# FIBRINOGEN PRECIPITATION BY STREPTOCOCCAL M PROTEIN*

## I. Identity of the Reactants, and Stoichiometry of the Reaction

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(Received for publication, February 1, 1965)

An active factor in streptococci which precipitated various mammalian plasmas and human and bovine fibrinogens was previously described (1). This fibrinogen-precipitating factor (FPF) was present in crude acid extracts of several strains of Group A streptococci. The identity of FPF and streptococcal M protein was suggested by: (*a*) absorption of M protein extracts with fibrinogen resulted in loss of precipitation with homologous anti-M antisera; (*b*) absorption of M extracts with homologous anti-M antisera resulted in loss of precipitation with fibrinogen; and (*c*) treatment of M extracts with pepsin or trypsin resulted in loss of precipitation with both anti-M antiserum and fibrinogen.

The present report describes experiments confirming the identity of FPF and M protein. Precipitates were prepared by reaction of human fibrinogen with a partially purified preparation of streptococcal M protein known to contain FPF. Immunization of rabbits with washed precipitates resulted in bactericidal, mouse protective, and "long-chain" antibodies directed against homologous streptococci. Immunoelectrophoretic analysis of sera from these rabbits was carried out to determine the presence of other plasma proteins in M fibrinogen (M fib.) precipitates. Finally, a rational stoichiometry of the reaction between M protein and fibrinogen was determined with radioactively labeled reactants.

### *Materials and Methods*

*Streptococci.*—Two strains of Type 1 streptococci were used. Strain T 1/155/4[1] was stored in the lyophilized state, cultured as needed in Todd-Hewitt broth, and serially passed through mice to enhance or maintain virulence. The $LD_{50}$ of T 1/155/4 was between five and ten organisms and only occasional mouse passage was required to maintain this level; this strain was employed in bactericidal, mouse protection, and "long-chain" tests for type-specific antibody. Strain T 1/2788 was obtained as phenolized bacterial cells,[2] which were washed five times in saline before use. One Type 12 strain, SF42,[1] was stored in the lyophilized state and used to

* This investigation was supported by grant HE-07288 from the United States Public Health Service.

‡ Research Career Development Awardee of the United States Public Health Service.

[1] Obtained through the courtesy of Dr. Rebecca C. Lancefield of The Rockefeller Institute.

[2] Kindly supplied by Merck, Sharpe, and Dohme Laboratories, West Point, Pennsylvania.

test type specificity of antisera. Virulence was enhanced or maintained by mouse passage when necessary.

*Preparation of Streptococcal M Protein.*—Partially purified M protein was prepared from both strains of Type 1 streptococci by the method of Lancefield and Perlmann (2). Boundary electrophoresis of these preparations at pH 8.6 revealed a single peak with sufficient boundary spreading to suggest some heterogeneity. No attempts at further purification were made.

*Preparation of Fibrinogen.*—Human fibrinogen (lot 18)[3] was purified by the method of Laki (3); purified material contained 93 per cent clottable protein and was used in 0.15 M NaCl buffer at pH 7.2.

*Isotopic Labeling of Proteins.*—Fibrinogen and streptococcal M protein were lightly labeled with $I^{131}$ according to the method described by Biozzi, *et al.* (4). To 40 mg of protein solution kept at 0 to 5° C, 0.2 ml of 0.5 per cent $Na_2CO_3$ and 0.8 ml of $KI_3$ solution cntaining 0.6 mc of $I^{131}$ were added with stirring. The $KI_3$ solution contained 123 mg per cent $I_2$ (w/v) and 157 per cent KI (w/v). After 1 hour, the solution was dialyzed in the cold for 48 hours until negligible amounts of radioactivity appeared in the dialysate. The amount of iodine bound to protein was calculated to be between 1 and 2 atoms per molecule. No change in ability of labeled fibrinogen (fib.*) preparations to clot was observed during the period used. Precipitin reactions of labeled M protein (M*) with homologous antiserum remained unimpaired. Radioactivity was assayed in a well type scintillation counter.

*Protein Determination.*—Concentrations of protein solutions were determined by micro-Kjeldahl analyses except for radioactive solutions or precipitates; these were measured by the Lowry modification of the Folin-Ciocalteu method using human serum albumin as a standard. Details of stoichiometry experiments are described with the results.

