OPSONIC ANTIBODIES EVOKED BY HYBRID PEPTIDE COPIES OF TYPES 5 AND 24 STREPTOCOCCAL M PROTEINS SYNTHESIZED IN TANDEM

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M proteins project as α-helical coiled-coil fibrils from the cell surface of *Streptococcus pyogenes* (1, 2). These surface fibrils render the organisms resistant to ingestion and killing by blood phagocytes (3). The antiphagocytic effect is neutralized by type-specific antibodies directed against the M protein. In addition to the type-specific antibodies, certain M proteins evoke crossreactive immune responses against sarcolemmal membranes of cardiac tissue (4, 5) and muscle myosin (6). The latter findings have hampered efforts to develop M protein vaccines against rheumatogenic strains of *S. pyogenes* because of the fear that the vaccines may trigger rather than prevent acute rheumatic fever and rheumatic heart disease. In an attempt to avoid heart crossreactive epitopes, we prepared vaccines from native and chemically synthesized subpeptide fragments of M protein. We found that selected synthetic peptides copying known amino acid sequences of different M proteins evoked type-specific protective immunity against the related streptococci without stimulating heart crossreactive immune responses (7–13). In this paper, we report that hybrid peptides containing copies of selected regions of two different serotypes of M protein synthesized in tandem evoke protective but not heart crossreactive immune responses against both serotypes of *S. pyogenes.* Our findings may have bearing not only on the development of safe, multivalent M protein vaccines to protect against the many M serotypes of streptococci giving rise to acute rheumatic fever and rheumatic heart disease, but also on the development of multivalent hybrid vaccines against a variety of other infectious agents.

Materials and Methods

Natural and Synthetic Peptides of Streptococcal M Protein. Natural polypeptide fragments of M protein were isolated and purified from limited digests of types 5 and 24 M proteins

This work was supported by research funds from the U.S. Veterans Administration, and by research grants AI-10085, AI-13550, and AM-16506 from the U.S. Public Health Service. Address all correspondence to Dr. Edwin H. Beachey, VA Medical Center, 1030 Jefferson Avenue, Memphis, TN 38104.

as described (14, 15). The polypeptides designated pep M5 and pep M24 were judged to be pure by SDS gel electrophoresis and quantitative amino acid analysis (16). Synthetic copies of the COOH-terminus, 23–35, of the cyanogen bromide–derived fragment 7 (CB7) of type 24 M protein (17), and the NH_2-terminus, 1–20, of pep M5 (9) were the same as those used and reported in previous studies (9, 10). They are designated S-CB7(23–35)C and S-M5(1–20), respectively. In addition, two hybrid peptides, S-M5(1–20)-S-CB7(23–35)C and S-M5(1–20)-S-CB7(19–34) were synthesized by the solid-phase method of Merrifield (18) as previously described (10). The synthetic peptides were purified by gel filtration on columns of Sephadex G50, and further purified as needed by reverse-phase HPLC on Ultrasphere ODS (Whatman Inc., Clifton, NJ) (10). The purified peptides were analyzed for purity and composition by quantitative amino acid analysis and automated Edman degradation to the penultimate amino acid residue (8, 17).

Rabbit Immunization. Sets of three New Zealand White rabbits (2 kg) were each injected with a 50 nmol dose of the respective hybrid synthetic peptides emulsified in CFA as described (9, 10). Rabbits were bled before, and at 2-wk intervals after the primary immunizing dose. At 4, 8, and 10 wk, each rabbit was injected with a 50 nmol s.c. booster dose of the same peptide in PBS (0.02 M phosphate with 0.15 M NaCl, pH 7.4). The preparation of antisera against S-M5(1–20), S-CB7(23–35)C, pep M5, and pep M24 was previously reported (9, 10). All sera were heat-inactivated at 56°C for 30 min and stored in sterile vials at 4°C.

Assays of Anti–M Protein Antibodies. The rabbit sera were assayed for antibodies against M protein by ELISA, opsonophagocytic assays, and indirect bactericidal assays, as described (19). The serotype specificity of the antibodies raised against the hybrid peptides was determined by assaying the antisera against types 6 and 19 streptococci, as well as against types 5 and 24 organisms in opsonophagocytic assays (19).

Assays for M Protein Epitopes. The presence of protective M protein epitopes was determined by the ability of natural and synthetic polypeptides to inhibit the opsonization of types 5 and 24 streptococci by antisera raised against the hybrid peptide S-M5(1–20)-S-CB7(23–35)C. The antisera (0.1 ml) were mixed with an equal volume of PBS containing 100 μM synthetic peptide. After incubation for 30 min at 37°C, the mixture was used in opsonophagocytosis tests of types 5 and 24 streptococci (19).

