Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2203–2207, April 1984
Medical Sciences

Epitope-specific protective immunogenicity of chemically synthesized 13-, 18-, and 23-residue peptide fragments of streptococcal M protein

(synthetic peptides/vaccine conjugates/protective immunity/rheumatic fever)

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Communicated by Robert Austrian, December 19, 1983

ABSTRACT **The ability of chemically synthesized subpeptides of type 24 streptococcal M protein to evoke protective antibodies in rabbits was investigated. We synthesized copies of the COOH-terminal 13, 18, and 23 amino acid residues of cyanogen bromide fragment 7 (CB7) of pepsin-extracted type 24 M protein, except that methionine was substituted for homoserine as the COOH-terminal residue. An additional residue of cysteine was added at the COOH terminus of the 13-residue peptide. Each of the peptides, designated S-CB7-(23–35)-Cys, S-CB7-(18–35), and S-CB7-(13–35), when conjugated to lysylated tetanus toxoid with glutaraldehyde, was capable of stimulating formation of protective anti-type 24 M protein antibodies in rabbits. The smallest peptide, S-CB7-(23–35)-Cys, elicited immune responses equally as strong, if not stronger, than those to the longer peptides. A single Lys/Gly substitution in this 13-residue peptide resulted in its failure to stimulate protective antibodies. None of the antisera reacted with heterologous serotypes of M protein and none reacted with frozen sections of human heart tissue. These results indicate that a chemically synthesized peptide fragment corresponding to as few as 13 amino acid residues of streptococcal M protein is capable of evoking protective anti-streptococcal antibodies without evoking antibodies crossreactive with cardiac tissue.**

For >50 years, attempts have been made to develop safe and effective vaccines against strains of group A streptococci that give rise to rheumatic fever and rheumatic heart disease (1, 2). Most of these efforts have been obstructed by severe toxic reactions to almost any streptococcal product introduced into the human host. Some of these products have been shown to give rise to antibodies that crossreact with host tissues, especially those of the heart (3, 4). Although it has long been established that the M protein on the surface of group A streptococci contains the protective antigen(s) of these organisms, the fear has been that the isolated M protein may include potentially harmful tissue crossreactive antigens that give rise to, rather than prevent, rheumatic fever. This concern is based on the finding that certain rheumatogenic streptococci produce M proteins that are closely associated with an antigen crossreactive with heart (5). Indeed, recently it has been established that two different M protein molecules contain, within their covalent structures, an epitope that evokes a protective anti-streptococcal antibody that also crossreacts with a sarcolemmal protein of human heart tissue (6).

In an attempt to identify protective as opposed to tissue crossreactive epitopes, we have undertaken studies of the protective immunogenicity of various native and chemically synthesized peptides of streptococcal M protein. We (6–11) and others (12, 13) have found that the large polypeptide fragments of M protein obtained by limited pepsin digestion of various types of whole streptococci (pep M5, pep M6, pep M19, and pep M24) are highly immunogenic when administered with various adjuvants, including a synthetic moiety of peptidoglycan called muramyl dipeptide (MDP) (14). Further cleavage of pep M24 with cyanogen bromide has yielded seven peptide fragments, CB1–CB7 (15), each of which retained type-specific protective immunogenicity (16–18). Recently, the chemically synthesized peptides S-CB3 and S-CB7, each of which is identical to its natural counterpart of the pep M24 molecule, except that the COOH terminus of each of the synthetic peptides is methionine instead of homoserine, were shown also to be immunogenic, eliciting high titers of opsonic antibody without stimulating the development of antibodies that crossreacted with heart tissue (14, 18, 19). More recently, we found that a synthetic copy of the NH_2-terminal region of pep M5 was similarly immunogenic (20).

In the present study, we have investigated the immunogenicity of yet smaller synthetic peptide fragments of type 24 M protein. These included chemically synthesized copies of the COOH-terminal 13, 18, and 23 residues of S-CB7. We present data to show that, when covalently linked to tetanus toxoid and injected as an emulsion in complete Freund's adjuvant (CFA), each of these synthetic subpeptides evoked type-specific anti-streptococcal antibodies without stimulating crossreactive immunity to cardiac tissue.

