HETEROGENEITY OF TYPE-SPECIFIC AND CROSS-REACTIVE ANTIGENIC DETERMINANTS WITHIN A SINGLE M PROTEIN OF GROUP A STREPTOCOCCI*

By JAMES B. DALE,‡ ITZHAK OFEK, AND EDWIN H. BEACHEY§

From the Veterans Administration Medical Center; and the Departments of Medicine and Microbiology, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104

M protein on the surface of group A streptococci renders the organism resistant to phagocytosis in the nonimmune host. Antibody directed against this surface antigen is opsonic for homologous-type organisms and promotes phagocytosis and killing in the immune host. The immune response to the M protein antigen has been thought to be universally type specific, resulting in a unique population of protective antibodies against each serotype of M protein. Increasingly sensitive immunological assays, however, have pointed toward significant cross-reactivity among different serotypes.

Cross-protection between M serotypes was first reported in 1939 by Hirst and Lancefield (1), who immunized mice with crude acid extracts of M protein. Later, Wiley and Wilson (2) described cross-protection between the M proteins of certain type 14 and 51 strains. Their data suggested the presence of two distinct protective M protein antigens in the cross-reactive strains; however, the possibility that the antigens resided in a single protein molecule was not excluded. Wiley and Bruno (3) later described a number of cross-reactions, some of which were protective, among types 33, 41, 43, 52, and "Ross" after injection of rabbits with heat-killed organisms. Unidirectional, cross-protective antibody between types 55 and 60 was shown by Bergner-Rabinowitz et al. (4) to develop in humans and rabbits. These studies suggested that some of the M antigens shared certain protective (or opsonogenic) determinants.

Several investigations have suggested that streptococcal M protein is composed of heterogeneous subunits, each of which possesses antigenic type specificity (5–12). Although whether or not the multiple determinants were identical or antigenically distinct was not clearly established, the observations that certain antisera precipitated M protein but were not opsonic (10, 11), whereas others were opsonic but did not precipitate M protein (11, 13), suggested the existence of distinct antigenic sites. More recently, Fischetti (14), with a sensitive radioimmunoassay, obtained data showing heterogeneity of the immune response to a single serotype-6 M protein. He presented evidence to suggest that some of the heterogeneous determinants were shared with other M serotypes and, furthermore, demonstrated a correlation of cross-reactivity

* Supported by program-directed research funds from the U. S. Veterans Administration and by research grants AI-10085 and AI-13550 from the U. S. Public Health Service.

‡ Recipient of a U. S. Public Health Traineeship Award (GR-AM07317-02).

§ Recipient of a Medical Investigatorship Award from the Veterans Administration.

among different serotypes of M protein with the presence of several common tryptic peptides, thus suggesting certain structural similarities among the types examined (15).

Our investigation was undertaken to study the antigenic diversity of a single polypeptide of M protein known to be composed of repeating primary structures (12). Purified pepsin extracts of types-5, -6, and -24 M proteins (pep M5, pep M6, and pep M24)[1] were used as antigens in the enzyme-linked immunosorbent assay (ELISA) to analyze the immune response in humans immunized with one of these preparations, pep M24. We present evidence to show that immunization with pep M24 can lead to the production of IgG class antibodies that cross-react with pep M6 and pep M5, and that a portion of the cross-reactive antibodies afford cross-protection. Moreover, immunoinhibition studies with cyanogen bromide peptide fragments derived from pep M24 indicate (*a*) that the M protein molecule is antigenically heterogeneous, possessing several distinct as well as identical type-specific antigenic determinants, and (*b*) that pep M24 contains an inaccessible cross-reactive determinant that becomes exposed only after fragmentation of the intact molecule.

Materials and Methods

M Protein. M proteins from limited peptic digests of intact types-5, -6, and -24 group A streptococci were obtained in pure form as judged by sodium dodecyl sulfate (SDS)-gel electrophoresis and amino terminal sequence analysis as described elsewhere in detail (16–18). The purified peptic extracts (pep M5, pep M6, and pep M24) were assayed for the presence of type-specific precipitating and opsonic antigens by capillary precipitation and opsonization inhibition tests, respectively (16, 19). Cyanogen bromide (CNBr) peptides were prepared from pep M24 and purified as previously described (12, 18).