Results

The compositions of the hybrid peptides, S-M5(1–20)-S-CB7(23–35)C and S-M5(1–20)-S-CB7(19–34) were confirmed by quantitative amino acid analysis. Automated Edman degradation to the penultimate residue gave the amino acid sequence of S-M5(1–20)-S-CB7(23–35)C shown in Fig. 1.

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              5                   10
Ala-Val-Thr-Lys-Gly-Thr-Ile-Asn-Asp-Pro-Glu-Ala-
←──────────────── M5 ─────────────────

         15                  20
Ala-Lys-Glu-Ala-Leu-Asp-Lys-Tyr-Arg-Lys-
─────────────────────────────→ ←──────
    25                 30               34
Ala-Asp-Leu-Glu-Lys-Ala-Leu-Glu-Gly-Ala-Met-Cys
──────────────── M24 ────────────────→
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FIGURE 1. Amino acid sequence of S-M5(1–20)-S-CB7(23–35)C.

The COOH-terminal cysteine residue was added so that the peptide could form dimers and thereby potentially increase its immunogenicity. The sequence of S-M5(1–20)-S-CB7(19–34) was found to be identical except for an additional four residues, Ala-Leu-Ala-Ala, between the tyrosine residue at position 20 and the arginine residue at position 21, and the deletion of the COOH-terminal methionine and cysteine residues. The latter peptide was synthesized in an attempt to

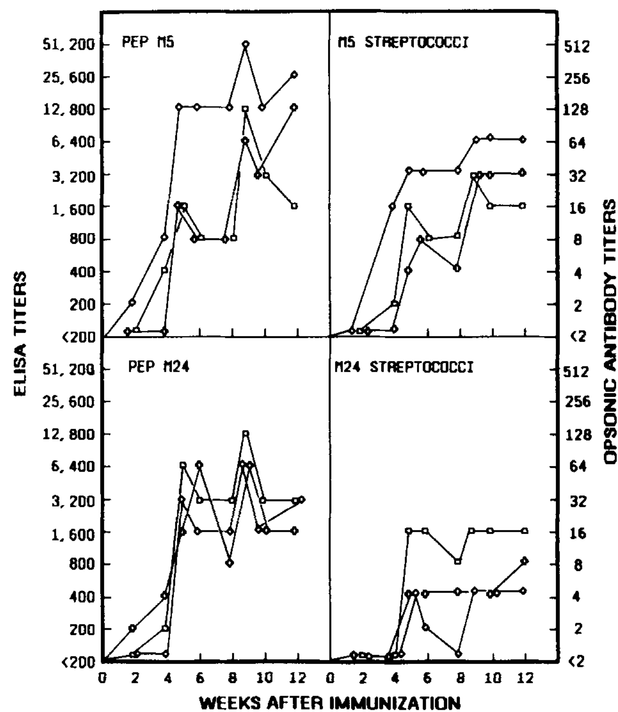

FIGURE 2. Immune responses in three rabbits (□, ⬡, ◇) as measured by ELISA against purified pepsin extracts of types 5 and 24 M proteins (pep M5 and pep M24) (12, 13) (*left*) or by opsonic antibody assays against types 5 and type 24 streptococci (*right*) after immunization with 50 nmol of S-M5(1–20)-S-CB7(23–35)C emulsified in CFA, followed 4, 8, and 10 wk later by the same dose in PBS. Each symbol represents a different rabbit. Sera collected at 2-wk intervals were assayed for antibodies to types 5 and 24 M proteins (pep M5 and pep M24) adsorbed to the walls of plastic cuvettes by ELISA. ELISA titers are expressed as the reciprocal of the highest dilution of antiserum giving an absorption >0.1 at 405 nm. Opsonic antibodies were assayed as described in Materials and Methods. The results of the opsonophagocytosis tests were confirmed by indirect bactericidal tests performed as previously described (19). The M protein type specificity was confirmed by the failure of the M5-M24 hybrid peptide immune sera to promote phagocytosis and killing of heterologous types 1, 6, 18, and 19 streptococci.

maintain the maximum α-helical potential of the hybrid peptide should this secondary structural feature play a role in protective immunogenicity.

