PROTECTIVE IMMUNOGENICITY AND T LYMPHOCYTE SPECIFICITY OF A TRIVALENT HYBRID PEPTIDE CONTAINING NH2-TERMINAL SEQUENCES OF TYPES 5, 6, AND 24 M PROTEINS SYNTHESIZED IN TANDEM

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The serotype-specific M protein emanating as α-helical coiled-coil fibrils from the surface of Streptococcus pyogenes is the major virulence determinant of these organisms (1–2). The fibrinogen binding and antiopsonic properties of these fibrils is overcome only by type-specific antibodies directed against epitopes of M protein not masked by fibrinogen (3–7). In addition to the protective epitopes, certain M proteins contain autoimmune epitopes that can evoke immune responses against host tissues, especially cardiac tissue (8–12). Because the nonsuppurative sequelae of streptococcal infections, acute rheumatic heart disease, is manifested by damage to cardiac tissues, efforts have been made to avoid these epitopes in M protein vaccine preparations. Our approach has been to synthesize peptide copies of selected regions of various M proteins in an attempt to devise protective vaccines free of autoimmune epitopes (13–14). In this way, we have shown that the NH2-termini of types 5 and 6 M proteins contain protective, but not tissue crossreactive, epitopes (15, 16). One problem with the approach has been the high degree of type specificity of the highly variable NH2-terminal sequences of these M proteins. In an attempt to overcome this problem, we have begun synthesizing short peptide sequences of different serotypes of M protein in tandem, with the hope that the tandem hybrids would evoke broadly protective immunity against many serotypes of S. pyogenes (17).

Recently, we reported (17) the protective immunogenicity of a bivalent peptide containing the NH2-terminal sequence of type 5 M protein synthesized in tandem with the COOH-terminal sequence of cyanogen bromide fragment seven (CB7)1 of type 24 M protein. In this paper, we report the type-specific protective immunogenicity of a trivalent peptide copying the NH2-terminal sequences of types 5, 6, and 24 M proteins synthesized in tandem. We show that the trivalent hybrid linked via a COOH-terminal cysteine residue to keyhole limpet hemo-

1 Abbreviations used in this paper: CB7, cyanogen bromide fragment 7; KLH, keyhole limpet hemocyanin.
cyanin (KLH) evokes high titers of antibodies against each of the respective natural M protein serotypes. The immune sera are shown to opsonize all three serotypes of streptococci and to lack tissue crossreactive antibodies. Moreover, the trivalent peptide is shown to sensitize rabbit T lymphocytes to respond to each of the natural M proteins as well as the synthetic, trivalent peptide. These findings have bearing on the construction of safe and effective vaccines against a variety of different serotypes of *S. pyogenes*, especially against the strains that trigger acute rheumatic fever and rheumatic heart disease.

**Material and Methods**

**Natural and Synthetic Polypeptides of Streptococcal M Proteins.** Polypeptide fragments of M proteins were isolated and purified from limited peptide digests of types 5, 6, and 24 streptococci as described (18, 19). The polypeptides designated pep M5, pep M6, and pep M24 were judged to be pure by SDS-PAGE and quantitative amino acid analysis (20). Synthetic copies of the NH2-terminal ends of types 5, 6, and 24 M proteins were synthesized separately and in tandem by the solid-phase method of Merrifield (21) as described (15). A COOH-terminal cysteine residue was added to each peptide for the purpose of coupling to carriers. 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) (15). The purified peptides were analyzed for purity and composition by quantitative amino acid analysis and automated Edman degradation to the penultimate amino acid residues (14, 22). The monovalent synthetic peptides are designated SM5(1-10)C, SM6(1-11)C, and SM24(1-12)C. The tandem peptide is designated SM5(1-10)-SM6(1-11)-SM24(1-12)C or SM5-6-24C.