MATERIALS AND METHODS

Preparation of Streptococcal M Protein Peptides. Polypeptide fragments of M protein were isolated and purified from limited peptic digests of whole type 5, type 6, and type 24 *Streptococcus pyogenes* as described (7, 8, 15). The purified polypeptides, designated pep M5, pep M6, and pep M24, were judged to be pure by $NaDodSO_4$ gel electrophoresis and amino acid analyses (15). A polypeptide (S-CB7) identical to native CB7 of pep M24, except that, at the COOH terminus, methionine was substituted for homoserine, was synthesized by a solid-phase method (21) on a benzhydryla-

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Abbreviations: CFA, complete Freund's adjuvant; CB1–CB7, cyanogen bromide peptides of pep M24; MDP, muramyl dipeptide for *N*-acetylmuramyl-L-alanyl-D-isoglutamine; pep M5, pep M6, pep M19, and pep M24, peptic extracts of types 5, 6, 19, and 24 streptococcal M proteins, respectively; S-CB3, chemically synthesized cyanogen bromide peptide 3; S-CB7-(1–35), -(13–35), -(18–35), -(23–35)-Cys, and -(23–32)-Lys-Ala-Met-Cys, chemically synthesized peptide fragments and substituted derivatives of cyanogen bromide peptide 7; S-M5-(1–20) and -(20–40), chemically synthesized peptides of type 5 M protein.

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mine resin (Beckman). Cleavage of the synthetic peptides from this resin with hydrofluoric acid resulted in a carboxamide group at the COOH terminus. After gel filtration (Bio-Gel P-6) and preparative reversed phase HPLC (Whatman Magnum 9 ODS), the peptide was judged to be homogeneous by analytical reversed phase HPLC and amino acid analysis (14). To obtain subpeptides of S-CB7, samples of the peptidyl resin were removed at various stages during the synthesis. Peptides S-CB7-(1–35), -(13–35), and -(18–35) prepared in this way were chosen for our studies. In addition, two pep M24 peptides, S-CB7-(23–35)-Cys and S-CB7-(23–32)-Lys-Ala-Met-Cys, were synthesized according to our specifications by Peninsula Laboratories (San Carlos, CA). The crude, deblocked synthetic peptides were purified by reversed phase HPLC on Ultrasphere ODS2 (Whatman). The synthetic copies of pep M5 protein, S-M5-(1–20) and S-M5-(20–40), were prepared in the same way for another study (20) and were used as control peptides in the present investigation.

Analytical Methods. Quantitative amino acid analyses were performed as described (22). Automated Edman degradations were performed with a Beckman Sequenator (model 890C) according to the principles first described by Edman and Begg (23). The slow peptide-DMAA (071472) program of Beckman Instruments was used (24). The phenylthiohydantoins were identified by HPLC (25). Arginine derivatives were identified as their parent amino acids after hydrolysis with 55% HI (26). Repetitive yields of 97% were obtained during automated Edman degradation.

Conjugation of Synthetic Peptides with Tetanus Toxoid. The chemically synthesized peptides were conjugated to lysylated tetanus toxoid with glutaraldehyde as described (20, 27). The conjugated peptides were stored frozen in 0.02 M phosphate/0.15 M NaCl, pH 7.4 (P_i/NaCl) at −70°C. In some experiments, we employed S-CB7 polymerized with glutaraldehyde as described (14, 27).

Immunization of Rabbits. To determine the immunogenicity of the synthetic subpeptides of S-CB7, New Zealand White rabbits (2 kg) were injected subcutaneously with a 25-nmol dose of synthetic peptide conjugated to tetanus toxoid and emulsified in CFA as described (17, 20). Rabbits were bled before and at 2-week intervals after the initial injection. At 4 and 10 weeks, each rabbit was injected subcutaneously with a 25-nmol booster dose of the respective conjugated peptide in P_i/NaCl, and sera were collected 2 weeks after each booster dose. Antisera against pep M5, pep M6, and pep M24 were prepared by immunizing rabbits with 3-nmol doses of the respective pep M proteins (8). All sera were stored at 4°C.

Assays for Anti-M Protein Antibodies. The rabbit sera were assayed for anti-M protein antibodies by ELISA, immunoprecipitation tests in agar gels, opsonophagocytic antibody assays, and passive protection tests in mice as described (8, 15–18). The serotype specificity of the antibodies to the synthetic peptide fragments of pep M24 was determined by assaying the antisera against pep M5, pep M6, and pep M19 by ELISA and by opsonophagocytic assays of M types 5, 6, and 19 streptococci.

