranes were reacted with a 1:1,000 dilution of rabbit pep M5 antiserum in TS-BSA buffer. After incubation for 2 h at room temperature, the membranes were washed six times in TS buffer and resuspended in TS-BSA buffer. M protein was visualized by reacting the nitrocellulose with rabbit polyclonal or mouse monoclonal antibodies against pep M5 (6). The nitrocellulose was then washed six times as described above and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G. The nitrocellulose sheet was washed with 0.02 M Tris–0.33 M NaCl (pH 7.5) and placed in 100 ml of this buffer containing 100 μl of 30% hydrogen peroxide, followed by the addition of 60 mg of horseradish peroxidase color development reagent (Bio-Rad Laboratories, Richmond, Calif.) dissolved in 20 ml of cold (−20°C) methanol. The molecular weight of λM5-derived M antigen was determined in SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (27).

Assays for cardiac tissue cross-reactive epitopes in λM5 lysates. Cardiac cross-reactive epitopes in λM5 were determined by reacting electroblotted λM5 lysate protein with rabbit anti-pep M5 antibodies (as described above) eluted from human cardiac sarcolemma (5). The presence of heart-reactive antibody in rabbit anti-λM5.4 lysate antisera (see below) was determined by using antibodies purified over a column of pep M5 conjugated to cyanogen bromide-activated Sepharose 4B. The affinity-purified antibodies were then reacted with SDS-polyacrylamide gel electrophoresis and electroblotted human heart sarcolemmal membranes (5). Rabbit antibodies were visualized by using peroxidase-conjugated goat anti-rabbit immunoglobulin as above.

Preparation of anti-λM5.4 lysate antiserum. New Zealand White rabbits (2 kg) were immunized intracutaneously with 10 mg (dry weight) of λM5.4 and λS5.2 lysates emulsified in complete Freund adjuvant (2). Serum was obtained before immunization and at 2-week intervals. Additional injections of 10 mg of λM5.4 and λS5.2 lysates in 0.5 ml of PBS (pH 7.2) were given intravenously on weeks 2, 4, and 8. The antibody titers of the immune sera against M protein were determined by ELISA by using pep M5 as the antigen (5) and by opsonization tests of homologous type 5 streptococci (3).

Absorption and inhibition assays of test sera. A 1-ml sample of a 1:10 dilution of each test serum employed in these studies was preincubated twice with 2 mg (dry weight) of λS5.2 by slow rotation at 37°C for 1.5 h, followed by centrifugation at 10,000 × *g* for 15 min in a Beckman Microfuge model 12 to remove any antibodies directed against lambda bacteriophages or *E. coli*. For the inhibition studies, 1-ml samples of diluted (1:10) rabbit antisera were incubated twice with 2 mg of λM5.4 lysate, 2 mg of human heart sarcolemmal membranes, or 1 mg of crude pep M5 and then centrifuged before use in the various antibody assays described above.

RESULTS

Immunochemical characterization of λM5 M protein. Fifteen recombinant λ bacteriophages expressing group A streptococcal M protein antigen(s) in *E. coli* K-12 were isolated from a gene bank of M type 5 *S. pyogenes* on the basis of their ability to react with anti-pep M5 antisera in immunoblot screening experiments (13). The λM5 lysates formed precipitates with rabbit pep M5 antisera, exhibiting precipitin lines of identity with each other (Fig. 1). The λM5 lysate, λM5.1, displayed antigenic identity with pep M5. Neither the λS5.2 (M protein-negative) recombinant (Fig. 1E) nor the host strain, *E. coli* LE392 (Fig. 1F), formed precipitin lines with the anti-pep M5 antiserum.

To determine whether the cloned gene product contained protective antigenic determinants, opsonization inhibition tests were performed (Fig. 2). The protein-containing lysates inhibited the opsonization of type 5 streptococci by pep M5 antiserum in a dose-related fashion, reaching a maximum of 100%. Similar results were obtained in ELISA inhibition tests; all of the antibodies against pep M5 were inhibited by the λM5 protein (data not shown). These data indicate that the M5 antigens expressed by λM5 contain all of the im-

munoprotective determinants contained in the pep M5 polypeptide fragment.