Each of three rabbits injected with 50 nmol of S-M5(1–20)-S-CB7(23–35)Cys, followed by booster injections with the same dose in saline at 4, 8, and 10 wk, developed high titers of serum antibodies against both type 5 and type 24 M protein as measured by ELISA, and against type 5 and type 24 *S. pyogenes* as measured by opsonophagocytic tests (Fig. 2). The immune sera were able to promote not only phagocytosis but also killing of both types 5 and 24 streptococci (Fig. 2), indicating that the antibodies evoked by the synthetic hybrid peptide recognized protective antigenic determinants of both M serotypes exposed on the surfaces of the respective serotypes of streptococcal cells.

Similar results were obtained with the free synthetic peptide S-M5(1–20)-S-CB7(19–34). Even though the latter peptide lacks a COOH-terminal cysteine residue and the potential to produce dimeric hybrid molecules, it nevertheless

TABLE I

Type-specific Inhibition of Opsonic Antibodies against Types 5 and 24 Streptococci in Sera of Rabbits Immunized with M5-M24 Hybrid Peptide

Serum used in opsonization tests	Opsonization (percentage of neutrophils with ingested streptococci)	
	Type 5 streptococci	Type 24 streptococci
Preimmune	2	0
Pooled (three rabbits) immune anti-S-M5(1-20)-S-CB7(23-35)C		
Uninhibited	80	88
Inhibited with:		
Type 5 streptococci	2	92
Type 24 streptococci	82	0
S-M5(1-20)	0	88
S-CB7(23-35)C	78	0
S-M5(1-20)-S-CB7(23-35)C	2	0

Equal volumes of serum obtained at 12 wk from each of the three immunized rabbits (see Fig. 2) were pooled and used in opsonization tests as described in Material and Methods. Preimmune sera obtained before the first immunizing dose were similarly pooled. The tests of inhibition of opsonization were performed by preincubating 0.2 ml samples of twofold dilutions of the pooled immune serum for 30 min at 37°C with pellets of type 5 or 24 streptococci obtained by centrifuging 5 ml of an 18-h Todd-Hewitt broth culture of each of the organisms. After removing the organisms by centrifugation and membrane filtration (0.45 μm), the absorbed sera were employed in the opsonization tests. The soluble inhibitors were dissolved at a concentration of 100 μM in PBS, and 0.1 ml of the solutions were incubated with 0.1 ml of pooled immune serum for 30 min at 37°C before adding the mixture to the phagocytosis tests mixtures. The results are expressed as percentage of neutrophils ingesting streptococci after incubation with human blood, as described in Fig. 2.

was capable of evoking opsonic antibodies against both type 5 and type 24 streptococci without conjugation to an additional carrier protein. The ELISA titers, measured at 6 wk against pep M24 were 51,200, 6,400, and 12,800, and against pep M5 were 400, 6,400 and 12,800 in each of three rabbits, respectively. Moreover, the undiluted sera of the immunized rabbits promoted the opsonization of both types 5 and 24 streptococci as follows: 4, 32, and 52% for type 5 streptococci, and 98, 72, and 84% for type 24 streptococci.

Interestingly, when either of the hybrid peptides was covalently linked to tetanus toxoid with glutaraldehyde (9) before immunization, the immune responses to type 5 streptococci were essentially the same, whereas those to type 24 streptococci were totally suppressed. For example, opsonic antibody titers against type 5 streptococci 6 wk after immunization with S-M5(1–20)-S-CB7(23–35)C were 1:2, 1:4, and 1:16 in each of three rabbits, whereas those against type 24 streptococci were all <1:2; the undiluted immune sera failed to opsonize type 24 streptococci.

The specificity of the opsonic antibodies raised against the unconjugated peptide S-M5(1–20)-S-CB7(23–35)C was determined by immunoabsorption and inhibition tests (Table I). Absorption of type 5 or type 24 opsonic antibodies by the respective serotypes of whole streptococci was entirely type-specifc. Similarly,

the peptide S-M5(1–20), corresponding to a region of type 5 M protein, inhibited opsonization only of type 5 streptococci, whereas S-CB7(23–35)C, corresponding to a region of type 24 M protein, only inhibited opsonization of type 24 organisms. The hybrid peptide used to immunize the rabbits inhibited opsonization of both serotypes (Table I).

None of the rabbits immunized with either of the synthetic hybrid M protein peptides either as the free peptides or as tetanus toxoid conjugates developed antibodies crossreactive by immunofluorescence tests with sarcolemmal membranes or by ELISA with purified muscle myosin. These results indicate that the M protein regions incorporated into the hybrid peptides contain protective but not heart crossreactive epitopes.