Assays for M Protein Epitopes. The presence of M protein epitopes on the synthetic subpeptides was detected by the inhibition of ELISA and by the inhibition of the type-specific opsonization of homologous M serotypes of *S. pyogenes* (18, 19). ELISA inhibition tests were performed by incubating a constant dilution of antiserum with serial dilutions of soluble synthetic peptide in P_i/NaCl (16, 18). The mixtures were incubated at 37°C for 30 min and then added to cuvettes coated with pep M5, pep M6, pep M19, or pep M24 (16, 17). In some experiments, the subpeptide S-CB7-(23–35)-Cys was covalently linked to bovine serum albumin-coated cuvettes via cysteine through a bifunctional crosslinker (3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (Sigma).

Immunofluorescent Tests. Each of the rabbit antisera was examined for immunologic crossreactivity with sarcolemmal membranes as described in detail (6).

RESULTS

Automated Edman degradation confirmed the sequences of S-CB7-(13–35), S-CB7-(18–35), and S-CB7-(23–35)-Cys to be identical to the corresponding regions of S-CB7-(1–35) except that S-CB7-(23–35)-Cys contained an additional residue of cysteine at the COOH terminus (Table 1). The sequence of a fourth peptide identical to S-CB7-(23–35)-Cys, except that the glycine residue at position 33 was substituted with lysine, also was confirmed by automated Edman degradation. The latter peptide is referred to as S-CB7-(23–32)-Lys-Ala-Met-Cys and was employed to study the epitope specificity of the immune response to the unsubstituted peptide, S-CB7-(23–35)-Cys.

Immunogenicity of Synthetic Subpeptides. None of the synthetic subpeptides was found to be immunogenic in rabbits when injected subcutaneously in P_i/NaCl or emulsified in CFA. However, when covalently linked to lysylated tetanus toxoid and emulsified in CFA, each of the peptides, except S-CB7-(23–32)-Lys-Ala-Met-Cys, elicited high titers of antibodies against the uncleaved pep M24 molecule as measured by ELISA in at least two of three immunized rabbits; one rabbit responded less well to linked S-CB7-(13–35) (Fig. 1). The tetradecapeptide S-CB7-(23–35)-Cys elicited responses equally as strong, if not stronger, than those to the longer peptides in each of three rabbits tested. In contrast to the strong immune responses to S-CB7-(23–35)-Cys, the same peptide with a single Lys/Gly substitution at position 33 evoked only weak or no responses as measured either by ELISA against pep M24 or by opsonophagocytic assays using type 24 streptococci (Fig. 1).

The antisera evoked by each of the peptides formed immunoprecipitates with the uncleaved pep M24 molecule in agar gel diffusion tests (data not shown). In addition, the antisera elicited by even the smallest peptide produced precipitin lines of identity with glutaraldehyde-polymerized S-CB7-(1–35) (14) and pep M24 (Fig. 2). None of the immune sera re-

Table 1. Covalent structures of synthetic peptides of type 24 streptococcal M protein

Synthetic peptide	Amino acid sequence						
	5	10	15	20	25	30	35
S-CB7-(1–35)*	N F S T A D S A K I K T L E A E K A A L A A R K A D L E K A L E G A *M*						
S-CB7-(13–35)	L E A E K A A L A A R K A D L E K A L E G A *M*						
S-CB7-(18–35)	A A L A A R K A D L E K A L E G A *M*						
S-CB7-(23–35)-Cys	R K A D L E K A L E G A *M C*						
S-CB7-(23–32)-Lys-Ala-Met-Cys	R K A D L E K A L E *K A M C*						

Each amino acid sequence was confirmed by sequential Edman degradations. The single-letter code for amino acid residues (28) is used to conserve space and for ease of comparison. Italicized residues indicate substitutions or additions with respect to native CB7.

*The sequence of S-CB7-(1–35) was confirmed in a previous study (19).

