INFECTION AND IMMUNITY, Sept. 1975, p. 679-686

Vol. 12, No. 3
Printed in U.S.A.

Guanidine Extraction of Streptococcal M Protein

HAROLD RUSSELL* AND RICHARD R. FACKLAM
Center for Disease Control, Atlanta, Georgia 30333

Received for publication 10 February 1975

A new method of extracting M protein from streptococcal cell walls has been presented. The extracting agent was guanidine-hydrochloride, a protein denaturant. The crude guanidine extract was further purified by ammonium sulfate and pH 5 fractionation and by hydroxyapatite column chromatography. Three major protein peaks were eluted from the hydroxyapatite column with 0.01, 0.1, and 0.3 M phosphate buffer, respectively. Protein fractions eluted at 0.1 and 0.3 M phosphte concentrations contained antigens that precipitated with homologous M-protein specific antisera, whereas the 0.01 M phosphate fraction had no immunological activity. The fraction eluted with 0.3 M phosphate was electrophoretically homogeneous in sodium dodecyl sulfate-acrylamide gels and elicited the production of bactericidal antibodies in rabbits. The 0.1 M phosphate buffer eluant was electrophoretically heterogeneous and did not elicit the production of bactericidal antibodies in rabbits.

Streptococcus pyogenes, Lancefield's group A streptococcus, can cause pharyngitis and impetigo, and, in a small percentage of cases, acute rheumatic fever or acute glomerulonephritis may follow (9). M proteins, constituents of the group A streptococcus, play a major role in the pathogenicity of the organism. They impede phagocytic destruction of group A streptococci in susceptible hosts. Antibodies specific for M protein types will opsonize homologous group A streptococci and lead to their phagocytosis in vivo and in vitro. The M protein antibodies produced in man and experimental animals are type specific.

Lancefield first demonstrated the extraction of M proteins from streptococcal cells with hot HCl in 1928 (11). Since that time, numerous attempts have been made to extract M proteins from streptococcal whole cells and cell walls by milder extraction methods. Milder extraction methods are favored in order to study the physical and chemical structure of M protein molecules.

This presentation describes a new method of extracting M protein from streptococcal cell walls. Modifications of the M-protein purification procedure introduced by Johnson and Vosti (10) will be also presented. Some of the immunological and physiological characteristics of the purified antigen are described.

MATERIALS AND METHODS

Bacterial strain. Lancefield's strain 12/27/1 (CDC-SS-635) of group A streptococcus, type M-12, was used in these studies. It was obtained from the Streptococcus Section of the Center for Disease Control (CDC), Atlanta, Ga. The strain was cultured in the presence of human blood to select an M-rich clone for large-scale cultivation (2).

Cultivation of bacteria. Organisms were cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with glucose, $NaHCO_3$, and Na_2HPO_4 as described by Fox (6). A colony was transferred from a blood agar plate to a tube containing 5 ml of broth and incubated for 8 h. Three milliliters of the 8-h culture was transferred to 30 ml of broth and incubated for 16 h. The latter culture was used as the inoculum for 300 ml of broth, which was subsequently used to inoculate 3 liters of broth (10%, vol/vol). The culture was incubated for 16 h at 35 C.

Cell walls. Cell walls were isolated by the method of Bleiweis et al. (3) with minor modifications. Washed bacteria from 20 liters of broth (40 g [wet weight]) were suspended in 200 ml of distilled water. Twenty-four milliliters of bacterial suspension and 24 g of size 12 glass beads (0.17 to 0.18 mm) (Bronwill Scientific, Rochester, N.Y.) were added to a 60-ml serum bottle. The stoppered bottles were shaken on a Braun homogenizer (Bronwill Scientific) at 4,000 oscillations/min with sufficient CO_2 delivered to the chamber to prevent heating. The cell walls were washed three times with cold distilled water and suspended in 0.01 M phosphate buffer, pH 7.3. The latter suspension was treated with ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) (10 μg/ml) and lyophilized.