**Rabbit Immunization.** Sets of three New Zealand White rabbits (2 kg) were each injected with a 50-nmol dose of the trivalent peptide SM5-6-24, either in the unconjugated state or conjugated to KLH using succinimidyl 4-(N-maleimido-methyl)-cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL) and emulsified in CFA as described (23). 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 preparation in PBS (0.02 M phosphate with 0.15 M NaCl, pH 7.4). 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 and opsonophagocytic assays as described (24). The serotype specificity of the antibodies raised against the hybrid peptide was determined by ELISA and phagocytosis inhibition studies, using each of the serotypes of whole streptococci as absorbents or the monovalent or trivalent peptides as soluble inhibitors. The antisera (0.1 ml) were mixed with an equal volume of washed whole streptococci, incubated for 30 min at 37°C, and centrifuged at 12,000 g for 15 min to remove the bacteria. The absorbed sera were then used in opsonophagocytosis tests against types 5, 6, and 24 streptococci. The antisera (0.1 ml) were similarly incubated with 100 µM synthetic peptide, and after incubation for 30 min at 37°C, the mixtures were used in the opsonophagocytosis tests.

**Lymphocyte Cultures.** Mononuclear cells were isolated from whole, heparinized (100 U/ml) rabbit blood by Ficoll-Hypaque gradient centrifugation, washed three times, counted, and resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), and Hepes buffer (25 mM). Cells (2 × 10⁶) were cultured in 96-well round-bottomed tissue culture plates (No. 3799; Costar, Cambridge, MA) in a total volume of 200 µl RPMI 1640 supplemented with 5% heat-inactivated FCS (Grand Island Biological Co.). Cells were incubated with pep M proteins or KLH at concentrations of 10 µg/ml or with 10 nmol synthetic peptides that had been dried onto the wells in sterile distilled water before the experiments (25). The cells were cultured for 5 d at 37°C. 6 h before harvesting, 1 µCi of [³H]thymidine (sp act 2 Ci/mmol; Research Products International Corp., Prospect, IL) in 25 µl of culture medium was added to each well. Cultures were harvested with a
Figure 1. Amino acid sequence of trivalent M protein peptide.

Thr-Val-Thr-Arg-Gly-Thr-Ile-Ser-Asp-Pro-Arg-Val-

Thr-Arg-Ser-Gln-Thr-Asp-Thr-Ser-Glu-Lys-Cys

Results

We synthesized a trivalent hybrid peptide copying the NH₂-terminal 10 residues of pep M5, 11 residues of pep M6, and 12 residues of pep M24. The composition of the hybrid peptide was confirmed by quantitative amino acid analysis. Sequence analysis by Edman degradation to the penultimate residues confirmed the sequence as shown in Fig. 1.

The hybrid peptide is designated as SM5(1-10)-SM6(1-11)-SM24(1-12)C or SM5-6-24C. The COOH-terminal cysteine residue was added for the purpose of coupling to a carrier. The composition of the additional monovalent peptides SM5(1-10)C, SM6(1-11)C, SM24(1-12)C were similarly confirmed by amino acid analysis. These synthetic peptides were used in the following studies.

Immunogenicity of Trivalent Peptide SM5-6-24C. Immunization of rabbits with SM5-6-24C covalently linked through its COOH-terminal cysteine residue to KLH evoked high titers of antibodies against the respective M proteins as measured by ELISA using pep M5, pep M6, or pep M24 as antigens (Table I). Four of the seven immunized rabbits produced antibodies against all three M proteins. ELISA inhibition experiments demonstrated that the hybrid peptide inhibited all of the antibody reactivities with pep M5, pep M6, or pep M24 (Fig. 2). The subpeptides SM5(1-10)C, SM6(1-11)C, and SM24(1-12)C completely inhibited the reactivity of the anti-SM5-6-24C serum only against the homologous serotype of pep M protein, although the highest concentration of SM5(1-10)C tested partially inhibited reactivity of the antiserum with pep M6 (Fig. 2).