Coomassie blue staining of SDS gels of λM5 phage lysates demonstrated that the lysates contained numerous polypeptides (Fig. 3A). Subsequent immunoblot analyses showed that the M protein antigens expressed by 14 of the 15 λM5 bacteriophages resided in five polypeptides with relative molecular weights (M_r) of 57,900 (57.9K), 55.4K, 52.9K, 40.0K, and 32.6K (Fig. 3B). One recombinant, λM5.8, expressed M protein antigen in five peptides which each had an M_r 2K to 3K larger (M_r 61.0K, 58.6K, 55.6K, 41.9K, and 34.3K; Fig. 3C) than the corresponding peptides expressed by the other 14 M protein-positive recombinants. Similar patterns to those obtained with anti-pep M5 polyclonal sera for λM5.4 and λM5.8 antigenicity were observed when a mouse monoclonal antibody (hybridoma IIC5.6) against pep M5 was used in the immunoblot analyses (Fig. 3D and E, respectively). Neither the λS5.2 M-negative control (Fig. 3F) nor the sonic lysates of the propagating strain of *E. coli* (Fig. 3G) contained material that reacted with the anti-pep M5 antibodies.

Cardiac cross-reactive epitopes in λM5 lysates. Previous studies of the immunochemical properties of pep M5 have demonstrated the presence of cardiac tissue cross-reactive epitopes in the pepsin-extracted M protein molecule (5). To determine whether the cloned M protein contained these heart cross-reactive epitopes, immunoblots of the phage lysates were reacted with affinity-purified heart-reactive pep M5 antibodies that were adsorbed to and eluted from sarcolemmal membranes of human cardiac sarcolemma. The immunoblotted λM5 phage lysates contained multiple cross-reactive molecular species (Fig. 4). The cross-reactive species corresponded in molecular weight to the five major polypeptide antigens of λM5.4 and λM5.8 lysates as demonstrated with unfractionated pep M5 antiserum (see Fig. 4B and C, respectively). Again, λM5.8 expressed cross-reactive antigenic determinants of slightly larger molecular weight (M_r of ca. 2K to 3K; Fig. 4C). (It should be noted that the 55.4K and 52.9K bands of λM5.4 lysate, as well as the 58.6K

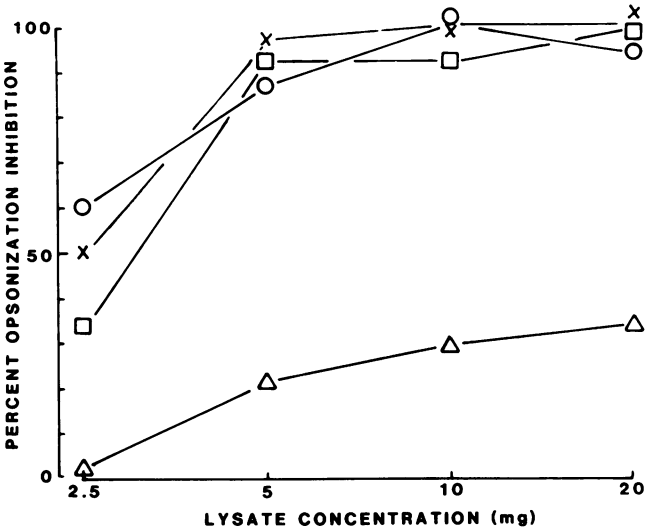

FIG. 2. Inhibition of opsonic antibodies directed toward type 5 streptococci by λM5.1 (○), λM5.2 (×), λS5.2 (△), and λM5.4 (□) lysates.