Antigen extraction. Six grams (dry weight) of washed cell walls was suspended in 100 ml of 6 M guanidine-hydrochloride (Eastman Kodak Co., Rochester, N.Y.). The pH was adjusted to 2.0 with 5 N HCl. The suspension was mixed on a magnetic stirrer (Curtin Scientific Co., Houston, Tex.) for 30 min at 25 C. Then the pH was adjusted to 7.2 with 5 N NaOH, and the suspension was centrifuged at 15,000

$\times g$ for 20 min. The supernatant was set aside, and the sediment (cell wall debris) was extracted two more times with 100 ml of 6 M guanidine-hydrochloride as previously described. The cell wall debris was discarded after the third extraction. All supernatants were combined and dialyzed against running water for 14 to 16 h. During dialysis of the supernatant, a white precipitate formed. It was removed by centrifuging at 15,000 $\times g$ for 20 min. The supernatant, referred to as crude M-protein extract, was kept at 4 C until further fractionation. The sediment was discarded.

Ammonium sulfate fractionation. The crude M-protein extract was further fractionated by precipitation between 33 and 60% saturation with $(NH_4)_2SO_4$ as described by Lancefield and Perlmann (14). The fraction that was insoluble at 60% $(NH_4)_2SO_4$ saturation was dissolved in 0.02 M sodium phosphate buffer, pH 6.8, and desalted on a G-50 fine Sephadex column. The column was eluted with 0.02 M sodium phosphate buffer, pH 6.8. The desalted, 60%-saturated $(NH_4)_2SO_4$-insoluble fraction is referred to as the partially purified M protein extract.

pH 5.0 fractionation. The partially purified M protein extract was dissolved in 0.1 M sodium acetate buffer, pH 5.0. The mixture was centrifuged at 15,000 $\times g$ for 15 min to remove precipitated protein. The precipitate was washed with 0.1 M acetate buffer, pH 5.0, until the wash fluid read less than 0.01 optical density at 280 nm units in a Beckman spectrophotometer (Beckman Instruments, Fullerton, Calif.). The supernatant and all wash fluids were combined, adjusted to pH 7.2, and kept at 4 C until ready for fractionation with hydroxyapatite. This fraction is referred to as the pH 5-soluble crude M-protein extract.

Column chromatography. The method of column chromatography was that of Johnson and Vosti (10). The pH 5-soluble crude M-protein extract was dialyzed overnight against 0.01 M sodium phosphate buffer, pH 6.8. The dialyzed material (41 mg of protein in 10 ml of 0.01 M phosphate buffer) was applied to a hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) column (12.5 by 1.4 cm) that had been equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The bed volume of the hydroxyapatite column varied from 8 to 15 ml. The column was eluted with sodum phosphate buffer, of increasing molarity, by stepwise elution. Concentrations of 0.01, 0.1, and 0.3 M were used. Thirty fractions, 3 ml each, were collected after the introduction of each molar concentration of buffer. Absorbance of the effluent material was monitored at 280 nm on a LKB Uvicord II photometer (LKB Instruments, Inc., Rockville, Md.). The column was washed with each elution buffer until there was no absorbance of effluent at 280 nm. The column flow rate was regulated to 0.33 ml/mn by a LKB 12,000 Varioperpex peristaltic pump (LKB Instruments, Inc.). All column chromatography was performed at 25 C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis with a discontinuous buffer system was performed by the method of Davis (4). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a continuous buffer system was performed by the method of Maizel (16). All gels contained 7.5% polyacrylamide and were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.). All electrophoresis runs were made at 25 C.

Animal inoculations. Rabbits were inoculated with protein (500 μg to 1.6 mg) eluted from the hydroxyapatite column previously described. Antigens were mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) (1:1; vol/vol) and injected subcutaneously on the backs of rabbits at multiple sites. Three rabbits were utilized for each antigen preparation. The rabbits were bled at weekly intervals. Some of the rabbits received booster inoculations of antigen after 6 and 15 weeks of immunization. The minimum amount of protein administered to any of the rabbits was 500 μg. The maximum total dosage was 1.6 mg of protein per rabbit.

Indirect bactericidal tests. The opsonic capacity of antisera elicited by hydroxyapatite fractions in rabbits was measured by the technique of Lancefield (12).

Opsonization inhibition. The efficacy of certain hydroxyapatite fractions to neutralize bactericidal antibodies and allow M-rich group A streptococci to resist phagocytosis was measured by a modified method of Beachey and Cunningham (1). Antigen (0.2 ml) was mixed with 0.2 ml of antisera specific for type M-12 streptococci (CDC, Atlanta, Ga.). The mixture was incubated at 37 C for 30 min and kept at 4 C overnight (14 to 16 h). The mixture was then centrifuged at 3,000 $\times g$ for 15 min, and the supernatant antisera was used in the indirect bactericidal test to determine if bactericidal antibodies had been remove by precipitation or neutralized by antigen. Controls were run with mixtures of type M-12 antigen and heterologous antisera (M-14) to determine the type specificity of the antigen.