Human Serum. Sera used in this study were obtained from healthy volunteers who received immunizing doses of pep M24. The immunization protocols have been reported in detail elsewhere (17). Briefly, volunteers between the ages of 22 and 58 (mean age: 29) who did not have histories or evidence of cardiac or renal disease, rheumatic fever, or atopic allergies were given intradermal skin tests with 0.1–1.0 μg of pep M24 and pep M6. Negative skin-test individuals who were chosen for the vaccination studies received an alum-precipitated pep M24 vaccine in doses of 100–200 μg injected subcutaneously (17). Sera were collected from all vaccinated subjects immediately before the first dose and at 2-wk intervals thereafter. In addition, a serum sample was obtained from Dr. Ofek, that had a documented type-24 streptococcal pharyngitis. The latter serum was obtained 8 wk after the pharyngitis. All sera were heat inactivated at 56°C for 30 min before use in the ELISA or opsonic tests (see below).

ELISA. The ELISA was performed according to the principles of Engvall and Perlmann (20) and as described by Russel et al. (21). The various M protein antigens were adsorbed to 12- × 75-mm polystyrene tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at a concentration of 5 μg/ml in 0.1 M carbonate buffer at pH 9.6. 1 ml of antigen solution was added to each tube, incubated for 3 h at 37°C, and stored at 4°C until used. Before use, the tubes were washed three times with 0.15 M NaCl (saline) supplemented with 0.15% Tween-20 (saline-Tween). Serial, twofold dilutions of the test serum in 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS) supplemented with 0.05% Tween-20 (PBS-Tween) were added to the antigen-coated tubes and incubated at 37°C for 3 h. The tubes were then washed thoroughly as above, and 1 ml of alkaline phosphatase-conjugated rabbit anti-human IgG (Microbiological

[1] *Abbreviations used in this paper:* CB, cyanogen bromide-derived peptide(s) of pepsin extract of type-24 M protein; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; PBS, 0.02 M phosphate/0.15 M NaCl, pH 7.4; PBS-Tween, PBS supplemented with 0.05% Tween; pep M5, pepsin extract of type-5 M protein; pep M6, pepsin extract of type-6 M protein; pep M24, pepsin extract of type-24 M protein; saline, 0.15 M NaCl; saline-Tween, saline supplemented with 0.05% Tween-20; SDS, sodium dodecyl sulfate.

Associates, Walkersville, Md.), diluted 1:500 in PBS-Tween, was added and incubated overnight at 37°C. After washing the tubes once more with saline-Tween, 1 ml of *p*-nitrophenyl phosphate (1 mg/ml, Sigma Chemical Co., St. Louis, Mo.) in 0.1 M carbonate buffer (pH 9.6) that contained 1 mM $MgCl_2$ was added. The reaction was allowed to proceed for 1 h at 37°C and was stopped by the addition of 0.1 ml of 1 M NaOH. Absorbance was read at 400 nmol in a Junior Coleman spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). The titer of the test serum was recorded as the inverse of the last serum dilution that gave an absorbance >0.1.

Inhibition experiments were performed by making serial, 10-fold dilutions of soluble antigen in 0.5 ml PBS to which was added 0.5 ml of a constant dilution of human serum. The mixture was incubated at 37°C for 30 min and then added to the antigen-coated tubes. The dilution of human serum used was one that was known to yield an absorbance of 0.6–0.8 at 400 nmol in the absence of absorbing antigen.

Opsonophagocytic Assay. In vitro phagocytosis tests for type-specific opsonic antibody were performed as previously described (16). The assay mixtures consisted of 0.4 ml of fresh blood supplemented with 10 U/ml of heparin, 0.05 ml of a standard suspension of streptococci, and 0.1 ml of test serum. The ratio of streptococcal colony-forming units:leukocyte was ~10:1. The percentage of neutrophils that ingested streptococci (percent phagocytosis) was estimated by microscopic counts of stained smears prepared from the assay mixtures after incubation for 30 min. A positive opsonic antibody response was recorded if in three separate tests the serum produced phagocytosis of 20% or greater, whereas phagocytosis of the same organism in the presence of nonimmune rabbit serum was <0.5 that of the test serum in each assay.