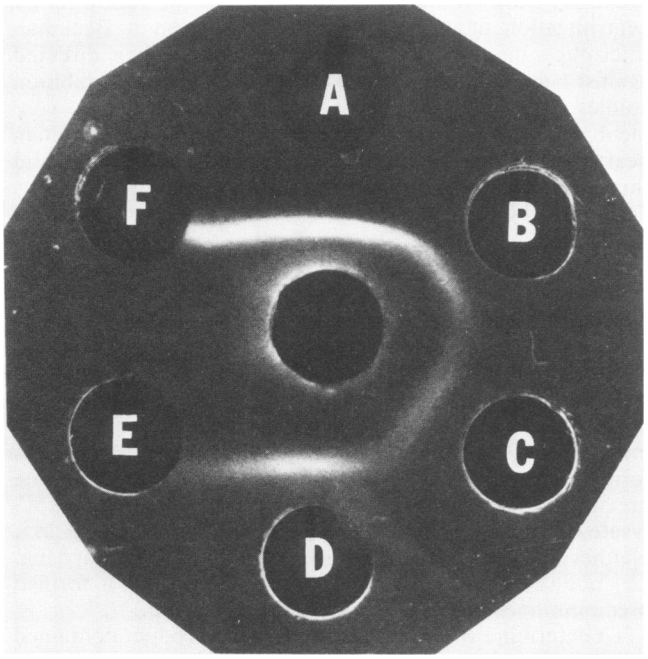

FIG. 1. Ouchterlony double-diffusion analysis of λM5 lysates. When reacted with rabbit antiserum raised against pep M5 (center well), the λM5 lysates (λM5.1 [B], λM5.2 [C], and λM5.4 [D]) formed precipitin lines of identity with each other, whereas λM5.1 exhibited homology with pep M5 (A). Note that the M protein-negative control lysate (λS5.2 [E]) and the propagating strain of *E. coli* (F) did not display any antigenic homology with λM5 lysates or pep M5.

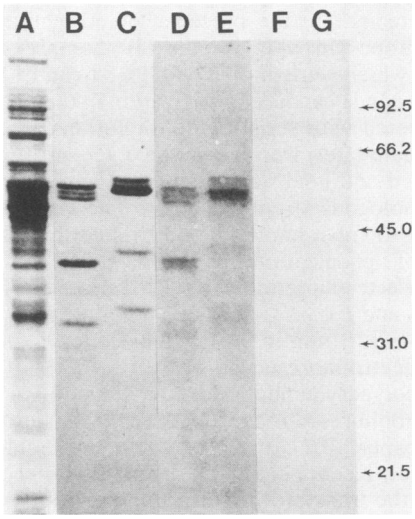

FIG. 3. SDS-polyacrylamide gel electrophoresis and immunoelectrophoretic blot analysis of λM5 lysate antigenic determinants. (A) Coomassie blue staining of λM5.4 revealing the numerous polypeptides of the crude lysate. (B) Western blot analysis of λM5.4 lysate demonstrating the five major anti-pep M5 reactive determinants (M_r 57.9K, 55.4K, 52.9K, 40.0K, and 32.6K). (C) The anti-pep M5 reactive epitopes of the λM5.8 lysate also resided in five polypeptides (M_r 61.0K, 58.6K, 55.6K, 41.9K, and 34.3K). The antigenicity of λM5.4 (D) and λM5.8 (E) lysates was demonstrated with mouse monoclonal anti-pep M5 antibodies. Note that the λS5.2 lysate (F) and *E. coli* K-12 strain LE392 (G) failed to show M protein-like antigenic material.