Protein determination. Protein was measured by the method of Lowry et al. (15). Human serum albumin was used as an arbitrary protein standard.

Immunological methods. Microagar gel diffusion tests were performed by the method of Wadsworth (21). Capillary precipitin tests were performed by the method of Swift et al. (18).

RESULTS

Antigen extraction. The total yield of guanidine-extractable protein from 6 g of cell walls was 437 mg. Approximately 75% of the total yield was released in the first extraction mixture, and the other 25% was released by two consecutive extractions, 20 and 5%, respectively. The crude guanidine extracts from streptococcal cell walls yielded positive capillary precipitin reactions and agar gel precipitin lines of identity with HCl extracts when reacted with M-12-specific antisera (Fig. 1).

The crude guanidine extraction method has been performed on group A streptococcal M types 1, 3, 4, 5, 6, and 11. Crude extracts yielded positive capillary precipitin reactions with the

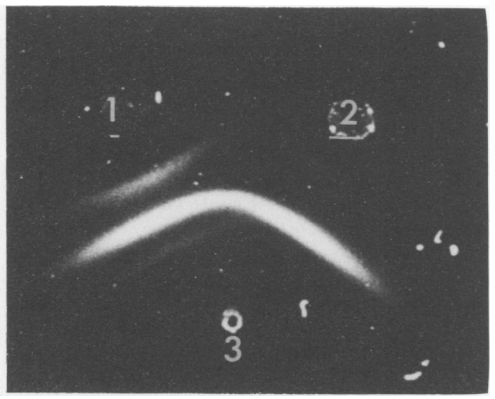

FIG. 1. *Gel diffusion pattern illustrating a line of identity between a guanidine extract and a hot HCl extract of group A M-12 streptococci. (1) Crude guanidine extract; (2) crude HCl extract; (3) group A M-12 antisera.*

corresponding M-specific antisera (Biological Reagents Branch, CDC, Atlanta, Ga.). However, this report deals only with the further fractionation and immunological properties of the M-protein antigen extracted from type M-12 cell walls.

pH 5.0 fractionation. A precipitate formed when the pH of the crude M protein extract was adjusted to 5.0. This was expected since the reported isoelectric point of several M proteins is about 5.3 (13). However, when the precipitate was washed with 0.1 M acetate buffer (pH 5.0) and resolubilized at pH 7.2, the resulting solution had no capillary precipitin or opsonic inhibitory activity when reacted with M-12-specific antisera. The pH 5.0-soluble fraction possessed the immunological activity. It gave a positive capillary precipitin reaction and had opsonic inhibitory activity. The precipitin activity is found in the pH 5-insoluble fraction of a hot HCl extract. The pH 5.0-soluble or -insoluble fractions under investigation did not react with streptococcal group A antisera.

Column chromatography. Three protein peaks, with maximum absorption at 280 nm, were eluted from the hydroxyapatite column. One protein peak was eluted for each concentration of phosphate buffer; the same elution profile was obtained with a continuous gradient.

Fractions were pooled and concentrated by lyophilization. The fractions were designated a, b, and c on the basis of elution pattern. Fraction a was eluted with 0.3 M phosphate buffer, fraction b with 0.1 M phosphate buffer, and fraction c, the portion of extract that did not adhere to the hydroxyapatite when washed, with 0.01 M phosphate buffer.

Fractions a, b, and c had absorbance ratios (280/260 nm) of 1.78, 1.5, and 1.1, respectively. The yield of total protein from the hydroxyapatite column was 24.6 mg or 60% of initial protein applied to the column. Fraction a contained 7.5% of the eluted protein; fraction b, 27.6%; and fraction c, 24%.

Polyacrylamide gel electrophoresis. Figure 2 shows that fraction a material (right) migrated as a homogeneous band when subjected to SDS-acrylamide gel electrophoresis, whereas fraction b material (left) migrated into two major and three minor bands.

Fractions a and b were also subjected to electrophoresis in a discontinuous buffer system (4). Fraction a migrated as two major and three minor bands (Figure 3). Fraction b migrated in several (approximately 12) bands distributed throughout the electrophoresis tube. Fraction c was not subjected to electrophoresis in either system.