Streptolysin O and Streptozyme Antibody Assays. These assays were performed as previously described (22). The streptozyme reagent was purchased from Wampole Laboratories, Div. Carter Wallace, Cranbury, N. J.

Results

After immunization, all 12 individuals developed greater than fourfold rises in pep M24 antibody titers 8 wk after the initial injection, whereas two individuals developed fourfold or greater rises in titer against the heterologous pep M6 and pep M5 antigens (Fig. 1). None of the vaccinees developed a rise in antibody titer to streptolysin O or streptozyme reagent (data not shown). The two cross-reactive sera and one of the type-specific sera were used in the following studies.

Inhibition of Cross-Reactive Sera with Pep M Proteins. Both of the sera that showed rises in cross-reactive antibody titers against pep M5 and pep M6 in the ELISA also showed low levels of opsonic antibodies against type-6, but not type-5, streptococci (Table I). These results suggested that type-6 M protein shared a common antiphagocytic determinant with pep M24. To further investigate this possibility, one of the cross-reactive sera was preincubated with the homologous or heterologous pep M proteins. The opsonization of type-6 streptococci by the pep M24 immune serum was blocked only by pep M6 (Table II). Surprisingly, the antibody was not inhibited by the immunizing antigen, pep M24. Neither pep M5 nor pep M6 inhibited opsonization of the vaccine strain of type-24 streptococci.

In contrast to the inhibition of opsonization tests, ELISA showed that antibody against pep M24 was partially inhibited by pep M5 and pep M6, although 1,000–10,000 times more of the heterologous antigens was required to achieve inhibition comparable to that of the homologous pep M24. Antibody against pep M6 was inhibited by pep M5 and pep M6 but not at all by pep M24 (Fig. 2). Pep M24 partially inhibited antibody against pep M5, and pep M6 inhibited 100% of this antibody. Interestingly, pep M6 was ≃10 times more effective in blocking antibody against pep M5 than the homologous antigen.

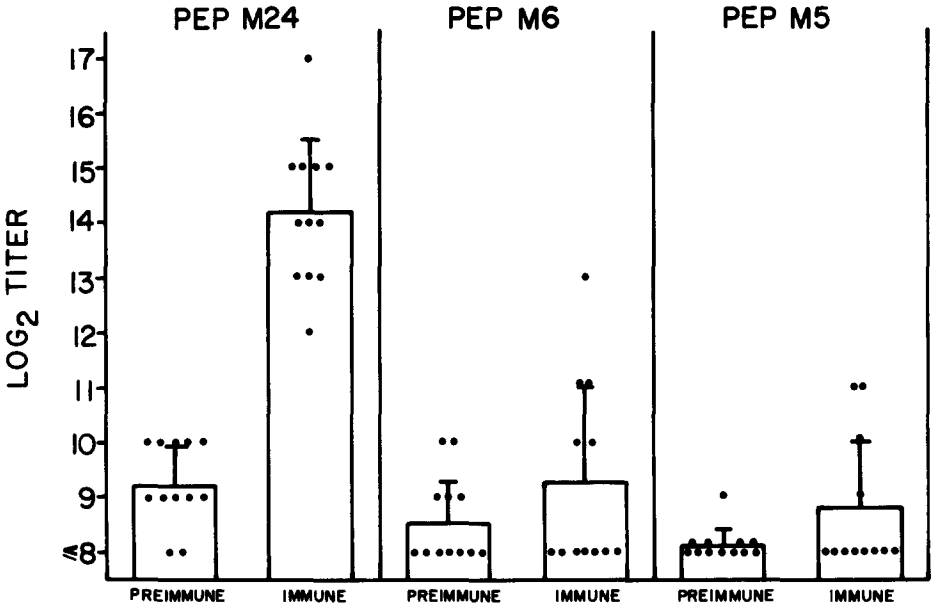

FIG. 1. The primary immune response to pep M24, pep M6, and pep M5 after immunization with pep M24 in human volunteers as measured by the ELISA. Bars represent mean titers ± SD.

