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Peptic Digestion of Streptococcal M Protein

I. Effect of Digestion at Suboptimal pH upon the Biological and Immunochemical Properties of Purified M Protein Extracts

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Purified streptococcal M protein vaccines often produce non-type-specific immunotoxic reactions in the skin or the blood of humans. In an attempt to free the type-specific M antigen (TSM) of such non-type-specific (NTSM) immunotoxic properties, purified M protein preparations were subjected to brief periods (5 to 10 min) of enzymatic digestion in dilute solutions of pepsin (20 µg/ml) at pH 5.0. Such peptic digestion abolished the ability of M protein to aggregate platelets in human platelet-rich plasma and to precipitate plasma fibrinogen. It greatly reduced (up to 16-fold) the capacity of M protein to react with the NTSM complement-fixing antibody that is normally present in human serum. In contrast, it had no effect upon the reactivity of the type-specific M antigen; the pepsin-treated M protein retained its ability to inhibit type-specific streptococcal opsonization by homologous M antibody, and, moreover, retained its ability to elicit type-specific opsonic antibody responses in rabbits.

Previous efforts to purify streptococcal M protein vaccines have failed to yield a completely homogeneous antigenic preparation that reacts only with its homologous-type antibody in human serum. All of the purified fractions of M protein we studied possessed immunotoxic properties either in the skin (1, 14, 22) or in the blood (2–6, 25) of human hosts regardless of the presence or absence of type-specific serum antibody. This indicated the presence of an M-associated non-type-specific antigenic moiety(ies), hereafter called NTSM. Intensive efforts by the conventional purification methods of gel filtration, ion-exchange chromatography, and disc electrophoresis, however, have failed to separate completely NTSM from the type-specific M (TSM) determinant in purified M protein fractions (1, 14, 17, 25). This has hampered the use of such M protein preparations as vaccines against streptococcal infections in humans. Therefore, we have sought other methods for purification of the type-specific M antigen.

In the present paper (presented in part at the Annual Meeting of the American Society for Microbiology, May, 1973, Miami, Fla.) we report the effect of mild enzymatic digestion with pepsin upon the immunochemical properties of purified M protein extracts. Our data indicate that such digestion greatly reduced NTSM activity of M protein without affecting its type-specific seroactivity or immunogenicity.

MATERIALS AND METHODS

Streptococci. The strains of *Streptococcus pyogenes* used in this study included M types 12 (SF42) and 30 (D24), both obtained originally from Rebecca Lancefield, the Rockefeller University, New York. In addition, an M type 24 strain isolated from a patient with acute rheumatic fever was used. The organisms were stored lyophilized or frozen at −70 C in Todd Hewitt Broth (THB) supplemented with 20% normal rabbit serum (NRS). Frequent passage of the streptococci through mice or through human blood (7) maintained optimal production of M protein.

Extraction and purification of M protein. Streptococci rich in M protein were grown in 50-liter batches in THB for 16 h at 37 C. After chilling to 4 C, aqueous penicillin G was added to a final concentration of 2×10^4 U/ml. The organisms were then collected by continuous-flow centrifugation and washed in 0.02 M phosphate-buffered 0.9% NaCl at pH 7.4 (PBS). M protein was extracted from cell walls by an alkaline method previously described by Fox and Wittner (13). Streptococci were ruptured with glass beads in a Braun MSK homogenizer (A. H. Thomas, Philadelphia, Pa.) and the cell walls were separated from unruptured whole cells and membranes by differential centrifugation by the method of Bleiweis, Karakawa, and Krause (8). The cell walls were washed three times in PBS by centrifugation at $9,000 \times g$ and six times by centrifugation at $5,000 \times g$ (13).