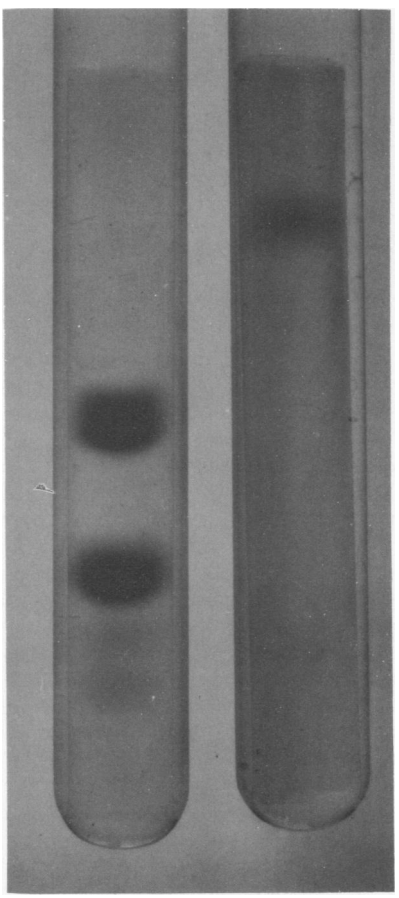

FIG. 2. *SDS-polyacrylamide gel electrophoresis of fraction a (right) and b (left) obtained by hydroxyapatite column chromatography. Sample migration was from cathode (top) to anode (bottom).*

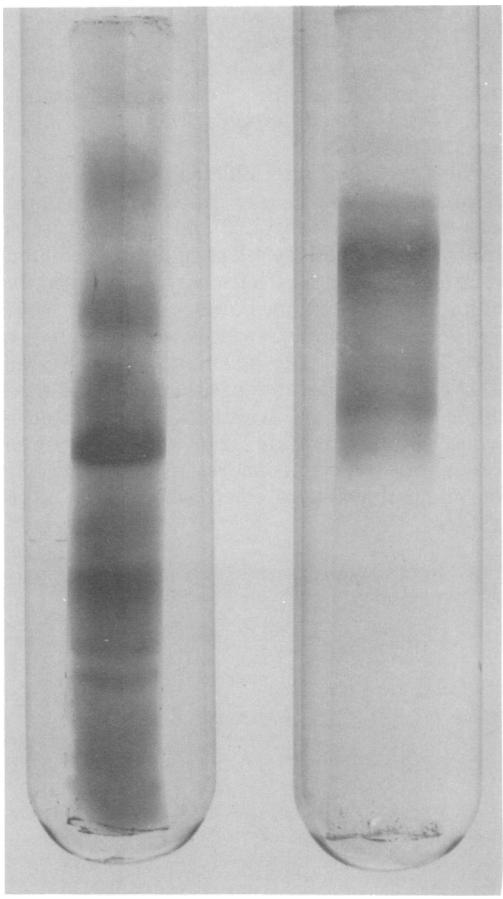

FIG. 3. *Discontinuous buffer system polyacrylamide gel electrophoretic patterns of hydroxyapatite fractions a (right) and (left).*

Immunogenicity. Table 1 presents bactericidal test results from antisera obtained after rabbits were immunized with the three fractions. The rabbits received an initial antigen dose of 100 μg of protein. They were given a 250-μg protein booster after 6 weeks and a 150-μg booster after 15 weeks. Two of the three rabbits immunized with fraction a produced bactericidal antibodies, which is an indication that fraction a contains M protein. Bactericidal antibodies to fractions b or c were not detected in sera of six rabbits immunized with these fractions.

Figure 4 illustrates the persistence of bactericidal antibodies in one of the rabbits immunized with fraction a. Bactericidal antibodies did not persist, but the rabbit showed a typical anamnestic response when it received an antigen booster. Strain 12/27/1 multiplies five times in each bactericidal test run in our laboratory. Two weeks after immunization fraction a antiserum inhibits only one generation of growth, but after 22 weeks total inhibition is observed.