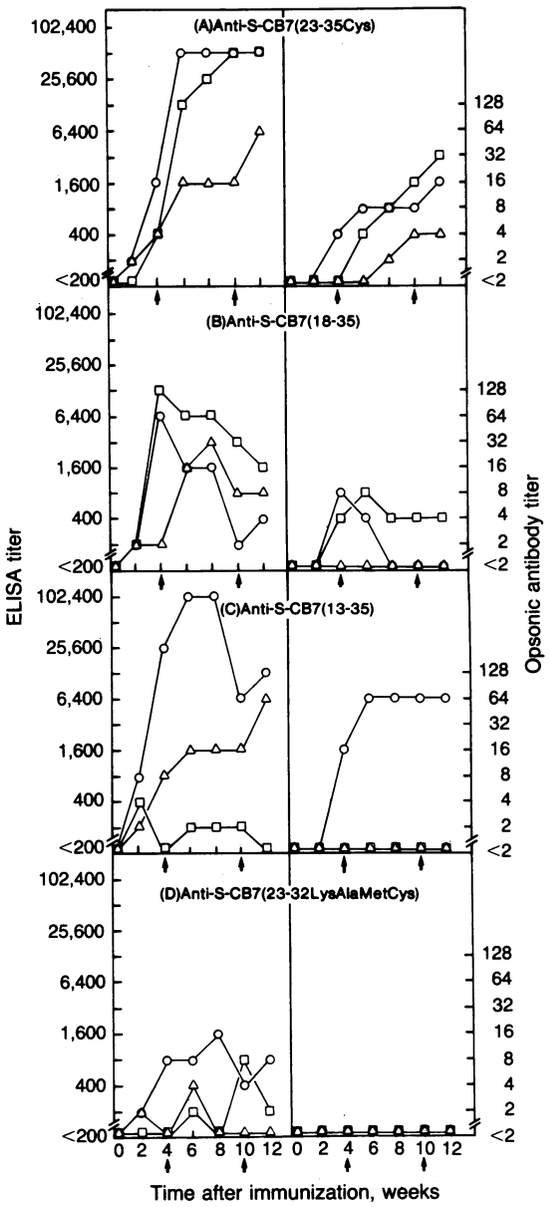

FIG. 1. Immune responses of rabbits against tetanus toxoid-conjugated synthetic peptide fragments of type 24 streptococcal M protein as measured by ELISA against pep M24 (left) and by opsonic antibody assays (right). Sets of three rabbits (△, □, and ○) were immunized with 25 nmol of tetanus toxoid-conjugated S-CB7-(23–35)-Cys (*A*), S-CB7-(18–35) (*B*), S-CB7-(13–35) (*C*), or S-CB7-(23–32)-Lys-Ala-Met-Cys (*D*) emulsified in CFA. Booster injections of 25 nmol of the respective peptide conjugates in P_i/NaCl were given as indicated by the arrows. In control experiments, none of the immune sera reacted with pep M5, pep M6, or pep M19 in ELISA or opsonized heterologous serotypes 5, 6, or 19 streptococci, indicating the serotype specificity of the immune response.

acted in ELISA against heterologous pep M5, pep M6, or pep M19 M proteins or by opsonophagocytic assays with type 5, 6, or 19 streptococci (data not shown). Moreover, neither the preimmune or immune sera reacted with frozen sections of cardiac tissue or with sarcolemmal membranes isolated therefrom.

Passive Mouse Protection with Anti-S-CB7-(23–35)-Cys. Two of the three rabbit antisera against S-CB7-(23–35)-Cys, obtained after two booster immunizations, promoted killing of all of the type 24 streptococci inoculated into fresh human blood (Table 2). A pool of these two rabbit sera was capable of passively protecting mice challenged with type 24 streptococci (Table 3). These results indicate that a peptide containing as few as 13 amino acid residues of M protein is capable of evoking protective anti-streptococcal antibodies.

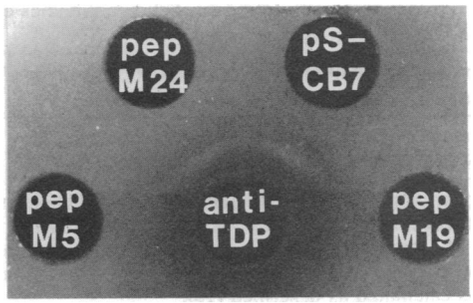

FIG. 2. Double-immunodiffusion in agar gel of anti-S-CB7-(23–35)-Cys (anti-TDP) with glutaraldehyde-polymerized S-CB7-(1–35) (pS-CB7) and the uncleaved natural proteins (pep M24, pep M5, and pep M19). Note arc of identity between pS-CB7 and pep M24 and absence of precipitin lines with the heterologous serotypes.