*Immunological Techniques.*—Precipitin tests were performed with the capillary tube technique (5). The detailed method used for indirect bactericidal tests has been described previously, (6) and summarized below. A *normal* human serves as the donor; *i.e.* donor blood alone will not inhibit the growth of the inoculated microorganisms. To 0.05 ml of serum from the animal to be tested, 0.3 ml of normal, freshly drawn donor blood is added and 0.1 ml of organisms introduced. After rotation for 3 hours, a 0.1 ml sample is plated to determine the number of surviving bacteria; in the presence of a suitable donor and type-specific, anti-M antibody, there is a reduction in the number of bacteria. Conversely in the absence of type-specific antibody, the inoculum increases by a factor of 32 to 64. Positive and negative control sera were used in each test.

Lengthening of the streptococcal chain while growing in anti-M antibody has been described by Stollerman and Ekstedt (7). This phenomenon is sensitive to relatively small amounts of type-specific antibody. Details of the method have been described previously (8). Briefly, an inoculum of Type 1 organisms is placed in serum to be tested for anti-M antibody. After 3.5 hours incubation at 37°C, thirty chains are counted, the mean chain length and standard deviation calculated and compared with mean chain length of streptococci grown in normal rabbit serum. The procedures used in mouse protection tests are described with the experiments.

*Immunoelectrophoresis.*—A modification of Scheidegger's technique (9) for agar gel immunoelectrophoresis was employed. After washing in normal saline, the slides were dried and stained with amidoblack 10B by the method of Uriel and Scheidegger (10).

*Experimental Animals.*—New Zealand white, male rabbits weighing between 2 and 3 kg were used for all immunizations.

## RESULTS

*Production of Type-Specific Antibodies by Immunization with M Fibrinogen Precipitates.*—To 50 mg of purified fibrinogen dissolved in 10 ml of buffered

[3] Obtained from E. R. Squibb & Sons, New York, New York through the courtesy of the American Red Cross.

saline was added 3.0 ml of a 1 per cent solution of M protein. After stirring, the mixture was allowed to react at 4°C for 18 hours and the resulting precipitate washed five times with 40 ml of buffer. The precipitate was then resuspended in saline so that the suspension contained 4 mg/ml of protein by Kjeldahl analysis. The saline suspension was then emulsified in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit) and 0.5 ml of the emulsion injected into four sites in each of 2 rabbits. The supernatant fluid from the final wash of

TABLE I

*Indirect Bactericidal Tests with Sera from Rabbits Immunized with M-Fibrinogen Precipitates and Final Wash of Precipitates*

| Antigen used in immunization | Test mixture | | No. of colonies of Type 1 streptococci | | | |
|---|---|---|---|---|---|---|
| | Serum | Whole blood | Inoculum, after 3 hours' growth in test mixture | | | |
| | | | 500 | 174 | 42 | 16 |
| M-fibrinogen precipitates | Rabbit 1 | Normal human | ∞* | ∞ | 3250 | 1950 |
| | " 1 absorbed‡ | " " | 522 | 77 | 4 | 0 |
| | " 2 | " " | ∞ | ∞ | 2750 | 450 |
| | " 2 " | " " | 618 | 154 | 0 | 0 |
| Final wash of M-fibrinogen precipitate | Rabbit 3 | Normal human | ∞ | ∞ | 2650 | 640 |
| | " 3 absorbed‡ | " " | ∞ | ∞ | 2250 | 940 |
| | " 4 | " " | ∞ | ∞ | 3300 | 900 |
| | " 4 " | " " | ∞ | ∞ | 2100 | 760 |
| | | Normal human | | | | |
| None | Normal rabbit | | ∞ | ∞ | 3600 | 870 |
| | " " absorbed‡ | " " | ∞ | ∞ | 2500 | 1010 |

* Indicates innumberable colonies.

‡ Rabbit sera absorbed with 50 μg increments of human fibrinogen until no more precipitate. Equal fibrinogen increments added to normal rabbit serum and to serum from rabbits immunized with final wash of M-fibrinogen precipitate.

the precipitate was concentrated by lyophilization to 4 ml and then dialyzed *vs.* normal saline. After dialysis, 2 ml of the final wash fluid was emulsified in complete Freund's adjuvant and 0.5 ml of the emulsion injected into four sites of each of 2 rabbits. Two weeks later, all rabbits were given 0.2 ml intradermal injections of their respective antigens at four sites. Animals were bled 2 and 4 weeks after beginning of immunization. Serum was separated and tested for the presence of type-specific antibodies.