The antisera raised against the trivalent hybrid peptide opsonized and killed types 5, 6, and 24 streptococci (Tables II and III). Four of the seven antisera opsonized all three serotypes, although the opsonization of type 5 streptococci by rabbit serum 8593 was weak, as demonstrated both by phagocytosis (Table II) and indirect bactericidal tests (Table III). All opsonized type 6 and all except one opsonized type 24 streptococci. The type specificity of the opsonic antibodies was demonstrated by opsonization inhibition tests (Table IV). Absorption of the antisera with the respective serotypes of whole streptococci removed only the opsonic antibodies against the homologous serotype. Similarly, each of the
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*The preimmune sera from each of the rabbits gave titers of <200 against each of the antigens tested in ELISA. The endpoint in each case is recorded as the reciprocal of the highest dilution of antiserum producing an absorbance reading ≥0.1 at 450 nm. Because dilutions of each of the antisera resulted in absorbancy curves that fell sharply (data not shown), indicating antibodies of high affinity, the endpoint value used was considered to be valid.*

**TABLE I**

ELISA of Sera of Rabbits Immunized with SM5-6-24C Conjugated to KLH and Emulsified in CFA

| Immune sera, rabbit number | ELISA titers against: | pep M5 | pep M6 | pep M24 | SM5-6-24C |
|---------------------------|-----------------------|-------|-------|--------|-----------|
| Preimmune*                | <200                  | <200  | <200  | <200   |
| 8507                      | 25,600                | 51,200| 51,200| >102,400|
| 8508                      | 3,200                 | 400   | 25,600| 51,200  |
| 8509                      | 200                   | 6,400 | 12,800| 52,200  |
| 8591                      | 51,200                | 102,400| 51,200| 102,400 |
| 8592                      | 200                   | 6,400 | 200   | 25,600  |
| 8593                      | 6,400                 | 51,200| 25,600| 51,200  |
| 8594                      | <200                  | 102,400| 400   | >102,400|

**Figure 2.** Inhibition of the reactions of anti-SM5-6-24C with immobilized pep M5 (left), pep M6 (middle), and pep M24 (right) by the component peptides SM5(1-10)C, SM6(1-11)C, and SM24(1-12)C and the trivalent hybrid peptide SM5-6-24C. Note that the hybrid peptide inhibited the reactions against each of the pep M proteins whereas the component peptides inhibited only the homologous reactions. The exception was the partial inhibition by SM5(1-10)C of the reaction of anti-SM5-6-24C with pep M6 (middle).

Synthetic subpeptides inhibited only the opsonic antibodies against the organisms bearing the parent serotype of M protein. The hybrid peptide inhibited opsonization of all three serotypes (Table IV).

**Absence of Autoimmune Epitopes in SM5-6-24C.** Because types 5 and 6 pep M proteins were previously shown to contain host tissue crossreactive epitopes (9-11), we examined the synthetic hybrid peptide for such epitopes. The trivalent hybrid peptide failed to inhibit the cardiac sarcolemmal and myosin crossreactive antibodies in any of the antisera previously raised (9-11) against types 5 and 6 M proteins. Moreover, none of the seven rabbits developed antibodies that crossreacted with human myocardium, as measured by immunofluorescence tests of frozen sections of human cardiac tissue, or myosin, as measured by ELISA or
**Table II**

**Opsonic Antibodies in Sera of Rabbits Immunized with SM5-6-24C Conjugated to KLH and Emulsified in CFA**

| Immune sera,* rabbit number | Opsonization: neutrophils with associated streptococci |
|-----------------------------|--------------------------------------------------------|
|                             | Type 5 | Type 6 | Type 24 |
| Preimmune pool†              | %      | %      | %       |
| 8507                        | 4      | 2      | 0       |
| 8508                        | 82     | 80     | 96      |
| 8509                        | 44     | 62     | 92      |
| 8591                        | 0      | 88     | 84      |
| 8592                        | 66     | 86     | 78      |
| 8593                        | 2      | 84     | 2       |
| 8594                        | 38     | 82     | 72      |

*Immune sera were obtained 1 wk after an initial injection of 50 nmol of SM5-6-24C emulsified in CFA followed by booster injections of the same dose in PBS at 4 and 8 wk. The type specificity of the M5-M6-M24 hybrid peptide was confirmed by the failure of the immune sera to promote phagocytosis and killing of heterologous types 1, 12, and 19 streptococci.

