TOXIC EFFECTS OF STREPTOCOCCAL M PROTEIN ON PLATELETS AND POLYMORPHONUCLEAR LEUKOCYTES IN HUMAN BLOOD*

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Immunization against Group A streptococcal infections has been hampered by toxic properties of preparations of M protein employed as vaccines (1, 2). Intradermal injections of purified preparations of M protein in man have produced local inflammatory responses resembling cell-mediated reactions (1, 3-6). Because the reactions to both rare and common M serotypes occur with similar frequency and intensity (1), it has been suggested that the toxicity is due to impurities shared by many M protein preparations. Theoretically, therefore, hypersensitivity to the type-specific M antigen when isolated in pure form should be encountered only in the host specifically immune to the homologous type. This concept is also supported by the resistance to phagocytosis of the intact M-rich streptococcus in fresh, nonimmune human blood in vitro (7). In such preparations the surface of the organism appears nonreactive with platelets and white blood cells (8).

In our previous studies of delayed allergy to purified M protein in guinea pigs (2), we found it difficult to produce cell-mediated hypersensitivity that was entirely type specific. Such hypersensitivity could be demonstrated in young animals early in the course of the immune response but later, cross-reactivity to M protein of other serotypes was usually encountered. These cross-reactions were considered to be due to shared determinants among various serotypes of M protein (9-11) or, perhaps, to a trace contaminant of a potent toxic substance such as cell-wall mucopeptide (12, 13).

Subsequently, we have attempted to separate the M determinant from antigens responsible for the inflammatory reactions by absorption experiments with type-specific M antiserum (8). In the course of these studies it became apparent that highly purified M protein preparations have potent in vitro cytotoxic effects upon platelets and polymorphonuclear leukocytes (PMN).1

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1 Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; PRP, plate-rich plasma; THB, Todd-Hewitt broth.
Moreover, homologous type antiserum specifically absorbed these cytotoxic properties. This report describes these experiments and presents evidence to suggest that the toxic effects are due, at least in part, to a moiety of M protein which carries the type-specific M determinant.

Materials and Methods

Streptococci.—The strains of Group A streptococci used in this study included M Types 6 (S43), 12 (SF42), and 30 (D24) which were obtained from Dr. Rebecca Lancefield, The Rockefeller University, New York. In addition, an M 24 strain isolated from a patient with acute rheumatic fever and an M 56 strain from a patient with acute glomerulonephritis (14), were used. Stock cultures of each serotype were stored, lyophilized, or frozen (−65°C) in defibrinated sheep blood or in Todd-Hewitt broth (THB) supplemented with 20% normal rabbit serum.

Extraction and Purification of M Protein.—Streptococci rich in M protein were grown in 30–50 liter batches in THB, collected by continuous-flow centrifugation, washed in phosphate-buffered 0.9% NaCl, extracted with hot HCl, and purified by ribonuclease digestion and column chromatography as previously described (2). In addition to the methods previously described, purified M protein fractions were obtained by gradient elution from columns of carboxymethyl-cellulose according to the method of Fox and Wittner (15).

Titration of the Type-Specific M Antigen.—Serial twofold dilutions of M protein solutions were made in 0.02 M phosphate-buffered 0.9% NaCl at pH 7.4. Precipitin tests of each dilution were made with standard type-specific antisera obtained from The Communicable Disease Center, Atlanta, Ga., by the capillary tube method of Swift, Wilson, Lancefield (16). The reciprocal of the highest dilution of a 1 mg/ml solution of M protein producing a type-specific precipitate was designated the "M titer." The immunochemical purity of M fractions was also checked, as previously described (2), by double-diffusion tests in agar gel with homologous-type unabsorbed antisera. Antisera were produced in rabbits by immunization with whole, heat-killed Group A streptococci according to the method of Lancefield (17).

Human Blood.—Fresh blood samples were obtained by venipuncture from healthy volunteers and from the umbilical cord of infants during uncomplicated delivery of healthy mothers. The blood samples were immediately mixed with 10 units/ml of heparin to prevent clotting. All samples were used in tests within 2 hr after collecting. Cell-rich plasma rather than whole blood was used in some experiments and was obtained by allowing the erythrocytes in fresh, heparinized blood samples to sediment for 1 hr at room temperature (24°C) in the plastic collecting syringe, held in vertical position, needle-end at the top. The collecting needle was replaced with a needle bent U-shaped. While maintaining the syringe in the vertical position, the supernatant cell-rich plasma was carefully ejected through the bent needle into sterile plastic tubes.