As shown in table I, none of the sera proved capable of imparting bactericidal properties to normal human blood. However, capillary precipitin tests with Type 1 M protein, 0.1 mg/ml, revealed moderate precipitates in serum 1 and 2. Precipitins for human fibrinogen were also present in these sera. Normal

human blood, used as a source of polymorphonuclear leukocytes in the bactericidal tests also contains fibrinogen; the probable presence of immune precipitates was therefore investigated as an inhibiting factor in the bactericidal tests. One ml aliquots of serum from rabbits immunized with M-fibrinogen precipitates was absorbed by serial addition of 50 $\mu$g amounts of dried fibrinogen until no further precipitation was noted. Equal fibrinogen increments were added to normal rabbit serum and to serum from rabbits immunized with final wash of M-fibrinogen precipitates. The ability of the absorbed sera from rabbits immunized with M-fibrinogen precipitates to impart bactericidal properties to normal human blood is shown in Table I. Failure of "absorbed" control sera to

TABLE II

*Specific Protection of Mice by Sera from Rabbits Immunized with M-Fibrinogen Precipitates*

| Serum | Group A streptococci used | Culture dilutions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ |
| | | Mouse No. | Mouse No. | Mouse No. | Mouse No. | Mouse No. | Mouse No. | Mouse No. | Mouse No. |
| | | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 |
| Rabbit 1 | T1/155/4 (Type 1) | 1 1 1 1 | 1 S S S | S S S S | S S S S | S S S S | ——— | ——— | ———* |
| | SF 42 (Type 12) | ——— | ——— | ——— | ——— | 2 3 2 2 | 2 2 2 2 | 2 2 2 2 | S 2 S S |
| Rabbit 2 | T1/155/4 (Type 1) | 1 1 1 1 | 1 2 S S | S S S S | S S S S | S S S S | ——— | ——— | ——— |
| | SF 42 (Type 12) | ——— | ——— | ——— | ——— | 1 1 1 1 | 1 1 1 1 | 1 2 1 S | 2 2 S S |
| Normal rabbit | T1/155/4 (Type 1) | 1 1 1 1 | 1 1 1 1 | 1 1 2 1 | 1 1 S 1 | 1 2 S S | S S S S | S S S S | ——— |
| | SF 42 (Type 12) | ——— | ——— | 1 1 1 1 | 1 1 1 1 | 1 1 1 1 | 1 2 3 S | 3 1 S S | ——— |

Numeral indicates day of death; S indicates survival for 10 days.
* Indicates dilution not tested.

produce bacterial effects in the test system rules out the possibility that specific antibodies were added with the absorbing fibrinogen preparation.

Mouse protection tests were performed with sera from rabbits 1 and 2 obtained 4 weeks after immunization. Swiss white mice, weighing 20 to 25 gm, were injected with 0.5 ml of serum intraperitoneally on the day before each challenge: control mice received normal rabbit serum. Mice were challenged intraperitoneally with 0.5 ml each of previously determined appropriate dilutions of 4- hour Todd-Hewitt broth cultures of the homologous type and one heterologous type of Group A streptococci. Table II shows that the immune serums protected mice against between 100 and 1000 $LD_{50}$'s of the virulent Type 1 streptococci.

To confirm the presence of M antibodies in the sera of immunized rabbits, growth of virulent Type 1 organisms in these sera was observed with reference

to chain length. The mean chain length and standard deviations obtained from the sera of 2 immunized rabbits are shown in Table III. The chain length of streptococci grown in these sera were significantly longer than in normal sera ($P < 0.001$). The results obtained with unabsorbed sera parallel those in the precipitin and bactericidal tests.

*Immunoelectrophoresis of Antisera Obtained by Immunization with M-Fibrinogen Precipitates.*—To determine if the washed precipitates produced by reaction of M protein and human fibrinogen contained other plasma proteins, the antisera produced in rabbits by immunization with these precipitates (*vide supra*) were studied by agar-gel immunoelectrophoresis.