† The preimmune pool contained equal amounts of sera obtained from each of the rabbits before immunization.

**Table III**

**Indirect Bactericidal Tests of Sera of Rabbits Immunized with SM5-6-24C Hybrid Peptide**

| Immune sera, rabbit number | Number of colonies of streptococci after 3 h growth in test mixtures |
|----------------------------|----------------------------------------------------------|
|                            | Type 5 (Inoculum 9) | Type 6 (Inoculum 13) | Type 24 (Inoculum 13) |
| Preimmune pool              | 2,020              | 3,554                | 2,200                 |
| 8507                        | 20                 | 115                  | 760                   |
| 8508                        | 155                | 250                  | 560                   |
| 8509                        | 2,140              | 190                  | 400                   |
| 8591                        | 490                | 485                  | 15                    |
| 8592                        | 2,090              | 185                  | 2,360                 |
| 8593                        | 1,010              | 90                   | 195                   |
| 8594                        | 2,100              | 95                   | 240                   |

Western immunoblots (data not shown). These results indicate that the NH₂-terminal regions of each of the M proteins incorporated into the tandem hybrid peptide contained protective but not autoimmune epitopes.

Cell-mediated Immune Responses in Rabbits Immunized with SM5-6-24C. Previous studies (13) have shown that synthetic peptide copies of M proteins are capable of priming lymphocytes in vivo to respond to subsequent in vitro challenge with the natural pep M proteins. In the present study, it was of interest to determine if the synthetic hybrid peptide had the ability to mimic T cell epitopes of the corresponding natural M proteins. Preimmune and immune (10 wk) lymphocytes were cultured in the presence of 10 μg/ml of KLH or pep M
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### TABLE IV

**Specificity of Opsonic Antibodies Raised in Rabbits Against SM5-6-24C Trivalent Hybrid Peptide**

| Streptococci opsonized with: | Opsonization: neutrophils with associated streptococci |
|-----------------------------|---------------------------------|
|                             | Type 5 | Type 6 | Type 24 |
| Preimmune serum             | %      | %      | %       |
| Anti-SM5-6-24C              | 76     | 78     | 74      |

Absorbed with:

- Type 5 streptococci: 0, 78, 72
- Type 6 streptococci: 74, 6, 70
- Type 24 streptococci: 78, 76, 2

Inhibited with:

- SM5(1-10) C: 0, 76, 72
- SM6(1-11) C: 78, 6, 76
- SM24(1-12) C: 78, 76, 2
- SM5-6-24 C: 0, 6, 4

### TABLE V

**In Vitro Blastogenic Responses of Lymphocytes from Rabbits Immunized with Synthetic Hybrid Vaccine SM5-6-24C-KLH**

| Lymphocytes stimulated with: | Preimmune | 10 wk | Preimmune | 10 wk | Preimmune | 10 wk |
|-----------------------------|-----------|-------|-----------|-------|-----------|-------|
|                             | 8662      | 8663  | 8665      |       |           |       |
| Control                     | 614 ± 99  | 859 ± 62 | 165 ± 74  | 2,096 ± 29 | 1,058 ± 243 | 2,132 ± 210 |
| KLH                         | 445 ± 43  | 2,675 ± 6,437 | 380 ± 81  | 80,452 ± 6,040 | 1,335 ± 224 | 48,454 ± 5,660 |
| pep M5                      | 5,069 ± 147 | 110,851 ± 18,534 | 3,385 ± 136 | 212,640 ± 7,450 | 7,083 ± 353 | 72,272 ± 467 |
| pep M6                      | 6,706 ± 745 | 26,488 ± 1,319 | 2,714 ± 249 | 37,697 ± 2,748 | 1,556 ± 197 | 6,225 ± 397 |
| pep M24                     | 2,611 ± 242 | 7,841 ± 850 | 3,058 ± 135 | 19,780 ± 3,306 | 5,262 ± 1,453 | 14,642 ± 3,222 |
| SM5-6-24C                   | 407 ± 150 | 857 ± 86 | 328 ± 71 | 28,595 ± 6,814 | 1,134 ± 537 | 3,175 ± 850 |
| SM5(1-10) C                 | 563 ± 70  | 643 ± 197 | 424 ± 24  | 615 ± 81 | 410 ± 61 | 1,441 ± 321 |
| SM6(1-11) C                 | 511 ± 88  | 550 ± 226 | 217 ± 42  | 1,787 ± 616 | 343 ± 58 | 824 ± 70 |
| SM24(1-12) C                | 603 ± 66  | 473 ± 119 | 542 ± 66  | 3,885 ± 1,214 | 522 ± 120 | 1,017 ± 396 |

*Immune lymphocytes were collected and cultured in vitro as described in Materials and Methods. Unstimulated control cultures were incubated without antigen.

Proteins in solution, or 10 nmol of unconjugated synthetic peptides that had been dried onto plastic plates (25). Immune lymphocytes from all three rabbits showed significant blastogenesis in response to in vitro challenge with all three pep M proteins, as measured by [H]thymidine incorporation (Table V). The lymphocytes of one of three rabbits (8663) responded to the hybrid synthetic peptide, whereas none responded to the individual component peptides (Table IV). Taken together, these results suggest that the hybrid peptide can prime lymphocytes in vivo to respond to epitopes of the natural M proteins in addition to the hybrid synthetic immunogen. The inability of the individual synthetic peptides to stimulate lymphocyte blastogenesis may relate to their molecular size, conformation, or mode of presentation. To test the latter hypothesis, each
peptide was covalently coupled to BSA and presented to the immune lymphocytes in solution. Under these conditions, lymphocytes from all three rabbits responded to the hybrid peptide, but none responded to the individual component peptides (data not shown), suggesting that the subpeptides were incapable of taking on the conformation required to stimulate T lymphocytes sensitized with the trihybrid peptide.

Discussion

It has previously been reported (17, 26) that peptide copies of virulent determinants of bacteria synthesized in tandem are capable of evoking immune responses against the natural protein. Houghten et al. (26) reported that 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 stimulated protective immune responses against both the heat-labile and heat-stable toxins of *Escherichia coli*. Recently, Beachey et al. (17) demonstrated that a 20-residue NH2-terminal peptide of type 5 M protein synthesized in tandem with a 13-residue peptide of type 24 M protein evoked opsonic antibodies against the respective serotypes of streptococci. However, to our knowledge, the present study is the first to show that a tandem peptide containing more than two distinct virulence determinants is capable of stimulating immune responses to each of the respective natural proteins on the surface of virulent microorganisms. The question as to whether a short peptide flanked on each end by different peptides containing distinctly different immunospecificities would be seen in a natural conformation by the immune system is of some practical concern as well as of theoretical importance. Our studies clearly demonstrate that an 11-residue peptide of type 6 M protein, flanked on its NH2-terminal end by a 10-residue peptide of type 5 M protein and on its COOH-terminal end by a 12-residue peptide of type 24 M protein, is readily recognized by the immune system in the rabbit. In fact, the antibody responses as measured by opsonization of the respective serotypes were universal to the type 6 peptide; all of the rabbits immunized with the trivalent hybrid peptide developed opsonic antibodies against type 6 streptococci, whereas only four of seven developed antibodies against type 5 streptococci and six of seven against type 24 organisms. Thus, the potentially hidden position of the middle peptide in this case was not a detriment to recognition.