The washed cell walls were then digested with ribonuclease, extracted twice at 37 C with M hydroxylamine at pH 10.0 for 90 min, and fractionated by ammonium sulfate precipitation as described by Fox

and Wittner (13). The fraction which precipitated between 0.33 and 0.60 saturation with ammonium sulfate was dissolved in and dialyzed against PBS. The preparation was then dialyzed alternately against 0.03 M sodium acetate buffer at pH 4.0 and PBS at pH 7.4. The latter procedure decreased the content of nucleic acids as indicated by an increase in the absorbancy (A_{280}/A_{260}) ratio (average increase, 0.25). The partially purified extract was then dialyzed against deionized water and lyophilized. Hydroxylamine extracts were also made from whole streptococcal cells rather than cell walls and were partially purifed by the same method.

Analytical methods. Total protein content was estimated by the method of Lowry et al. (21). Methylpentose was estimated by the method of Dische and Shettles (11). Relative nucleic acid content was monitored by determining ultraviolet extinction ratios at 280 and 260 nm (24). Total hexose determinations were performed by the phenol-sulfuric acid method of Dubois et al. (12).

Acrylamide gel electrophoresis was performed by the method of Davis (10) using a Canalco model 1200 electrophoresis apparatus (Canalco, Rockville, Md.). Fifty-microgram samples were subjected to electrophoresis in a 7% acrylamide gel at 25 C for 1 h (3 mA/gel tube). The gels were removed and stained with aniline black and destained in 7% acetic acid.

Streptococcal antisera and titration of type-specific precipitating M antigen. Antisera to whole streptococci were produced by immunization of rabbits by the method of Lancefield (20). Standard M type-specific antisera were also obtained from the Center for Disease Control (Atlanta, Ga.). Antibody was stimulated against M proteins by subcutaneous injection of 1 mg of M protein in 0.5 ml of PBS emulsified with an equal volume of complete Freund adjuvant. Rabbits were bled at 2-week intervals after immunization. Sera were handled as described above.

Titration and detection of the M antigen by the type-specific capillary precipitin method was performed by the method of Swift et al. (23). Immunodiffusion and immunoelectrophoresis tests were performed as previously described (1).

Streptococcal phagocytosis and bactericidal tests. In vitro, timed phagocytosis and indirect bactericidal tests for type-specific M antibody were performed as previously described (4). In brief, the bactericidal test mixture consisted of 0.4 ml of fresh, heparinized (10 U/ml) human blood, 0.05 ml of a standardized suspension of phagocytosis-resistant streptococci, and 0.05 ml of test serum. The mixtures were incubated at 37 C in a rotator apparatus at 8 rpm for 3 h. The number of surviving colonies was estimated by preparing pour plates in 5% sheep blood agar. Timed phagocytosis test mixtures were similar to those used in the bactericidal tests, except that a greater number of streptococci were added (approximately 10 streptococcal units/leukocyte). The percentage of polymorphonuclear leukocytes containing ingested streptococci was estimated by microscope examination of stained smears prepared from a drop of test mixture at 15 and 30 min of incubation.

Titration of the M antigen by type-specific inhibition of opsonization. Opsonization inhibition tests were performed as previously described (2). The test consisted of incubating serial dilutions of 1 mg/ml solutions of M protein with type-specific antiserum for 30 min at 37 C. To remove any precipitates, the mixture was centrifuged at 1,200 × g for 30 min. Washed log-phase streptococci were treated with the absorbed serum for 15 min at 37 C and 15 min at 0 C. The organisms were then washed twice and resuspended in ice-cold THB. The resuspended organisms were added to whole human blood and rotated at 8 rpm at 37 C. Smears were prepared at 15 and 30 min, and the percentage of phagocytosis was estimated as described above. The results are expressed as the percentage of maximum phagocytosis as follows: % maximum phagocytosis = [(% phagocytosis in presence of absorbed antiserum)/(% phagocytosis in presence of unabsorbed antiserum)] × 100. In this way, experiments performed on different days using different blood could be compared. The reproducibility of this method has been previously reported (2). Control streptococci treated with NRS, M protein, or PBS retained their resistance to phagocytosis.