TABLE III

*Comparison of Chain Length of Streptococcus T1/155/4 Grown in Serum from Normal and Immunized Rabbits*

| Rabbit serum | Antigen used in immunization | Chain length | |
|---|---|---|---|
| | | Mean* | Standard deviation |
| No. 1 | M-fibrinogen precipitates | 15.8 | ±8.1 |
| No. 2 | M-fibrinogen precipitates | 18.7 | ±10.2 |
| No. 3 | Final wash of M-fibrinogen precipitate | 4.6 | ±1.9 |
| Normal serum‡ | None | 4.1 | ±2.0 |

* Mean of 30 chains counted.

‡ Average of means derived from 9 normal rabbit sera. Each mean calculated on the basis of 30 chains counted.

To the top well of each slide was added normal human serum; normal human plasma was placed in the bottom well and a current of 6 ma/slide applied for 2 hours. Voltages varied from 260 to 210v. After electrophoresis, the center trough was cut into each slide and filled with antiserum from rabbits 1 and 2 respectively. After 24 hours' incubation at room temperature in a moist chamber, the slides were washed for 24 hours in saline, dried, and stained with amidoblack. Precipitin lines were never observed in the top portion of the slide, *i.e.* with human serum as shown in Fig. 1. Two lines close to each other were regularly seen in the bottom half of each slide. Both lines failed to develop when antisera absorbed with purified fibrinogen were used. The contamination of partially purified fibrinogen with plasminogen is well known, (11) and the second precipitin line is believed to represent such contamination. In no instance were any of the immunoglobulin fractions ($I_gA$, $I_gM$, $I_gG$) observed to produce precipitin lines with these rabbit sera.

*Stoichiometry of M-Fibrinogen Reactions.*—Experiments designed to determine the amount of precipitate produced by varying proportions of M protein

and fibrinogen were performed in a constant volume of 2.5 ml of 0.01 M phosphate-buffered saline. M protein was added to three sets of tubes containing

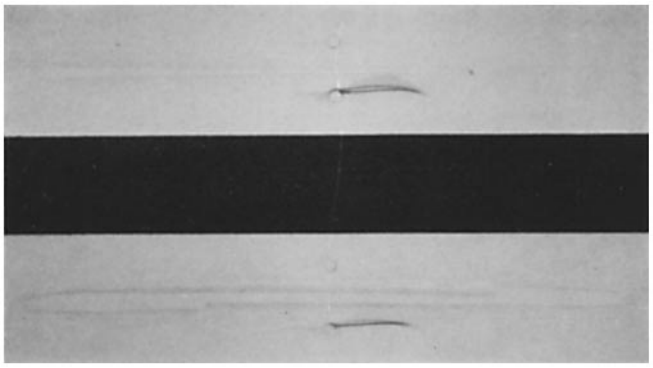

FIG. 1. Immunoelectrophoresis of human serum (top well, each slide) and human plasma, (bottom well, each slide) *vs.* rabbit antiserum 1 (top trough) and rabbit antiserum 2 (bottom trough).

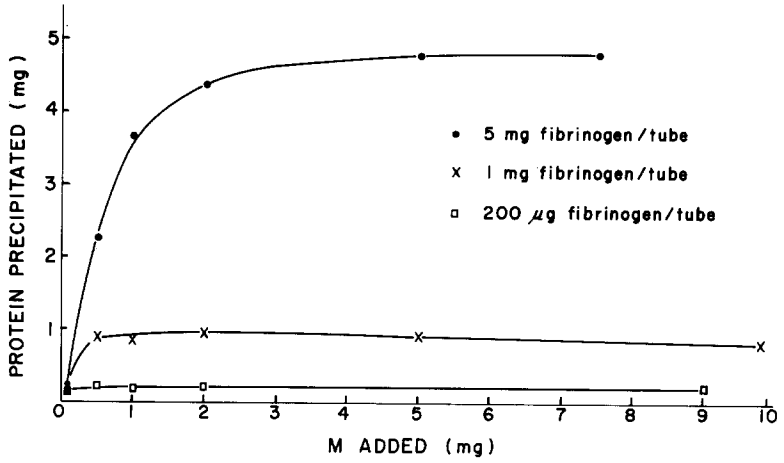


FIG. 2. Effect of addition of M protein to constant amounts of fibrinogen upon amount of protein precipitated.