Epitope Specificity of S-CB7-(23–35)-Cys. To determine the accessibility of the epitope(s) of S-CB7-(23–35)-Cys, we examined the capacity of the antibodies against this peptide to interact with the immunizing peptide, with the same peptide containing a lysine substitution at position 33 and with the uncleaved pep M24 as measured by ELISA. The antibodies recognized the immunizing agents the best but reacted in high dilution with the native pep M24 as well as with the lysine 33-substituted 23–35 subpeptide (Fig. 3*A*). In contrast, antibodies raised against the lysine 33–substituted peptide S-CB7-(23–32)-Lys-Ala-Met-Cys reacted the best with the substituted peptide but only poorly with pep M24 (Fig. 3*B*).

To assess further epitope specificity, homologous and heterologous peptides of various lengths were examined for their capacity to inhibit the interaction of anti-S-CB7-(23–35)-Cys with pep M24 or with S-CB7-(23–35)-Cys linked to bovine serum albumin in ELISA. As can be seen in Table 4, when pep M24 was used as the solid-phase antigen, each of the homologous type 24 peptide fragments, but none of the heterologous type 5 synthetic M protein fragments, was capable of inhibiting antibody binding to a high degree. Each of the peptides tested, except S-M5-(1–20) and S-M5-(20–40), inhibited binding by 100% at a concentration of 10 μM. In contrast, there was variability in the capacity of the peptides to inhibit the binding of the antibody to S-CB7-(23–35)-Cys-bovine serum albumin (Table 4). Only the homologous peptide S-CB7-(23–35)-Cys was capable of completely inhibiting the interaction at a concentration of 40 μM. Each of the oth-

Table 2. Indirect bactericidal tests of anti-tetradecapeptide S-CB7-(23–35)-Cys against type 24 *S. pyogenes*

Rabbit serum	Number of colonies of type 24 streptococci after 3-hr growth in test mixtures	
	Inoculum 42	Inoculum 8
Preimmune (control)*	>2000	892
8293	0	0
8294	1248	148
8295	0	0

The test mixtures each consisted of 0.4 ml of fresh, heparinized (10 units ml^{-1}) human blood, 0.05 ml of streptococcal inoculum suspended in P_i/NaCl, and 0.05 ml of preimmune or immune serum as described (10). After incubation at 37°C by rotation end-over-end at 8 rpm for 3 hr, blood agar pour plates were prepared from each mixture to determine growth of colony-forming units (10).

*The preimmune serum consisted of a pool of serum collected from each of the three rabbits (8293, 8294, and 8295) before immunization with the tetradecapeptide.

Table 3. Protection by anti-tetradecapeptide antibodies of mice challenged with M type 24 streptococci

Serum used to passively immunize mice	LD_{50} in mice challenged with type 24 streptococci*
Preimmune	500 (3/15)
Anti-S-CB7-(23–35)-Cys	400,000 (12/15)

*The number of survivors per number of mice challenged with type 24 streptococci is given in parentheses. In each group of 15 mice, five sets of 3 mice were challenged with increasing doses of type 24 streptococci ranging from 50 to 400,000 colony-forming units. The LD_{50} was calculated as described (10).

er peptide fragments of S-CB7 was considerably less inhibitory at the same dose, ranging between 40% and 47% inhibition. Interestingly, the uncleaved pep M24 molecule was the least inhibitory, with 10 μM inhibiting the reaction only 27% (Table 4). These results suggest that at least one epitope of S-CB7-(23–35)-Cys is less accessible in the larger synthetic peptides and is least accessible in the uncleaved parent polypeptide.

Epitope specificity of the antibody response was further evaluated by comparing the ability of S-CB7-(23–35)-Cys and its lysine 33-substituted analogue to inhibit the interactions of the immune sera with the uncleaved pep M24 molecule. As can be seen in Fig. 4*A*, S-CB7-(23–35)-Cys was a much better inhibitor than S-CB7-(23–32)-Lys-Ala-Met-Cys of the interaction of anti-S-CB7-(23–35)-Cys with pep M24. In contrast, the two peptides showed similar patterns of the inhibition of the interaction of anti-S-CB7-(23–32)-Lys-Ala-Met-Cys with the uncleaved M protein molecule, although, interestingly, the unsubstituted 23–35 peptide was consistently more inhibitory than the immunizing lysine 33-substituted peptide at all concentrations tested (Fig. 4*B*).