TABLE I

Assays for Opsonic Antibodies Against Types-24, -6, and -5 Streptococci in Cross-Reactive Sera of Two Humans Immunized with Pep M24

Serum	ELISA titer against:			Opsonic antibodies (percent phagocytosis of streptococci)*		
	pep M24	pep M6	pep M5	type 24	type 6	type 5
					%	
R.F.						
Preimmune	1,024	512	<256‡	4	8	2
Immune	32,768	8,192	2,048	72	54	3
R.P.						
Preimmune	256	512	<256	0	6	0
Immune	32,768	2,048	2,048	69	62	2

* Opsonophagocytosis tests were performed as described in Materials and Methods.
‡ The lowest dilution of serum tested in these assays was 1:256.

Inhibition of Cross-Reactive Sera with Peptide Fragments of Pep M24. To investigate the possibility that the cross-reactive antibody that failed to react with the immunizing pep M24 antigen was raised against an inaccessible determinant in the uncleaved pep M24 protein, we tested the ability of peptide fragments derived by CNBr digestion (see below) to inhibit the cross-reactive antibodies against pep M6 (Fig. 3). A mixture of the larger CNBr-derived peptides of pep M24 (CB) fragments, CB1-2, resulted in >50% inhibition of the assay, whereas pep M24 did not block antibody at even higher molar concentrations. Preincubation with the smaller peptides, CB3-7, resulted in

TABLE II

*Inhibition of Cross-Reactive and Type-Specific Opsonic Antibodies in a Cross-Reactive Serum of a Pep M24 Vaccinee**

Streptococci	Inhibitor	Percent phagocytosis
		%
Type 6	None	52
	Pep M5	48
	Pep M6	6
	Pep M24	62
Type 24	None	42
	Pep M5	48
	Pep M6	44
	Pep M24	2

* Opsonic antibodies against type-6 and type-24 streptococci were assayed in timed phagocytosis tests (see Materials and Methods). The opsonic inhibitory activity of each of the pep M proteins was assayed by mixing 0.1 ml of diluted serum with 100 μg pep M protein dissolved in 0.1 ml PBS, incubating the mixture at 37°C for 30 min, and using the treated serum to preopsonize type-24 or type-6 streptococci as previously described (15).

minimal inhibition, and pep M6, the homologous antigen, was an effective inhibitor at very low concentrations. These results suggested that pep M24 possessed a buried determinant that cross-reacted with an exposed determinant(s) on pep M6.

Inhibition of Type-Specific Sera with Pep M24 and Peptide Fragments. Previous studies showed that two of the CNBr fragments of pep M24, CB1 and CB2, each contained ~90 amino acid residues and were both found to have NH_2-terminal amino acid sequences identical with the intact pep M24 (12, 18). The remaining five fragments, CB3, CB4, CB5, CB6, and CB7, ranged from ~35 to 40 amino acid residues in size and had amino acid sequences different from CB1 and CB2 but identical with each other through the first 20 residues from their NH_2-terminal ends. It was of interest to compare the immunochemical properties of these two classes of peptides that had totally different repeating sequences by using the highly sensitive ELISA.

Inhibition experiments were designed to block the binding of antibody to pep M24, with the parent protein and mixtures of the CNBr peptides as soluble inhibitors of serum from one of the pep M24 vaccinees that did not show cross-reactions with pep M5 or pep M6 and from an individual with a natural type-24 streptococcal infection (Fig. 4). As expected, the intact pep M24 was a very effective inhibitor of both sera. A mixture of the smaller CNBr peptides, CB3–7, was least inhibitory, requiring 50,000 times the concentration of the parent protein to reach 50% inhibition of both sera. The inhibition by the larger peptides, CB1–2, was of particular interest because of the differential pattern of inhibition of the two immune sera. The biphasic curve obtained by preincubation of the naturally immune serum with CB1–2 indicated that ~40% inhibition was achieved with much lower concentrations of these soluble peptides than that necessary to inhibit the serum of the vaccinee.