Platelet aggregation and fibrinogen precipitation. Platelet aggregation tests were performed by the method of Born (9) and as previously described (4, 6, 18). Platelet-rich plasma (PRP) was obtained by centrifugation of heparinized whole human blood at 226 × g. The supernatant was removed, and a small quantity of the PRP was centrifuged at 1,000 × g for 30 min to obtain platelet-poor plasma (PPP). Twenty-five to fifty microliters of M protein solutions (1 mg/ml) was added to 0.45 ml of PRP. The reaction was recorded on a Payton aggregometer module (model 300A, Payton Associates Ltd., Buffalo, N.Y.). Two micromoles of adenosine diphosphate was added to a sample of PRP in all experiments to prove platelet viability.

Fibrinogen precipitation was performed by the method of Kantor and Cole (16). Human PPP was mixed with an equal volume of test solution in a capillary tube, and the reaction was observed after incubation at 25 C for 2 h.

Complement fixation. Complement fixation (CF) tests were performed by a microtechnique (3, 15, 25). The M antigen (1 mg/ml) was titrated over a range of twofold dilutions against pooled human serum with a known CF antibody titer of 1:20. Checkerboard titrations were performed, and the antigen titer was reported as the reciprocal of the highest antigen dilution that produced 50% lysis of sensitized sheep erythrocytes with pooled serum at a 1:20 dilution. The serum, composed of a pool from 20 normal humans, was tested for bactericidal antibodies against serotypes 12, 24, and 30 streptococci to demonstrate that type-specific M antibody was absent and that complement was fixed by NTSM rather than TSM antibody.

Pepsin digestion of M protein. Twenty micrograms of pepsin (2,800 U/mg, Worthington Biochemical Corp., Freehold, N.J.) was incubated with 1 mg of M protein at 37 C in 0.1 M acetate buffer at pH 5.0 for various time intervals as indicated (see Results). Pepsin activity was immediately neutralized after the appropriate time interval by adding 7.5% NaHCO₃ which raised the pH to approximately 8.

RESULTS

The NTSM reactivity of a partially purified, hydroxylamine-extracted type 24 M protein was abolished as measured by platelet aggregation and fibrinogen precipitation tests within 5 and 10 min of pepsin digestion, respectively (Table 1). As measured by the complement fixation test with normal human serum, however, a small amount of NTSM activity remained after 20 min of pepsin digestion (CF antigen titer 1:8 as compared to an initial titer of 1:128). In contrast, the type-specific opsonic inhibitory (OI) titer remained constant at 1:64 for at least 20 min of digestion (Table 1). Essentially the same results were obtained after such pepsin digestion of types 12 and 30 hydroxylamine-extracted M protein. Pepsin similarly abolished NTSM activity of conventional HCl extracts of these serotypes. However, since our previous studies (2) demonstrated that alkaline-extracted M24 was richer in TSM to begin with, we employed alkaline rather than acid M protein extracts for most of our studies.

Polyacrylamide disc electrophoresis of undigested M24 demonstrated one major and three minor protein bands (Fig. 1A and B). After 5 min of pepsin digestion, several additional bands appeared, one of which moved rapidly (Fig. 1C) and gradually disappeared upon further digestion (Fig. 1D and 1E).

Sephadex G-200 column chromatography of untreated M24 demonstrated elution of a predominant ultraviolet (280 nm) absorbing peak near the void volume followed by at least two additional peaks. Opsonic inhibitory activity was associated with the protein in each of the three elution peaks. NTSM activity was detected in the first two peaks (Fig. 2A). Pepsin
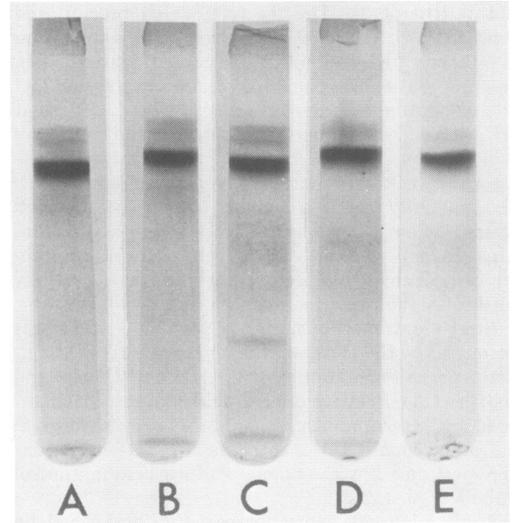