0.2 mg/tube, 1.0 mg/tube, and 5.0 mg/tube of fibrinogen respectively. Reaction mixtures were incubated at 4°C for 24 to 28 hours. Tubes were then centrifuged at 3000 *G* in the cold, supernates removed, and precipitates washed three times with cold buffer.

The effect of addition of increasing amounts of M protein to constant amounts of fibrinogen upon the amount of precipitate is shown in Fig. 2. M/fibrinogen

ratios in these tubes varied from 1/50 to 50/1. With increasing amounts of M protein, the amount of protein precipitated increased to a plateau. Great excess of M protein failed to alter total protein precipitated at each fibrinogen concentration. The results suggested a stoichiometric reaction between M protein and fibrinogen wherein precipitation was limited by the concentration of one of the reactants, or a solubility product of continuously variable reactants. To resolve these possibilities, separate experiments were performed with either M protein or fibrinogen lightly labeled with $I^{131}$. As in the previous experiments, increments of M protein were added to constant amounts of fibrinogen (1 mg/tube). In addition to protein assay, the contents of M* or Fib.* in supernates and precipitates were determined by radioassay. Total protein and labeled

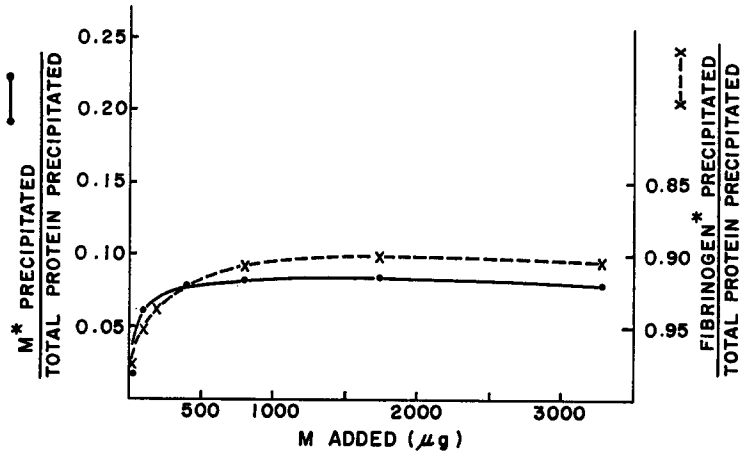


FIG. 3. Effect of addition of M protein to a constant amount (1 mg) of fibrinogen upon the ratio of reactants in the precipitate.

protein found in supernates and precipitates were required to be ±7 per cent of expected amounts.

In Fig. 3, the solid line indicates the fraction of protein in the precipitate which was identified as M protein and refers to the ordinate on the left; this fraction increased with increasing increments of M* protein until a plateau of 0.08 was reached. When labeled fibrinogen was reacted with unlabeled M protein, the fraction of fibrinogen in the precipitate is shown by the dotted line which refers to the ordinate on the right; this fraction decreased to a plateau of 0.9 with addition of increasing amounts of M protein. Results of both experiments are consistent with each other and illustrate two points; at low levels of M protein the precipitate is almost all fibrinogen, and a stable complex is reached which contains fibrinogen and M protein in a ratio of approximately

10:1. Amounts of protein precipitate were limited by concentration of reactants and no prozones were seen in either M excess or fibrinogen excess.

## DISCUSSION

The relationship of M protein to virulence and immunity in Group A streptococcal infections is well established (12). Presence of M protein upon the bacterial cell wall is a deterrent to phagocytosis and is believed to be the major mechanism of virulence of these organisms. A preliminary report (1) described the presence of fibrinogen precipitating factor in crude acid extracts of Group A streptococci which could not be separated from M protein. The work reported here confirms the identity of FPF and M protein by demonstration of type-specific anti-M antibodies in sera of rabbits immunized with M-fibrinogen precipitates. Whether the ability of M protein to react with fibrinogen plays a role in the resistance of M-containing streptococci to phagocytosis is unknown. Evidence against this possibility is the direct observation by Hirsch that human polymorphonuclear leucocytes phagocytose virulent streptococci in plasma (13).