To further define the differential pattern of inhibition of the two immune sera, the sera were preincubated with the purified peptides, CB1 and CB2 (Fig. 5). Each peptide was an effective inhibitor of the serum of the vaccinee, resulting in similar sigmoid curves that approached 100% inhibition. Inhibition of the naturally immune serum with CB2 showed a clearly defined, biphasic curve with plateaus at 30 and

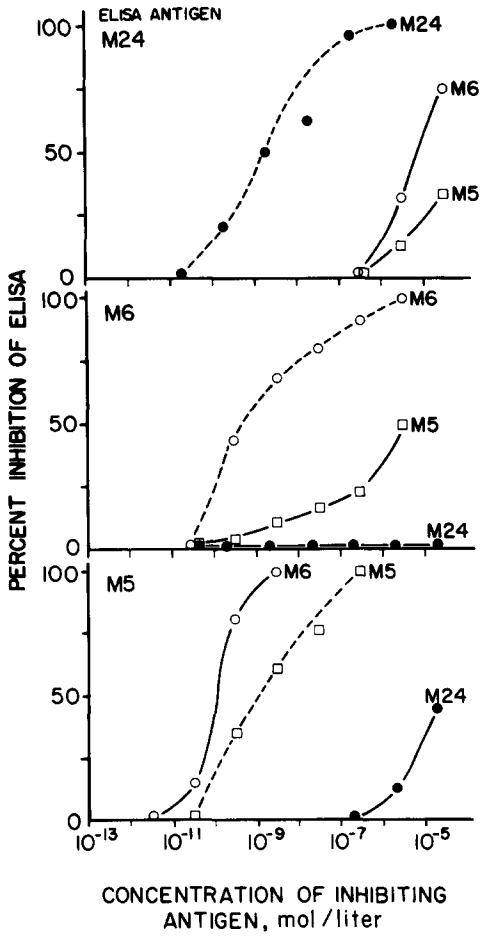

FIG. 2. Inhibition of cross-reactive serum antibodies from an individual immunized with pep M24. Increasing concentrations of pep M24 (●), pep M6 (○), and pep M5 (□) were used to inhibit antibody binding to each of the immobilized antigens. The dashed lines represent inhibition by the homologous antigen.

65% inhibition. Preincubation with CB2 achieved 30% inhibition at molar concentrations below those of the native antigen, thus suggesting enrichment of an antigenic determinant. Preincubation of the naturally immune serum with CB1 resulted in a monophasic curve that approached 100% inhibition at the highest concentration tested. High concentrations (up to 50 nmol) of the heterologous pep M6 and pep M5 were not inhibitory for either serum.

Discussion

The validity of the results obtained in the ELISA is dependent upon the purity of the antigen used. We believe that the pep M antigens prepared for these assays satisfy the most stringent criteria of homogeneity as judged by electrophoresis in SDS-gels, quantitative amino acid analysis, and amino terminal sequencing (12). Our previous

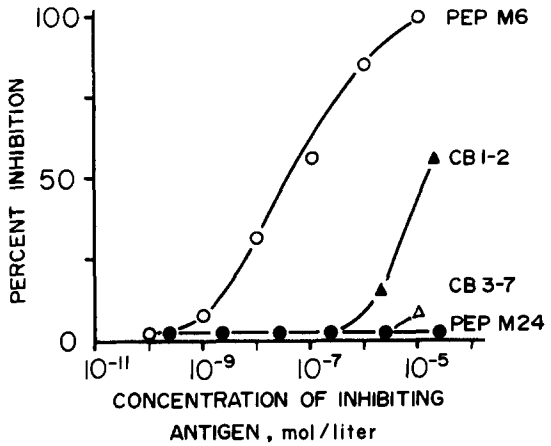

FIG. 3. Inhibition of cross-reactive serum antibodies from an individual immunized with pep M24. Increasing concentrations of pep M6 (○), pep M24 (●), CB1-2 (▲), and CB3-7 (△) were used to inhibit antibody against pep M6 in the ELISA.

data indicated that such preparations resulted in highly sensitive and type-specific assays by the ELISA (17, 23).