DISCUSSION

Our data demonstrate that a chemically synthesized peptide fragment containing as few as 13 amino acid residues of type 24 streptococcal M protein has the capacity, when covalently linked to a carrier, to evoke type-specific protective antibodies without stimulating simultaneously antibodies that crossreact with human heart tissue. These findings are of considerable interest with regard to the development of protective vaccines against strains of group A streptococci that give rise to acute rheumatic fever. Not only have the natural M proteins of group A streptococci been difficult to purify to homogeneity and to free of toxic properties but some of the

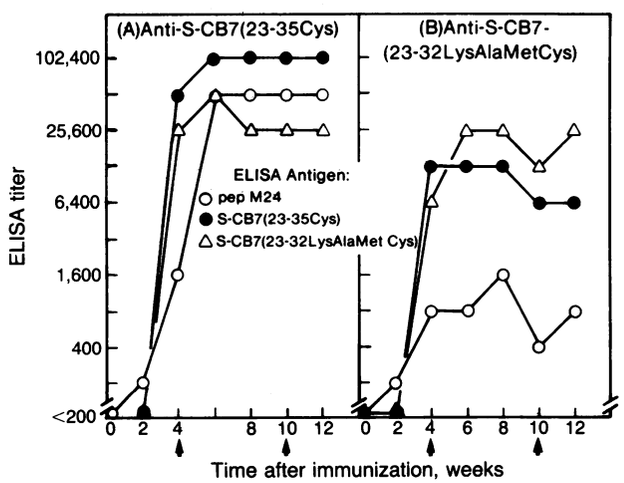

FIG. 3. ELISA of the interaction of anti-S-CB7-(23–35)-Cys (*A*) and anti-S-CB7-(23–32)-Lys-Ala-Met-Cys (*B*) with pep M24 (○), S-CB7-(23–35)-Cys (●), or S-CB7-(23–32)-Lys-Ala-Met-Cys (△).

Table 4. Capacity of various synthetic M protein peptides to inhibit the interaction of anti-S-CB7-(23–35)-Cys with pep M24 or the homologous peptide in ELISA

Test peptide	Concentration, μM	% inhibition of interaction with pep M24	% inhibition of interaction with S-CB7-(23–35)-Cys-bovine serum albumin
pep M24	1.2	42	0
	40.0	97	27
S-CB7-(1–35)	1.2	77	0
	40.0	100	47
S-CB7-(13–35)	1.2	80	10
	40.0	100	47
S-CB7-(18–35)	1.2	77	10
	40.0	100	40
S-CB7-(23–35)-Cys	1.2	77	34
	40.0	100	100
S-M5-(1–20)	1.2	0	0
	40.0	0	0
S-M5-(20–40)	1.2	0	0
	40.0	0	0

M proteins have been found to contain epitopes within their covalent structures that evoke antibodies crossreacting with a membrane protein of human cardiac sarcolemma (6).

In previous studies (7–10, 14–17), we have shown that native polypeptide fragments of M protein, ranging from M_r ≈4000 to M_r ≈40,000, each contain epitopes capable of eliciting protective immunity. More recently, we reported that synthetic copies of two different 35-residue peptides (S-CB3 and S-CB7) of type 24 M protein (18, 19) and of a 20-residue peptide of type 5 M protein (20) were capable of eliciting protective antibody. In none of these synthetic copies have we been able to detect tissue crossreactive epitopes.

Why the 13-residue M protein peptide S-CB7-(23–35)-Cys should be equally, if not more, immunogenic than the 18- or 23-residue peptides remains unclear; each of the longer peptides encompasses the entire structure of S-CB7-(23–35)-Cys. It must be remembered, however, that the latter peptide contains an additional residue of cysteine at its COOH terminus with the resultant potential for dimer formation through disulfide bonding. Such dimers would contain 28 amino acid residues, counting the two additional half-cystines, and, therefore, would actually be larger than either of the other two subpeptides. It should be noted also that the shortest peptide contains within its structure the most hydrophilic region of S-CB7 (18), as determined by hexapeptide hydrophilicity profiles according to the method of Hopp and