Opsonization inhibition. Table 2 shows that fraction a had strong opsonic inhibitory activity for strain M-12 (homologous strain) and only a small amount of inhibitory activity for a heterologous strain (M-14). Fractions b and c show a small amount of inhibitory activity for strain M-12. The latter fractions were not tested with the heterogous strain. The same amount of protein (172 μg) from each hydroxyapatite fraction (a, b, and c) was added to CDC M-12 and M-14 antisera prior to the bactericidal test.

Immunological activity. Table 3 summarizes the immunological data obtained from fractions a, b, and c. Fractions a and b gave a

TABLE 1. *Detection of opsonic antibodies against group A M-12 streptococci by the indirect bactericidal test*

Inoculum plus:	Colony-forming units from 2-h 10^{-5} dilution (of strain 12/27/1) of:		
	1:4	1:16	1:32
Inoculum	174	39	12
Normal rabbit serum	Laked	2,000	620
CDC M-12 antisera	8	0	0
Rabbit anti-a serum	40	12	0
Rabbit anti-b serum	3,088	700	144
Rabbit anti-c serum	3,200	1,056	416

Rabbits were immunized with protein fractions obtained by hydroxyapatite chromatography.

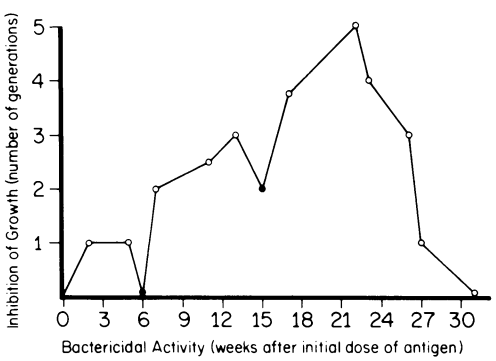

FIG. 4. *Rabbit immune response to M protein expressed as bactericidal activity of rabbit anti-hydroxyapatite fraction a serum. Note that approximately five generations of growth occur each time strain 12/27/1 (group A, M-12) is incubated in the presence of human blood for 3 h, i.e., standard conditions of the indirect bactericidal test which were used in our laboratory. An increase in number of generations inhibited indicates the presence of bactericidal antibodies.* ●, *Week of booster inoculations.*

TABLE 2. *Opsonic inhibitory activity of streptococcal M-12 hydroxyapatite fractions*

Inoculum plus:	Colony-forming units from 2-h 10^{-5} dilution (of streptococci) of:		
	1:4	1:16	1:32
Inoculum (M-12 strain)	108	68	32
Normal rabbit serum	Laked	5,400	1,216
CDC M-12 antisera	0	4	8
Fraction a + CDC M-12 antisera[a]	Laked	4,680	1,400
Fraction b + CDC M-12 antisera[a]	288	116	52
Fraction c + CDC M-12 antisera[a]	272	40	8
Inoculum (M-14 strain)	208	130	35
Normal rabbit serum	Laked	3,328	960
CDC M-14 antisera	12	4	4
Fraction a + CDC M-14 antisera[a]	244	28	24

[a] Streptococcal M-12 hydroxyapatite fractions (172 μg of protein) were added to CDC M-12 and M-14 antisera, and the resulting mixtures were used as antisera in the bactericidal test.

positive capillary precipitin reaction with CDC type-specific M-12 antiserum (produced with heat-killed whole cells, Biological Reagents Branch, CDC, Atlanta, Ga.). Fraction a yielded a weak precipitin reaction with CDC M-12 antiserum in a capillary precipitin test, whereas fraction b yielded a strong precipitin reaction. Fraction c had no precipitin, opsonic, or antigenic activity under the conditions tested. All capillary precipitin reactions were based on equivalent amounts of protein per test. Fractions a and b evoked strong capillary precipitin antibodies in rabbits. Figures 5 and 6 summarize the agar gel immunodiffusion results. A line of identity formed between fractions a and b when reacted with CDC M-12 antiserum (Fig. 5). However, the precipitating antibody in fraction a antiserum was not homologous to the precipitating antibody in CDC M-12 or fraction b antisera. Likewise, the precipitating antibody in fraction b antiserum was not homologous to CDC M-12 or anti-fraction a antisera (Fig. 6). These reactions remained consistent with Lancefield, guanidine, and "purified" antigens. We have what appears to be three separate precipitating activities. Only fraction a evoked bactericidal antibodies in rabbits, and only fraction a was able to neutralize the bactericidal activity in CDC M-12 or anti-fraction a antisera.