In this study, immunization with pep M24 antigen led to the production of antibody in two individuals that cross-reacted with pep M5 and pep M6 in the ELISA. Both of these also showed a rise in opsonic antibody against type-6, but not against type-5, streptococci. However, the cross-reactive opsonic antibodies against type-6 streptococci could be inhibited by the pep M6 antigen only. By the ELISA, pep M5 partially blocked antibodies against pep M6. Interestingly, however, these antibodies could not be blocked in either assay by the immunizing antigen, pep M24. The ability of the CB peptides to inhibit the cross-reactive antibody against pep M6 as determined by ELISA suggested that the cross-reactive antibodies were directed against a buried antigenic site in the pep M24 molecule, and that the buried site(s) must be the same as an exposed site(s) on pep M5 and pep M6. Similar buried determinants have been reported for other bacterial protein molecules. These include fragment A of diphtheria toxin (24), fragment A of cholera enterotoxin (25), and a portion of tetanus toxin (26). Nonbacterial proteins, including hemocyanin (27), serum albumin (28–30), immunoglobulin (31, 32) and myelin basic protein (33) also have been reported to contain inaccessible antigenic regions that became exposed only after fragmentation of the intact molecules. Only 2 of the 12 vaccinees responded immunologically to these apparently buried regions of pep M24, which suggested that during antigenic processing the inacessible cross-reactive region(s) of the M protein molecule may occasionally become exposed in vivo.

In regard to the immunological cross-reactions among the apparently homogeneous pep M protein serotypes, the covalent structural homologies recently recognized among pep M proteins may be of relevance (34, 35). Several of the amino acid residues were found to be common to pep M5, pep M6, and pep M24, which suggested that conservation of certain amino acids may account for the preservation of a specific tertiary structure in these three distinct M protein molecules. Whether or not these structures allow presentation of common antigenic moieties to account for the observed cross-reactions, however, remains to be proved.

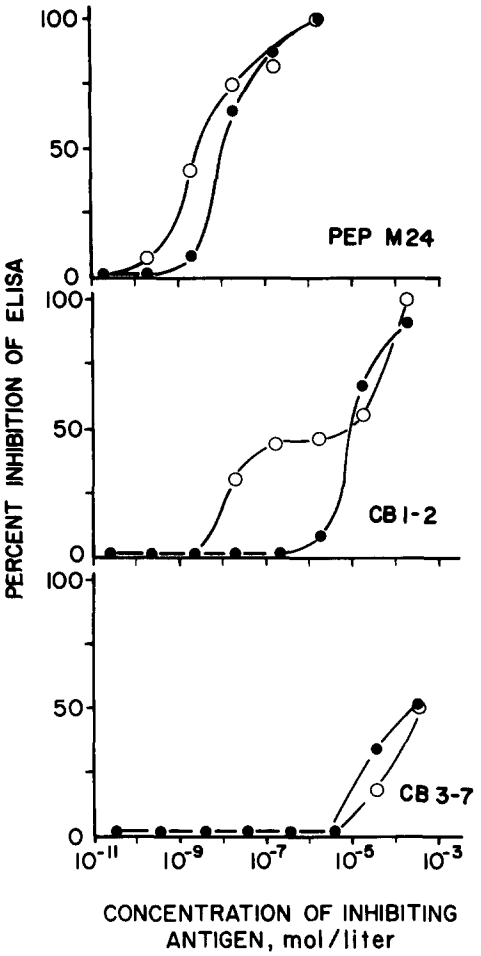

FIG. 4. Inhibition of antibodies from an individual vaccinated with pep M24 (●) and an individual with naturally acquired immunity to type-24 streptococci (○). Increasing concentrations of pep M24 (top), CB1-2 (middle), and CB3-7 (bottom) were used to inhibit the binding of antibody to pep M24 in the ELISA.