Agglutination of hemolytic streptocci by plasma or fibrinogen was described by Tillett and Garner (14). It seems probable that this reaction was due to interaction of M protein on the bacterial cell surface and fibrinogen. Reduction or loss of agglutination was observed with organisms heated to 56°C for 1 hour. Although this treatment is not likely to have destroyed M protein, some is released from the cell wall (15); this soluble M protein might explain decreased agglutination by competition with bound M protein for available fibrinogen. Agglutination of bacteria tends to facilitate phagocytosis, not to impede it, which also suggests that the properties of M protein as a determinant of virulence, and as a fibrinogen-precipitating factor, are unrelated.

Rabbit antisera prepared by immunization with M-fib. precipitates failed to impart bactericidal properties to normal human blood. These sera were shown by precipitin analyses to contain anti-M and antifibrinogen antibodies. When the latter were removed by absorption with fibrinogen, the sera conferred excellent bactericidal properties when mixed with normal human blood. Rothbard clearly demonstrated non-specific inhibition of the bactericidal test by the presence of antigen-antibody precipitates (16). He established that polymorphonuclear leukocytes became engorged with particulate material and appeared unable to ingest bacteria. Mouse protection tests were not blocked by the presence of antifibrinogen antibodies in the rabbit sera; mouse plasma did not precipitate with rabbit anti-human fibrinogen, and *in vivo* the number of polymorphonuclear leukocytes is not limited as in the bactericidal test. Growth in long chains of streptococci was also unimpaired by the presence of antifibrinogen antibodies. In this case antigen was not present.

Precipitation with human sera by acid extracts of Group A, Type 15 streptococci has been reported by Wood and Schramm (17). The streptococcal

factor bore similarities to M protein, but the authors believed it to be a separate substance. Kaplan reported faint precipitation of normal goat $\gamma$-2 globulin by a high concentration (50 mg protein per ml) of an acid extract prepared from Group A streptococci (18). The immunoelectrophoretic data reported above demonstrate antibodies to two plasma proteins in the sera of rabbits immunized with M-fibrinogen precipitates. No precipitin reactions were observed with *serum* proteins; antiserum absorbed with fibrinogen failed to precipitate with plasma. Clearly, the M protein preparation used in these experiments did not react with any serum protein. The plasma protein contaminating fibrinogen is thought to be plasminogen.

Human fibrinogen is a large molecule (mol wt 330,000 to 400,000) which is precipitated from solution by a variety of non-specific agents including heparin, protamine, and tosyl-arginine-methyl-ester (TAME) (19–22). Although the mechanism of aggregation of fibrinogen molecules by these agents or in the clotting process is complicated and unclear, alteration of hydrogen bounds appear necessary (11). In the course of conversion of fibrinogen to fibrin, at least two polypeptides, A and B, are enzymatically split off. Reaction with M protein does not result in release of these polypeptides (1). Acid-extracted M protein has a molecular weight of approximately 40,000 and a pK of 5.3 (22, 2). This isoelectric point is comparable to that of many serum proteins; it seems unlikely therefore that fibrinogen precipitation is due only to extremes of pK exhibited by M protein.

With increasing addition of M protein in fibrinogen solution, a constant amount of precipitate is reached, limited in amount by the starting concentration of fibrinogen and constant in M-fibrinogen proportion of approximately 1:10. The molar ratio of reactants is therefore close to 1.0. Although the mechanism of fibrinogen precipitation by M protein is unknown, alteration of surface charge on the fibrinogen molecule permitting aggregation is most likely. Extraction of M protein from M-fib. precipitates by high ionic strength salt solutions has not proven successful thus far.

The biological significance of a reaction between bacterial antigen and host plasma protein in a setting of clinical infection is unknown at present. Evidence indicating that M protein forms intravascular complexes with fibrinogen which lead to structural and functional renal lesions in experimental animals is presented in the paper that follows.

## SUMMARY

Evidence confirming the identity of fibrinogen-precipitating factor and streptococcal M protein is provided by the demonstration of bactericidal, mouse protective, and long chain–producing antibodies in the sera of rabbits immunized with washed M-fibrinogen precipitates. Two precipitin lines were observed in immunoelectrophoresis of human *plasma vs.* rabbit anti–M-fibrinogen

antiserum; no precipitin reactions were observed between human *serum* and rabbit anti-M-fibrinogen antiserum. The reaction between M protein and fibrinogen was stoichiometric; a constant equimolar ratio of reactants was observed in precipitates formed with a wide range of M protein concentrations.