Discussion

Recently, Houghten et al. (20) reported the stimulation of protective immune responses against both the heat-labile and heat-stable toxins of *Escherichia coli* by peroral administration of a hybrid peptide containing a 26 amino acid–residue copy of the labile toxin joined in tandem to an 18-residue copy of stable toxin. However, we believe our studies are the first to show that a synthetic hybrid peptide can raise antibodies that promote the phagocytosis and killing of two different bacterial pathogens. Although previous studies (7–13) have shown the feasibility of using chemically synthesized copies of M protein peptides as immunogens, the protective immunity evoked by these peptides has been restricted to the single M serotypes from which they were copied. It is true that one may produce multivalent vaccines by conjugating more than one synthetic peptide to the same carrier (21) or by polymerizing them to each other (22), but these approaches raise the problems associated with possible immunosuppression induced by repeated injections of the same carrier, or the potential toxicity of the polymerizing agent. Moreover, the orientation of the protective epitopes is more difficult to predict in such polymers. The tandem hybrids, on the other hand, have the advantage that the synthetic peptides can be tailored in a predictable way so as to render protective epitopes optimally immunoaccessible.

Of interest was the finding that the hybrid peptides were immunogenic in the absence of a carrier. Although one might explain the immunogenicity of the hybrid containing the cysteine residue at the COOH-terminus on the basis of its potential to form dimers 68 residues long, this explanation cannot account for the similar degree of immunogenicity of the hybrid peptide lacking the COOH-terminal cysteine residue. The latter peptide lacks both the cysteine and the adjacent methionine residues at the COOH-terminus, but has an additional four amino acid residues, Ala-Leu-Ala-Ala, in the middle of the molecule. This tetrapeptide is a part of the corresponding region of the natural CB7 fragment of type 24 M protein, and it was incorporated for the purpose of preserving the α-helical potential of the hybrid peptide should this structural feature play a role in protective immunogenicity. In another study (H. Gras-Masse, M. Jolivet, F. Audibert, E. Beachey, L. Chedid, and A. Tarter, manuscript in preparation), circular dichroism studies show that the latter hybrid has an α-helical structure of ~20%, whereas that of the former, containing the COOH-terminal cysteine residue, is $<6\%$ α helix. Because of the strong immunogenicity of the synthetic

hybrid containing virtually no α-helicity, it is obvious that conservation of this structural feature is not the key to protective immunogenicity of small peptide copies of streptococcal M proteins. It is possible, therefore, that each of the components of the synthetic hybrids serves as a carrier for the other to present the appropriate epitopes to the immune system in an optimally accessible and flexible conformation to stimulate the production of a repertoire of antibodies that will recognize the corresponding conformational adaptations of the natural M protein molecule (23).

The broader implication of our studies is that one may be able to synthesize tandem hybrids that contain protective epitopes of a variety of viral, bacterial, and other parasitic agents. However, there is a limitation to the number of different specificities that could be incorporated into a single-chain peptide by chemical synthesis. In the future, therefore, one may need to rely on the tools of molecular biology. Synthetic or cloned oligonucleotides encoding the desired epitopes may be fused in the appropriate reading frame in tandem to a carrier gene (24) that is then transferred into *Escherichia coli*. Such transformed bacteria would produce large quantities of long, multivalent hybrid vaccines capable of evoking broadly protective immunities against many serious infectious diseases, many of which are still fatal or produce sometimes devastating complications.

Summary

The protective immunogenicity of a hybrid peptide containing tandem copies of types 5 and 24 epitopes was investigated. Carboxy-terminal peptides of the cyanogen bromide–derived fragment 7 (CB7) of type 24 M protein were chemically synthesized, and then extended to include the first 20 residues of the amino-terminus of type 5 M protein. When emulsified in CFA and injected into rabbits without conjugation to a carrier, each of the synthetic hybrid peptides, designated S-M5(1–20)-S-CB7(23–35)C and S-M5(1–20)-S-CB(19–34), evoked opsonic antibodies against both types 5 and 24 streptococci without raising heart tissue–crossreactive immunity. These results suggest that tandem hybrid peptides may provide a new approach to the development of multivalent vaccines, not only to different serotypes of group A streptococci but perhaps also to a variety of other infectious agents.

We thank Edna Chiang, Mark Garrett, Quinn Cain, and Bob Cassel for expert technical assistance, and Johnnie Smith for expert secretarial assistance.

Received for publication 3 February 1986.

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