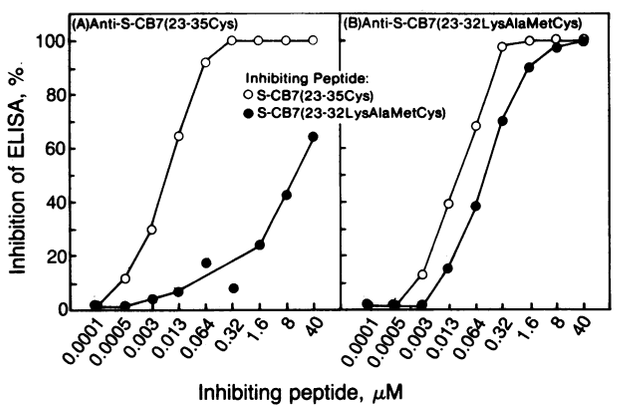

FIG. 4. ELISA of the inhibition of the interaction of anti-S-CB7-(23–35)-Cys (*A*) or anti-S-CB7-(23–32)-Lys-Ala-Met-Cys (*B*) with pep M24 by S-CB7-(23–35)-Cys (○) or S-CB7-(23–32)-Lys-Ala-Met-Cys (●).

Woods (29). Indeed, the latter investigators (29) predicted an antigenic determinant in type 24 M protein corresponding to a hexapeptide encompassing residues 23–28, which coincidentally represents the NH_2-terminal end of S-CB7-(23–35)-Cys. Perhaps such exposure at the end of the synthetic molecule renders the hexapeptide even more highly immunogenic.

Although the synthetic peptide S-CB7-(23–35)-Cys stimulated the production of high titers of type-specific antibodies directed against the uncleaved pep M24 and against the intact type 24 streptococci, not all of the antibodies were recognized by the natural M protein molecule. For example, pep M24 was much less effective than the immunizing peptide in inhibiting the interaction of anti-S-CB7-(23–35)-Cys with S-CB7-(23–35)-Cys in ELISA, indicating that this synthetic peptide may contain additional epitopes that are inaccessible or absent from the parent protein.

A comparison of the antigenic activity of S-CB7-(23–35)-Cys with a synthetic subpeptide containing a single Lys/Gly substitution as position 33 indicated that substitution of a noncharged with a charged amino acid residue in the COOH-terminal region reduced the molecule's ability to evoke protective antibody. The Lys/Gly substitution altered the peptide so that it no longer evoked protective antibodies, although it retained at least one epitope that partially inhibited the interaction of antiserum against the unsubstituted peptide with the natural M protein. Taken together, these results indicate that a peptide containing only 13 amino acid residues of M protein contains, nevertheless, more than one epitope and that one of the epitopes essential for stimulating protective immunity is influenced by a single amino acid substitution.

In a previous study (18) employing a combination of purified peptide fragments and a set of monoclonal antibodies, we demonstrated that a single amino acid substitution in the 35-residue repeating covalent structure of type 24 M protein markedly altered antigenic reactivity. A conservative Asp/Glu substitution at position 26 of CB4 and CB6, respectively, rendered the peptide unrecognizable by one of the protective antibodies but recognizable by a nonprotective one. It is apparent, therefore, that epitope specificity may be markedly altered by minor changes in the primary structure of the M protein molecule. Whether or not such minimal changes may account for antigenic crossreactivity among different serotypes of M protein remains to be determined.

The observation that single amino acid substitutions may result in major alterations in antigenicity of an infectious agent was first reported by Laver *et al.* (30). In their studies of the structure–function relationships of the influenza virus hemagglutinin, they demonstrated that the affinity of the interaction of a monoclonal antibody with type A influenza virus was reduced five orders of magnitude by a single Ser/Leu substitution in an antigenic determinant of the virus.

In only a few cases (18–20, 27, 31, 32) has it been shown that the antibodies evoked by synthetic peptides of infectious agents afford protective immunity against the related pathogen. In this respect, the synthetic fragments of streptococcal M protein, the structure and function of which have been well delineated, serve as excellent models for the study of the molecular mechanisms of host defenses against infectious pathogens in general.

We thank Drs. James B. Dale, Andrew H. Kang, Françoise Audibert, Michel Jolivet, and Hélène Gras-Masse for stimulating discussions and review of the manuscript. The expert technical assistance of Edna Chiang and Dan Monroe and secretarial assistance of Connie Carrier are gratefully acknowledged. These studies were supported by research funds from the U.S. Veterans Administration and by Grants AI-10085, AI-13550, and AM-16506 from the U.S. Public Health Service.

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