FIG. 1. Acrylamide gel electrophoresis of undigested M24 (A); M24 digested with pepsin at pH 5.0 for 0 min (B); 5 min (C); 10 min (D); and 20 min (E).

digestion for 5 min resulted in three to four peaks of smaller molecular size (Fig. 2B). The type-specific OI activity was present in a lower-molecular-weight species of protein and, moreover, was confined to a more narrow range of molecular size (Fig. 2B). Furthermore, NTSM activity as measured by CF tests could be detected only in the first two peaks eluted from the column to which the pepsin digest had been applied. It should be noted that the amount of NTSM activity was not based on quantitative analysis of each fraction of each peak. Therefore, small amounts of NTSM activity may have been undetectable by the methods employed.

Double diffusion tests in agar gel against type 24 streptococcal antiserum produced a precipitin line of identity between the untreated and the pepsin-digested M24 preparations (Fig. 3). The 5-min digest produced an additional faint precipitation line which fused with the single precipitin lines produced by the untreated M protein preparations. This may reflect the increased molecular heterogeneity after brief pepsin digestion that was observed also by polyacrylamide electrophoresis (see Fig. 1).

Thus, pepsin digestion appeared to split the multiple molecular structures of TSM into a smaller molecular species without affecting type-specific immunoreactivity.

Further evidence that TSM retained its activity was obtained in rabbit immunization stud-

TABLE 1. Effect of pepsin digestion upon non-type-specific reactivity of type 24 M protein

Digestion time (min)	Fibrinogen precipitation[a]	NTSM[b] antigen titers		TSM[b] titer: opsonization inhibition
		Platelet aggregation	Complement fixation	
0	++++	80	128	64
5	+	<10	16	64
10	0	<10	16	64
20	0	<10	8	64

[a] ++++, positive reaction of a 1:4 dilution of 1 mg/ml solution of M 24; +, positive undiluted; 0, negative undiluted.

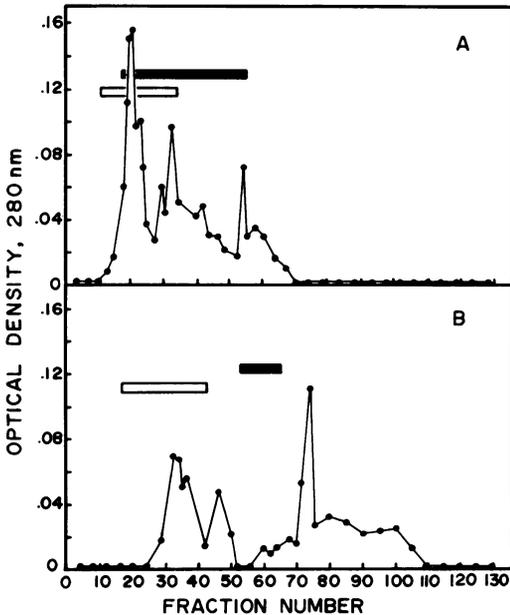[b] NTSM, non-type-specific M-associated antigen(s); TSM, type-specific M antigen.