An alternative explanation for the rise in antibody titers against the heterologous types-5 and -6 streptococci during pep M24 immunization is that the two volunteers had intercurrent infections with these serotypes. This possibility seems highly remote, however, because (*a*) types-5 and -6 streptococcal infections are relatively rare in the population under study (36), (*b*) the immune response occurred within an 8-wk period and the probability that 2 of 12 volunteers contracted such infections would be extremely small, and finally (*c*) neither of the subjects developed a rise in antistreptolysin O or streptozyme antibody titers.

Having recently established the partial covalent structure of pep M24 (12, 16, 18), we attempted to pinpoint the antigenic site(s) of the molecule responsible for type-specific immunity. Amino acid sequence analyses had revealed repeating identical sequences in pep M24 (12, 18). These repeating sequences were found to occur in two classes of peptides. The larger peptides, CB1 and CB2, were each composed of ~90 amino acid residues, and each contained amino acid sequences identical with each

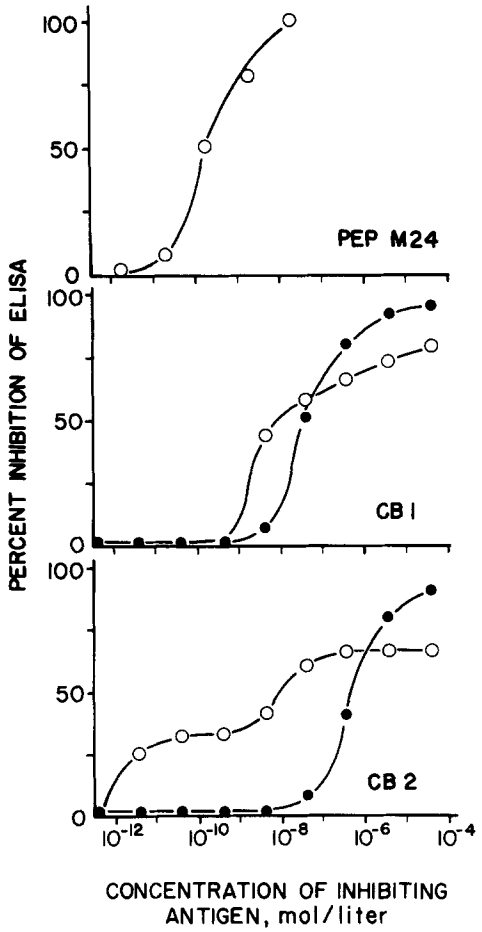

FIG. 5. Inhibition of serum from an individual vaccinated with pep M24 (●) and an individual with naturally acquired immunity to type-24 streptococci (○). Increasing concentrations of pep M24 (top), CB1 (middle), and CB2 (bottom) were used to inhibit antibody binding to pep M24 in the ELISA.

other and with the amino terminal region of the intact pep M24 molecule through at least the first 27 residues. The remaining five peptide fragments, CB3, CB4, CB5, CB6, and CB7 were smaller (35–40 amino acid residues) and had amino acid sequences different from CB1 and CB2 but identical with each other for the first 20 residues from their amino terminal ends (12). Each of the peptide fragments was shown to share type-specific antigenic determinants (12, 18).

It was of particular interest to determine if these peptides would inhibit type-specific antibodies from the immune sera of the pep M24 vaccinees. Our data obtained from inhibition studies of one of these sera and of a serum from an individual who had experienced a natural infection with type-24 streptococci indicated that pep M24 possessed more than one distinct type-specific antigenic determinant. The serum from the naturally infected individual contained one population of antibodies directed toward an antigen(s) present in CB2 but not in CB1 and another population of

antibodies directed toward an antigen(s) present in both of these peptides. The person vaccinated with pep M24 and the person with natural immunity to type-24 streptococci both had serum antibodies directed against the smaller peptide fragments, CB3–7.