## BIBLIOGRAPHY


1. Kantor, F. S., and Cole, R. M., A fibrinogen precipitating factor (FPF) of Group A streptococci., *Proc. Soc. Exp. Biol. and Med.*, 1959, **102,** 146.
2. Lancefield, R. C., and Perlmann, G., Preparation and properties of type-specific M antigen isolated from a Group A, type 1 hemolytic streptococcus., *J. Exp. Med.*, 1952, **96,** 71.
3. Laki, K., *in* Blood Clotting and Allied Problems, New York, Josiah Macy Jr. Foundation, 1951, 223.
4. Biozzi, G., Benacerraf, B., Stiffel, C., Halpern, B. N., and Mouton, D., Influence de la Quantité d'iode fixée sur les protéines sériques normales et modifies par la chaleur sur la phagocytose de ces solloides par les cellules du S.R.E., *Ann. Inst. Pasteur*, 1957, **92,** 89.
5. Swift, H., Wilson, A. T., and Lancefield, R. C., Typing Group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78,** 127.
6. Lancefield, R. C., Differentiation of Group A. streptococci with a common R antigen into three serological types, with special reference to the bactericidal test, *J. Exp. Med.*, 1957, **106,** 525.
7. Stollerman, G. H., and Ekstedt, R., Long chain formation by strains of Group A streptococci in the presence of homologous antiserum: a type-specific reaction., *J. Exp. Med.*, 1957, **106,** 345.
8. Kantor, F. S., and Cole R. M., Preparation and antigenicity of M protein released from Group A, type 1 streptococcal cell walls by phage-associated lysin., *J. Exp. Med.*, 1960, **112,** 77.
9. Scheidegger, J. J., Une micro-methode de l'immunoelectrophorese, *Internat. Arch. Allergy and Appl. Immunol.*, 1955, **7,** 103.
10. Uriel, J., and Scheidegger, J. J., Electrophoreses en gelose et coloration des constituants, *Bull. Soc. Chim. Biol.*, 1955, **37,** 165.
11. Laki, K., and Gladner, J. A., Chemistry and Physiology of the fibrinogen-fibrin transition, *Physiol. Rev.*, 1964, **44,** 127.
12. Lancefield, R. C., Current knowledge of type-specific M antigens of Group A streptococci., *J. Immunol.*, 1962, **89,** 307.
13. Hirsch, J., personal communication.
14. Tillett, W. S., and Garner, R. L., The agglutination of hemolytic streptococci by plasma and fibrinogen, *Bull. Johns Hopkins Hosp.*, 1934, **54,** 145.
15. Heidelberger, M., and Kendall, F. E, Protein fractions of a scarlatinal strain of streptococcus hemolyticus, *J. Exp. Med.*, 1931, **54,** 515.
16. Rothbard, S., Bacteriostatic effect of human sera on group A streptococci. III. Interference with bacteriostatic activity by blockage of the leukocytes, *J. Exp. Med.*, 1945, **82,** 119.

17. Wood, H. R., and Schramm, H., Precipitin reaction obtainable between human serum and extracts of group A, type 15 streptococci, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 870.
18. Kaplan, M. H., and Suchy, M. L., Immunologic relation of streptococcal and tissue antigens: II. Cross reaction of antisera to mammalian heart tissue with a cell wall constituent of certain strains of Group A streptococci, *J. Exp. Med.*, 1964, **119,** 634.
19. Godal, H. C., Precipitation of human fibrinogen with heparin, *Scand. J. Clin. and Lab. Invest.*, 1960, **12,** 56.
20. Mylon, E., Winternitz, M. D., and deSütö-Nagy, G. J., The determination of fibrinogen with protamin, *J. Biol. Chem.*, 1942, **143,** 21.
21. Abildgaard, U., and Godal, H. C., Aggregation of fibrin and fibrinogen by TAME, *Scand. J. Clin. and Lab. Invest.*, 1964, **16,** 531.
22. Pappenheimer, A. M. Jr., Williams, J. W., and Zittle, C. A., The antigenic structure of hemolytic streptococci of Lancefield group A. IX. Some physical properties of the M proteins, *J. Immunol.*, 1942, **43,** 61.