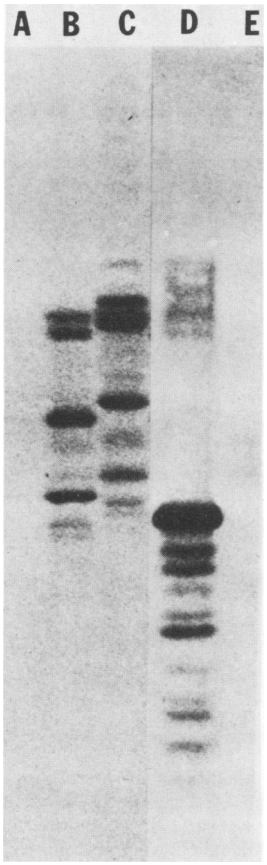

FIG. 4. Western blot analysis of human heart tissue cross-reactive epitopes expressed by λM5. Rabbit anti-pep M5 antibodies purified from human heart sarcolemmal membranes reacted primarily with the five major antigenic determinants of the λM5.4 (B) and λM5.8 (C) lysates. A partially purified pep M5 (D) served as a positive control; *E. coli* LE392 (A) and λS5.2 lysate (E) did not possess heart reactive antigens.

and 55.6K bands of λM5.8 lysate, appear as a single broad band when electrophoresed in an 8 to 18% continuous gradient gel.) All heart tissue cross-reactive epitopes in λM5 phage lysates were contained in polypeptides of greater molecular weight than those in the original (M_r, 30K) pep M5 immunogen (Fig. 4D). The M protein-negative control, λS5.2, and the *E. coli* K-12 LE392 host did not display any antigens that were shared with cardiac tissue epitopes (Fig. 4A and E, respectively).

Immunogenicity of λM5 M protein. To provide direct evidence for the presence of protective epitopes, rabbits were immunized with the M polypeptide expressed by the hybrid phage, λM5.4. Two animals developed antibodies against pep M5 as measured by ELISA (Fig. 5). Two of these antisera also opsonized type 5 streptococci (Fig. 6).

Cardiac tissue cross-reactivity of antisera raised against cloned M5 protein. The heart tissue cross-reactivity of the antibodies raised against λM5 lysates was examined in Western blots. The antisera were first purified by affinity chromatography over columns of pep M5-Sepharose 4B. The affinity-purified antibodies were then reacted with electroblotted sarcolemmal membranes (Fig. 7). The antibodies against the cloned M5 protein reacted primarily with five sarcolemmal membrane peptides of M_r 200K, 59K, 55K, 53K, and 27K as well as with several faintly staining peptides (Fig. 7C). These heart-reactive antibodies were completely inhibited by incubating the antibodies with pep M5 (Fig. 7D) or with cardiac sarcolemma (data not shown). Neither the preimmune serum nor antiserum raised against the control M protein-negative lysate (λS5.2) possessed any detectable heart cross-reactive antibodies.

DISCUSSION

We have shown that the gene encoding for type 5 group A streptococcal M protein can be successfully cloned into a lambda phage vector and expressed in *E. coli* (13). The type 5 M protein expressed by the recombinant phages exhibited all of the known immunological characteristics of the molecule purified from pepsin extracts (pep M5); it contained all of the immunoprotective determinants as well as the heart cross-reactive epitopes of pep M5. Most importantly, the cloned M protein was immunogenic in rabbits and evoked antibodies that opsonized streptococci and cross-reacted with human myocardium. The finding that the recombinant M protein is immunologically similar to pep M5 is not surprising in light of recent reports which suggest that the pep M protein represents a polypeptide fragment of the native molecule (9, 19). Fischetti et al. demonstrated that the pep M6 molecule is considerably smaller than the detergent (9)- or phage lysin (19)-released forms. In addition, protoplast-secreted type 12 M protein is larger than the pep M12 molecule (25). Most recently, Scott and Fischetti (20) and Fischetti et al. (10) have shown the recombinant type 6 M protein to have an M_r 4K to 6K larger than lysin-released M6. Based on these studies, it can be speculated that the M_r