DISCUSSION

Isolation of purified M protein from group A streptococci is essential for further chemical and immunological analysis of the antigens. It is also important to use methods that do not result in the degradation of the molecule. Guanidine, a protein denaturant, was used in the present studies to extract M protein from type

TABLE 3. *Biological activity of hydroxyapatite fractions of guanidine-extracted group A streptococcus M-12 antigen*

Fraction	Precipitation[a] with M-12 serum	Antigenic properties[b]		Opsonic[c] inhibition
		Precipitating-ab	Bactericidal-ab	
a (0.3 M PO_4)	± (weak)	+	+	+
b (0.1 M PO_4)	+	+	−	−
c (0.01 M PO_4)	−	−	−	−

[a] Precipitation between each purified fraction and standard M-12 antiserum (whole cell-produced M-12 antiserum containing confirmed homologous precipitins).

[b] Precipitating antibody = antibody evoked in rabbits with each fraction and Freund complete adjuvant (+, positive reaction between the antibody and the homologous antigen). Bactericidal antibodies = antibody evoked in rabbits with each fraction and adjuvant (+, positive opsonization of M-12 streptococci).

[c] Opsonic inhibition = an inhibition of opsonic activity of M-12 antiserum (whole cell-produced M-12 antiserum containing confirmed opsonins).

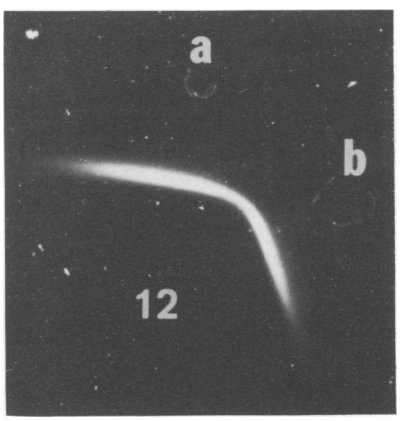

FIG. 5. *Gel diffusion pattern illustrating a line of identity between quanidine-extracted streptococcal type M-12 hydroxyapatite fractions a and b when reacted against CDC M-12 antisera. (12) CDC M-12 antisera; (a) hydroxyapatite fraction a; (b) hydroxyapatite fraction b.*