These results are consistent with those of Fischetti (14) who described the development of a family of type-specific antibodies to type-6 M protein in the immune sera of humans after natural streptococcal infections and in the sera of rabbits hyperimmunized with type-6 streptococci. By radiocompetitive assays he obtained evidence that suggested that opsonic antisera bound to the majority of the antigenic sites on the M6 molecule, whereas nonopsonic, cross-reactive antisera raised against heterologous serotypes of M protein reacted with only a small percentage of the antigenic sites recognized by M6 antiserum. Furthermore, the cross-reactions among M types were correlated with the presence of common peptide fragments, which suggested a certain degree of structural similarity among cross-reactive serotypes of M protein (15). The question as to whether or not certain of the cross-reactive determinants are buried or inaccessible in the intact M protein molecule, however, was not addressed. Our data suggest that certain of the cross-reactive determinants are inacessible in the intact molecule and are recognized by the immune system of only a small percentage of vaccinees in whom the buried antigenic site may become exposed during processing in vivo. Certain of the buried antigenic sites may prove to afford cross-protective immunity similar to that shown in our study between pep M24 and pep M6. Cross-reactions to such shared determinants may also account for the limited degree of heterologous protection recently reported by Wittner and Fox (37) who immunized mice with a trivalent vaccine that contained acid extracts of types-1, -3, and -12 M proteins. In addition to protection against the vaccine serotypes, the immunized animals were also protected against challenge infections with types-6 and -14 streptococci (37). Whether or not a single determinant among the many distinct type-specific determinants in an M protein molecule would be sufficient by itself to raise protective antibodies remains unknown. Recent studies in our laboratories,[2] on the immunogenicity of purified peptide fragments, however, suggest that opsonic antibodies can be raised against a limited number of the type-specific determinants in any given M protein molecule.

In conclusion, our studies indicate that (*a*) not all type-specific determinants of M protein are opsonogenic, perhaps because some are not exposed on the bacterial cell surface, (*b*) although M protein opsonogens are generally type specific, a few are common to more than one serotype, which indicates that more than one opsonogenic determinant may be exposed on the cell, and (*c*) some type-specific antigenic determinants that are not opsonogenic for the homologous type may be opsonogenic for another M type, which suggests that the cross-reactive opsonogen that is buried in one type of M protein may be present and exposed on the surface of another M type. Thus, continued identification of the antigens of various M proteins may yield a pool of small polypeptides, all representing opsonogens that ultimately can be used to induce broad protection against many serotypes with maximal economy of antibody production and with relatively small amounts of total protein injected.

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[2] Beachey, E. H., J. M. Seyer, and A. H. Kang. Primary structure of protective antigens of type 24 streptococcal M protein. Manuscript submitted for publication.

Summary

The heterogeneity of a pepsin extract of type-24 M protein (pep M24) was demonstrated by absorption of type-specific and cross-reactive human antisera with M protein fragments and heterologous serotypes of M proteins, pepsin extract of type-5 M protein (pep M5) and pepsin extract of type-6 M protein (pep M6). 2 of 12 individuals immunized with pep M24 developed significant rises in antibody titers against pep M5 and pep M6, as measured by the enzyme-linked immunosorbent assay. The same individuals also developed opsonic antibodies against type-6, but not type-5, streptococci, which suggested the development of cross-protective immunity. Inhibition studies of one of these sera with the heterologous pep M proteins showed that the cross-reactive antibodies against pep M6 could not be blocked by high concentrations of pep M24, the immunizing antigen; these antibodies could be blocked, however, by cyanogen bromide-derived peptide fragments of pep M24, which suggested that the cross-reactive antibody was raised against an inaccessible site(s) in the pep M24 molecule. Inhibition studies of type-specific immune sera with pep M24 and peptides derived therefrom indicated that the M protein molecule contained multiple distinct as well as identical type-specific antigenic determinants that are unequally distributed among the seven derived peptide fragments.

We thank Dr. Gene H. Stollerman and Dr. John N. Whitaker for stimulating discussions and helpful suggestions; Dr. Jerome Seyer for amino acid analyses; Edna Chiang, Robert Curtis, Sabra Seyer, and Elizabeth Jackson for excellent technical assistance; and Mrs. Johnnie Smith for secretarial assistance.

Received for publication 26 November 1979 and in revised form 21 January 1980.

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