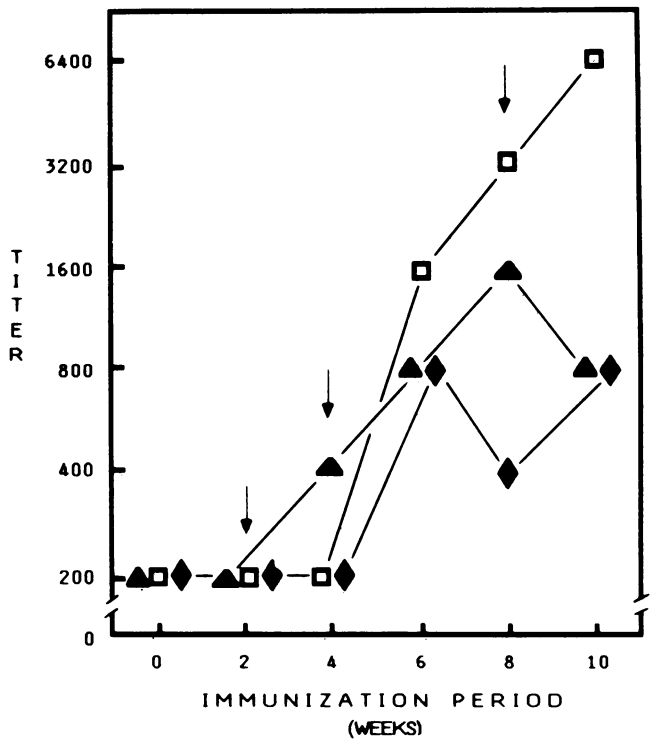

FIG. 5. ELISA determination of the immunogenicity of the λM5.4 lysate in rabbits. The λM5.4 lysate was capable of evoking the formation of anti-pep M5 antibodies in three rabbits (8325 [□], 8326 [▲], and 8327 [◆]). Additional injections of λM5.4 antigen are shown by the arrows.

57.9K λM5-derived polypeptide represents the intact type 5 M protein. Furthermore, the cloned M5 protein is significantly larger than the pep M5 fragment, with an M_r of ca. 23K (17), but similar in size to protoplast M protein (unpublished data; 25). The cloning and expression of the entire structural gene in *E. coli* will enable detailed immunochemical analyses of the intact M protein molecule.

In addition to the 57.9K polypeptide of M5, we also observed four lower molecular weight forms. These may represent degradative products of the M_r 57.9K polypeptide generated by proteolytic activity in the lysates. However, when the M protein gene was inserted into a plasmid and expressed by viable *E. coli* (13), these lower molecular weight forms were much less apparent. The susceptibility of M protein expressed by λ phage to proteolytic degradation is supported by the observation in Western blot analyses that treatment of the M protein produced by λM5 with pepsin at pH 5.8 resulted in a decrease in the intensity of staining of the M_r 57.9K, 55.4K, 52.9K, and 40.0K protein bands, with a concomitant increase in the intensity of staining of the 32.6K band (unpublished data).

One of the recombinant λM5 phages, λM5.8, expressed M5 polypeptides that were all approximately 2K to 3K larger in size than the corresponding antigens expressed by the other λM5 phages. Furthermore, there is evidence for the existence of more than one copy of the M5 determinant in the *S. pyogenes* chromosome (13). It is possible that one of the copies of the M5 gene is different, giving rise to the expression of an M5 determinant which varies in size from those cloned in the other λM5 recombinants isolated. Alternatively, the M5 determinant cloned in λM5.8 may have suffered an alteration, such as the fusion to another cistron or the insertion of a small DNA segment, that did not affect the translation reading frame during or subsequent to its cloning.