FIG. 2. *Sephadex G-200 column chromatography of (A) undigested M24, and (B) pepsin-treated (5 min) M24. A 3-mg sample of treated or untreated M24 in 1 ml of buffer was applied to a column (1.5 by 30 cm). Fractions (2 ml) were eluted with 0.9% NaCl buffered with 0.02 M phosphate at pH 7.4. Undiluted samples of each fraction were tested for type-specific antiopsonic activity as previously described (2). NTSM activity was measured by CF tests of checkerboard titrations of each fraction against a pooled human serum with a known NTSM antibody titer. The solid bars indicate the location of type-specific opsonic inhibitory activity and the open bars indicate NTSM activity.*

ies. A single 1.0-mg dose of either untreated or pepsin-digested (5 min) M24 evoked similar opsonic antibody responses which peaked 4 to 6 weeks after immunization (Fig. 4). This was confirmed both by timed phagocytosis and by the indirect bactericidal tests for type-specific M antibody.

DISCUSSION

Numerous attempts have been made to prepare highly purified streptococcal M protein which could be administered safely as vaccines against group A streptococcal infections (22). Our previous studies (3, 4, 5, 6) demonstrated the presence of an immunotoxic factor or factors which we were unable to separate from the type-specific M determinant by conventional purification methods. The work reported here

demonstrates that brief digestion of purified M protein preparations with dilute solutions of pepsin at suboptimal pH greatly reduced non-type-specific seroreactivity without significantly affecting its type-specific immunoreactivity or immunogenicity.

The exquisite sensitivity of streptococcal M protein extracts to proteolytic enzymes was first described by Lancefield in 1928 (19). She observed that, by altering digestion time and enzyme concentrations, non-type-specific antigenic fractions associated with her M protein preparation were much more rapidly destroyed than the type-specific M antigen. No further attempt was made, however, to determine the purity of the material obtained after such digestion. Our study demonstrates that, although most of the non-type-specific activity was destroyed by mild peptic digestion, the final
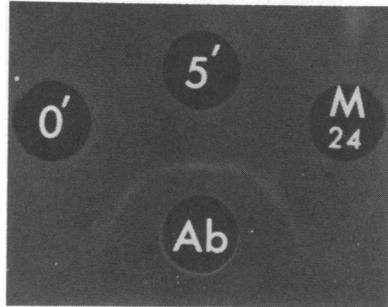

FIG. 3. *Agar gel immunodiffusion analysis of untreated type 24 M protein (M24) and M24 treated 0 and 5 min with pepsin. The lower well (Ab) contains unabsorbed antiserum from rabbits immunized with whole heat-killed type 24 streptococci. The concentration of M protein in each of the peripheral wells was 1 mg/ml.*
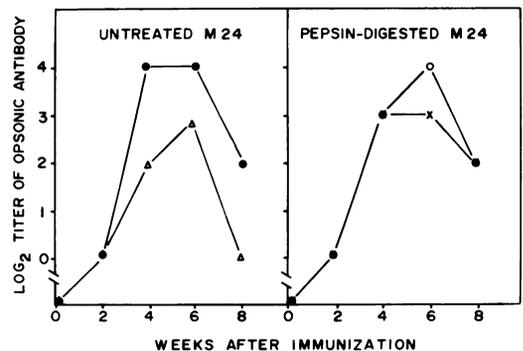

FIG. 4. *Type-specific opsonic antibody responses of rabbits immunized with untreated (● and △) or pepsin-digested (○ and ×) M24.*

material still contained some residual non-type-specific activity which could be detected only by the more sensitive complement fixation test. Moreover, this residual NTSM associated with TSM was capable of eliciting a weak non-type-specific antibody response in the rabbits immunized with the pepsin-treated M protein; these antibodies could be detected only by CF tests (unpublished observations). Tests to further identify the components of NTSM (3) involved in these reactions were not undertaken in the present study.

The gel filtration analysis, however, revealed that the pepsin-treated M protein contained a fraction rich in TSM which had no detectable NTSM by the qualitative methods employed. Further attempts have been made, therefore, to define the peptic digestion conditions whereby TSM might be extracted directly from intact streptococci (E. H. Beachey, et al., in press). From the evidence presented in the present paper, it was postulated that such enzymatic extracts of whole organisms should be rich in TSM but contain virtually no, or only minimal amounts of, NTSM to begin with as compared to conventional extracts.

ACKNOWLEDGMENTS

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