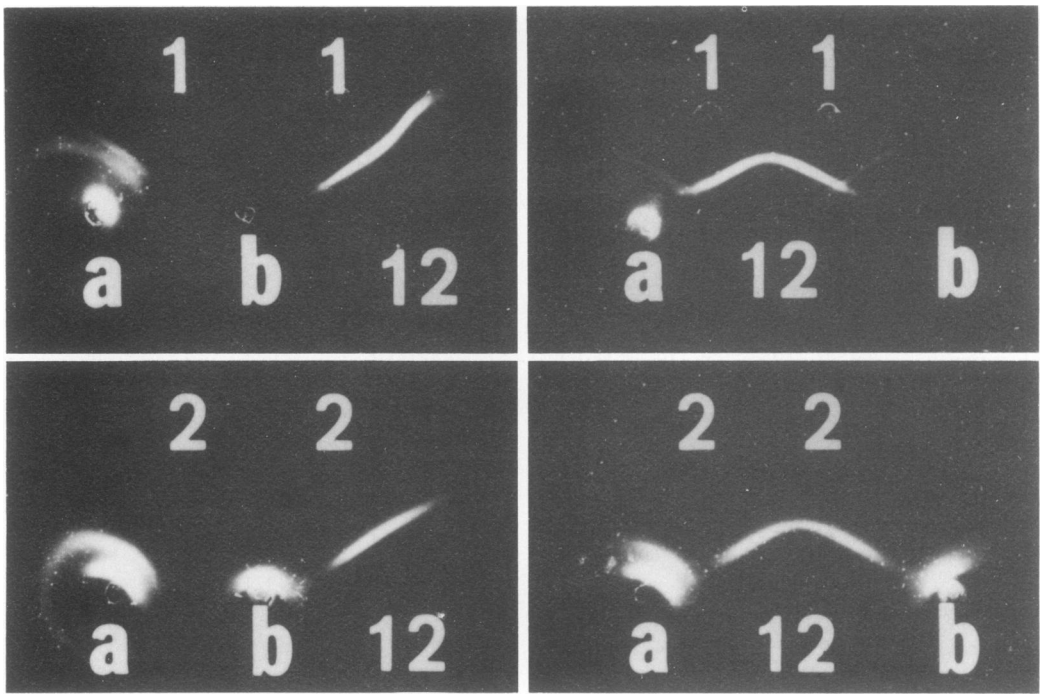

FIG. 6. *Gel diffusion patterns illustrating lines of nonidentity between the precipitating antibody in fraction a antisera and precipitating antibody in CDC M-12 and fraction b antisera (top left and right). The gel diffusion patterns (bottom left and right) represent lines of nonidentity between the precipitating antibody in fraction b antisera and precipitating antibody in CDC M-12 and fraction a antisera. (a) Fraction a antisera; (b) fraction b antisera; (12) CDC M-12 antisera; (1) fraction a (hydroxyapatite fraction); (2) fraction b (hydroxyapatite fraction).*

M-12 streptococcal cell walls. The protein denaturant is a strong electrolyte and dissolves most proteins readily. Electrostatic forces associated with proteins are minimal in the presence of this chemical, and it causes the polypeptide chains to form random coils (19). We postulated that if M protein is electrostatically bound to streptococcal cell walls, then elimination of most electrostatic forces would release the protein into the extracting medium. The use of guanidine was further justified since studies have illustrated that some guanidine-denatured enzymes (proteins) regain their native structure and biological activity when the denaturing agent is removed (5, 22).

It has not been established whether M protein is covalently or electrostatically bound to streptococcal cell walls (7). However, the work of V. A. Fischetti, E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie (Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, P242, p. 181) suggests that it is electrostatically bound, since they were able to extract it with a nonionic detergent. The present studies also suggest that M protein is electrostatically bound to cell walls since guanidine, a nonhydrolytic protein denaturant, was effective in extracting it from type M-12 streptococci.

The guanidine extraction procedure yields approximately the same amount of total protein as extracted by the Lancefield procedure. From 4 g of whole cells (wet weight), one guanidine extraction yields 3.8 mg of protein, whereas the Lancefield procedure extracts 4.1 mg of protein. The amount of precipitating antigen extracted is also very similar when compared by measuring the end point precipitating activity by diluting each extract and reacting the dilutions with CDC M-12 type-specific antiserum. It is difficult to compare our total yield of each fraction to the yields of other investigators since our product is different from that of others. Other investigators have not separated the biological activities; both fractions a and b have bactericidal as well as precipitating activities (20).

Although the isoelectric point of several types of M protein including type 12 is reported to be about 5.3 (13), protein extracted from type M-12 cell walls with guanidine was readily soluble at pH 5 to 7. Type M-12 protein extracted by the HCl method is insoluble at pH 5. This is an

indication that the M protein product extracted with guanidine is different from the product extracted with hot HCl. However, both products react with M-12-specific antisera.

A single protein band after SDS-polyacrylimide gel electrophoresis is not the sole criterion of protein purity, but it is certainly one criterion. Fraction a had a single protein band upon SDS electrophoresis. This illustrates the homogeneity of the M protein fraction with respect to molecular weight. The latter fraction elicited the production of bactericidal antibodies in rabbits but gave a very weak capillary precipitin reaction with type M-12-specific antisera. Fraction a also had opsonic inhibitory activity, i.e., it neutralized bactericidal antibodies (opsonins) and allowed M-12 streptococci to resist phagocytosis in the presence of human blood. Although there were multiple protein bands upon electrophoresis of hydroxyapatite fraction b in a discontinuous buffer system (4), most of the bands yielded a positive precipitin reaction with M-12-specific antisera. Fraction a yielded two major protein bands upon electrophoresis in the discontinuous buffer system; however, neither band reacted in immunodiffusion tests with M-12 antisera but did react weakly in a capillary precipitin test. The appearance of more than one protein band is not readily understood but could be due to aggregation or minor charge differences on the protein molecules. Fraction c was immunologically inactive. These studies suggest, as others have suggested (1, 8, 17), that the portion of M protein responsible for antiphagocytic activity is immunologically different from that portion responsible for immunoprecipitin activity. Guanidine extraction of cell walls followed by $(NH_4)_2SO_4$ fractionation and hydroxyapatite chromatography appears to be a means of separating the two M protein moieties.