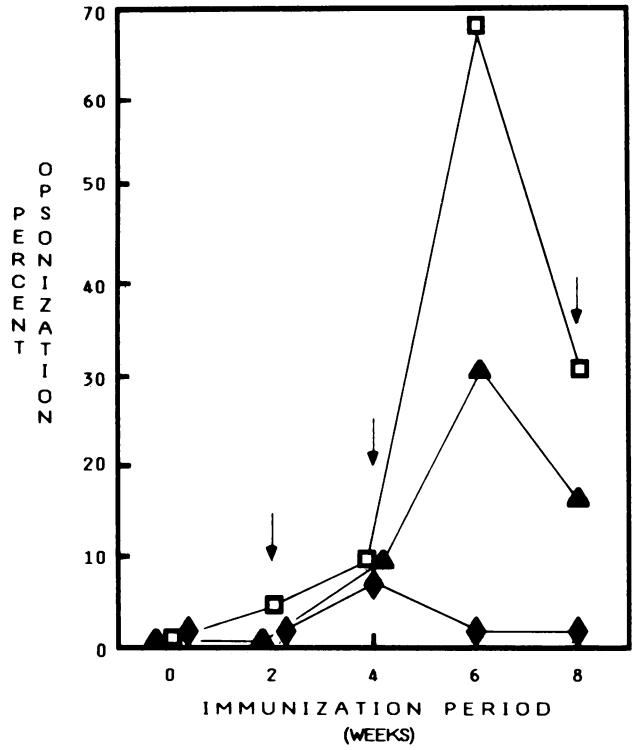

FIG. 6. Detection of immunoprotective epitope(s) in the λM5.4 lysate by opsonization. Of three rabbits (8325 [□], 8326 [▲], and 8327 [◆]) immunized with λM5.4, two (8325 and 8326) produced significant levels of antibodies which were opsonic for homologous organisms. Additional injections of λM5.4 antigen are shown by the arrows. Percent opsonization is the percentage of polymorphonuclear leukocytes with associated streptococci.

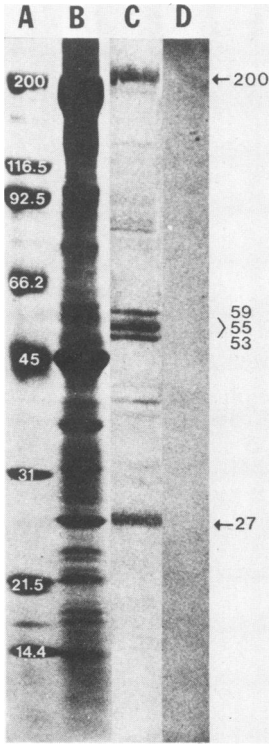

FIG. 7. Reactivity of anti-λM5.4 antibodies with human heart sarcolemmal membranes. Coomassie brilliant blue-stained molecular weight standards (A) and sarcolemmal membranes (B) are shown. (C) Anti-λM5.4 lysate antibodies reacted with electroblotted sarcolemmal membranes. (D) Anti-λM5.4 lysate antiserum absorbed with pep M5 before reaction with electroblotted sarcolemmal membranes.

The inhibition by λM5-expressed M protein of streptococcal opsonization and sarcolemmal immunofluorescence produced by antiserum raised against pep M5 provided indirect evidence for the presence of the immunoprotective and cardiac cross-reactive determinants in the λM5 lysates. Direct evidence was obtained by immunization experiments. In addition to the protective antibodies, the recombinant type 5 M protein evoked antibodies that cross-reacted with cardiac sarcolemma. The reaction of sarcolemmal membrane-specific anti-pep M5 antibodies with λM5 lysates indicated that the tissue cross-reactive moiety resided in each of the five major polypeptide fragments of the recombinant M protein. Moreover, the λM5 M protein stimulated the production of antibodies which reacted with five sarcolemmal membrane polypeptides. The anti-λM5.4 antibodies which cross-reacted with sarcolemmal membrane peptides could be absorbed completely with pep M5 and with the sarcolemmal membranes, indicating that the heart cross-reactive epitopes are exposed on the surface of intact heart membranes.

The present study has permitted the isolation and characterization of the antigenic and immunogenic regions of what appears to be a complete M protein molecule. Whether or not expressed material indeed represents the entire molecule

can be determined by nucleotide sequence analysis of the M protein structural gene. This will facilitate the identification of the immunologically significant determinants of the M protein molecule. Such information is essential not only for the development of a safe and effective streptococcal vaccine but also for the elucidation of those regions of M protein which evoke autoimmune responses.

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