It should be noted that the type 5 peptide contains a proline residue joined to the NH2-terminal arginine residue of the type 6 peptide, and that, in turn, the type 6 peptide contains a proline residue joined to the NH2-terminal valine residue of type 24 M protein. Because proline residues are known to be strong helix breakers, the central part of the molecule theoretically should be considerably more mobile, permitting the exposure of the M6 epitopes in the conformations required for recognition by the immune system as epitopes of the natural protein from which it was derived.

Our previous studies of a bivalent hybrid peptide of types 5 and 24 M proteins (17) demonstrated that the peptides were equally immunogenic with respect to the antibodies evoked against the natural protein, whether or not attempts were made to preserve the secondary structural potentials of the original proteins.
The present studies provide additional evidence that strict conservation of the secondary and tertiary structural features of the original proteins is not the key to protective immunogenicity of small peptide copies of streptococcal M proteins.

It should be noted that the peptides copied in the trivalent hybrid represent the NH₂-termini of types 5, 6, and 24 M proteins. Although the NH₂-terminal peptides of types 5 and 6 M proteins had previously been demonstrated to stimulate type-specific protective immune responses, these are the first studies to show similar protective properties of the NH₂-terminus of type 24 M protein. The region of type 24 M protein contained in the bivalent hybrid peptide previously reported from our laboratories (17) was a copy of the COOH-terminal 13 residues of CB7, an internal peptide fragment that is repeated five times in the M protein molecule (14). The presence of this repeating peptide may have accounted for the greater immunogenicity of the bivalent hybrid in the unconjugated form; the trivalent peptide reported in the present study was less immunogenic without conjugation to a carrier protein.

The generic implications of these studies is that one may selectively engineer a multivalent peptide vaccine containing protective epitopes of a variety of infectious agents. Although multivalent vaccine preparations have been made by polymerization of several synthetic peptides to each other with glutaraldehyde (27), this approach raises problems of potential toxicities associated with the polymerizing agent. Furthermore, the orientation and accessibility of protective epitopes is less predictable in such polymers. The tandem peptide approach has the advantage that one may tailor vaccine preparations that expose protective epitopes in a predictable and optimally immunoaccessible fashion.

Our studies clearly demonstrate that the hybrid M protein peptide is capable of priming T lymphocytes to respond to each of the respective natural M proteins as well as to the hybrid peptide itself in vitro. The failure of the individual component peptides to stimulate the primed lymphocytes either unconjugated or conjugated to BSA suggests that in the isolated state, they were unable to take on the conformation needed for recognition by the sensitized T cells. It appears, therefore, that the hybrid peptide confers structural features upon each of the component peptides more nearly resembling their presentations in the respective natural M proteins.

**Summary**

The protective immunogenicity of a hybrid peptide containing tandem copies of types 5, 6, and 24 M protein epitopes was investigated. An NH₂-terminal peptide of type 24 M protein was chemically synthesized and then extended to include NH₂-terminal peptides of types 6 and 5 M proteins yielding a 34-residue hybrid peptide containing a cysteine residue at its COOH-terminus. When conjugated via the cysteine residue to keyhole limpet hemocyanin (KLH), emulsified in CFA, and injected into rabbits, the synthetic hybrid evoked opsonic antibodies against types 5, 6, and 24 streptococci without stimulating tissue crossreactive immunity. The trivalent hybrid also was capable of priming T lymphocytes in vivo that responded to each of the native serotypes of M protein as well as to the synthetic hybrid peptide in vitro. The primed T cells failed to respond to the individual component peptides contained in the hybrid peptide,
suggesting that the hybrid peptide confers conformations resembling the presentations of each of the subpeptides in the respective serotypes of M protein. The brisk immune responses to the type 6 peptide contained in the middle of the tandem hybrid indicates that with judicious placement between proline residues, potentially hidden peptides are readily accessible to the immune system. These results suggest that synthetic tandem peptides can be tailored in a fashion in which each of the component sets of protective epitopes can be made optimally immunoaccessible and immunogenic.

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