Our results indicate that M protein is extractable from group A streptococcal M types 1, 3, 4, 5, 6, 11, and 12 with guanidine. When the guanidine extract of M-12 cell walls is further fractionated, an electrophoretically restricted protein is obtained (fraction a). The protein elicits the production of bactericidal antibodies in rabbits and yields a weakly positive capillary precipitin reaction. In contrast, fraction b is electrophoretically heterogeneous and gives a strong capillary precipitin reaction with M-12 specific antisera. The results strongly suggest that the portion of M protein responsible for antiphagocytic activity has antigenic determinants specific for opsonizing antibodies and some determinants specific for immunoprecipitin antibodies. However, it appears that the portion of M protein responsible for immunoprecipitin activity has only antigenic determinants specific for precipitating antibodies and none that react with antiphagocytic antibodies. The present study demonstrates a method for isolating the two moieties or at the least isolation of the opsonogen.

ACKNOWLEDGMENTS

We wish to thank John F. Obijeski for performing the SDS-polyacrylamide electrophoresis and Laura R. Edwards for skillful technical assistance.

LITERATURE CITED

1. Beachey, E. H., and M. Cunningham. 1973. Type-specific inhibition of preopsonization versus immunoprecipitation by streptococcal M proteins. Infect. Immun. **8**:19-24.
2. Becker, C. G. 1964. Selection of group A streptococci rich in M-protein from populations poor in M-protein. Am. J. Pathol. **44**:51-60.
3. Bleiweis, A. S., W. W. Karakawa, and R. M. Krause. 1964. Improved technique for the preparation of streptococcal cell walls. J. Bacteriol. **88**:1198-1200.
4. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404-427.
5. Epstein, C. J., R. F. Goldberger, and C. B. Anfinsen. 1963. The genetic control of tertiary protein structure: studies with model systems. Cold Spring Harbor Symp. Quant. Biol. **28**:439-449.
6. Fox, E. N. 1964. Antigenicity of the M proteins of group A hemolytic streptococci. J. Immunol. **93**:826-837.
7. Fox, E. N. 1974. M proteins of group A streptococci. Bacteriol. Rev. **38**:57-86.
8. Fox, E. N., and M. K. Wittner. 1968. Antigenicity of the M proteins of group A hemolytic streptococci. IV. Cross-reactivity between serotypes. J. Immunol. **100**:39-45.
9. Ginsburg, I. 1972. Mechanisms of cell tissue injury induced by a group of streptococci: relation to poststreptococcal sequelae. J. Infect. Dis. **126**:419-455.
10. Johnson, R. H., and K. L. Vosti. 1968. Purification of two fragments of M protein from a strain of group A, type 12 streptococcus. J. Immunol. **101**:381-391.
11. Lancefield, R. C. 1928. The antigenic complex of *Streptococcus haemolyticus*. J. Exp. Med. **47**:91-103.
12. Lancefield, R. C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. J. Exp. Med. **106**:525-544.
13. Lancefield, R. C. 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89**:307-313.
14. Lancefield, R. C., and G. E. Perlmann. 1952. Preparation and properties of type-specific M antigen isolated from a group A type 1 hemolytic streptococcus. J. Exp. Med. **96**:71-82.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
16. Maizel, J. V., Jr. 1971. Gel electrophoresis of protein, p. 191-192. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press Inc., New York.
17. Ofek, I., S. Bergner-Rabinowitz, and A. M. Davies. 1969. Opsonic capacity of type-specific streptococcal antibodies. Israel J. Med. Sci. **5**:293-296.
18. Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing group A hemolytic streptococci by M precipitin

reactions in capillary pipettes. J. Exp. Med. **78**:127-133.
19. Tanford, C. 1968. Protein denaturation, p. 159-161. *In* C. B. Anfinsen, Jr., M. L. Ansor, J. T. Edsall, and F. M. Richards (ed.), Advances in protein chemistry, vol. 23. Academic Press Inc., New York.
20. Vosti, K. L., R. H. Johnson, and M. F. Dillon. 1971. Further characterization of purified fractions of M protein from a strain of group A, type 12 streptococcus. J. Immunol. **107**:104-114.
21. Wadsworth, C. A. 1957. A slide microtechnique for the immune precipitates in gel. Int. Arch. Allergy Appl. Immunol. **10**:355-360.
22. White, F. H., Jr. 1961. Regeneration of native secondary and tertiary structures by air oxidation of reduced ribonuclease. J. Biol. Chem. **236**:1353-1360.