Bactericidal- and Phagocytosis-Inhibition Tests.—In vitro, indirect bactericidal and timed phagocytosis tests for type-specific M antibody were performed as previously described (18, 19). In brief, bactericidal test mixtures consisted of 0.4 ml of fresh, heparinized human blood, 0.05 ml of a standardized suspension of phagocytosis-resistant streptococci, and 0.05 ml rabbit antiserum against the homologous M type. The mixtures were incubated at 37°C in sterile rubber-stoppered test tubes by tumbling end-over-end in a rotator apparatus at 8 rpm for 3 hr. The number of surviving colonies was estimated by preparing pour-plates in 5% sheep blood agar. Phagocytosis test mixtures were similar to those used in bactericidal tests, except that a greater number of streptococci were added (approximately 10 streptococcal units/leukocyte). The percentage of polymorphonuclear leukocytes containing ingested streptococci (per cent of phagocytosis) was estimated by microscopic examination of stained (Wright’s-Giemsa) smears.
prepared from a drop of the mixtures at 15, 30, and 60 min of incubation at 37°C in the rotator apparatus.

The inhibitory effect of M protein preparations on each of the above tests was studied by incubating anti-M antiserum with various concentrations of homologous or heterologous serotypes of purified M protein fractions for 30 min at 37°C. Any precipitates which may have formed were removed by centrifugation at 1200 g for 20 min. The supernatant antiserum was then added to the test mixture containing homologous-type streptococci as described above.

Platelet-Leukocyte Aggregation Tests.—The clumping and lytic effects of purified M protein on platelets and polymorphonuclear leukocytes were studied by examining stained smears prepared from fresh blood-M protein mixtures by bright-field light microscopy or by examining, by phase-contrast microscopy, a drop of cell-rich plasma incubated with M protein.

Platelet aggregation was also studied by the turbidimetric method of Born (20). Platelet-rich plasma (PRP) was obtained by centrifuging fresh heparinized (10 units/ml) human blood at 226 g for 10 min at room temperature. 1 ml samples were transferred with a plastic pipette into siliconized 10 × 75 mm glass cuvettes. The PRP samples were stirred magnetically by adding a small teflon-coated iron bar to each tube. At 0.5-1 min intervals, the optical density was measured in a Coleman Jr. spectrophotometer adjusted to zero OD with distilled water at 600 μm. Each substance to be tested was added in a volume of 0.05 ml while the PRP was being stirred. The optical density of platelet-poor plasma, obtained by centrifuging PRP at 1000 g for 15 min, was subtracted from each reading before plotting the results.

PMN-Migration Tests.—The migration of human blood PMN in the absence and in the presence of various concentrations of M protein was studied by the capillary tube method according to Bryant et al. (21). Test mixtures were prepared from 0.45 ml of heparinized blood and 0.05 ml of M protein dissolved in 0.02 M phosphate-buffered 0.9% NaCl at pH 7.4. Immediately after mixing, sterile glass capillary tubes (internal diameter, 0.8 mm) were filled to approximately 70% capacity. The tubes were then sealed with sterile modeling clay which had been coated with a thin layer of sterile silicone jelly. The sealed capillary tubes were centrifuged at 500 g for 10 min, mounted upright on lantern slide frames (Polaroid), and incubated vertically for 2 hr at 37°C. At the end of incubation, the migration of PMN above the origin was estimated with an ocular micrometer on an American Optical dissecting microscope.

RESULTS

Cytotoxic effects of M protein preparations were first encountered when the bactericidal test for type-specific anti-M antibody was employed to assay small amounts of M protein (see Materials and Methods). This method was employed to identify the type-specific M determinant in our preparations by its capacity to inhibit opsonization of homologous-type streptococci by M antibody. In the course of these experiments, it was noted that the addition of an excess of M protein to the system inhibited phagocytosis non-type specifically. Stained smears of the test mixtures showed extensive platelet-PMN clumping and destruction. Systematic studies of the effects of M protein on blood samples in the absence of antiserum were, therefore, undertaken.

Morphologic Effects of M Protein on Platelets and Leukocytes of Normal Human Blood.—The addition of purified type 24 M protein to fresh normal human bloods in concentrations of 0.1 μg/ml or greater regularly caused clumping of platelets and leukocytes. Higher concentrations fused and lysed aggregated platelets and caused vacuolization and “smudging” of PMN (Fig. 1 a). These
Fig. 1. Blood smear stained with Wright's-Giemsa prepared after incubating fresh human blood for 15 min at 37°C with (a) M 24, 10 µg/ml, or (b) Type 24 streptococci, 2 × 10⁴ colony-forming units/ml. × 2500.
Fig. 2. Platelet-PMN clumping effects of various concentrations of purified type 24 M protein mixed with cell-rich human plasma. (a) Control, cell-rich plasma without M protein, (b) M 24, 0.1 μg/ml, (c) 1.0 μg/ml, (d) 10.0 μg/ml. Phase-contrast, X 700.
morphologic changes in platelets and PMN were not observed in phagocytosis tests when samples of blood from the same donors were mixed and incubated with live Type 24 Group A streptococci (10^9/ml) of the same strain from which the M protein was extracted (Fig. 1 b). Live streptococcal strains of other M serotypes, also rich in M proteins and highly resistant to phagocytosis, similarly were shown to produce no apparent effect when mixed with these bloods. An unencapsulated, M protein-negative strain (Type 1 av) of Group A streptococci which were highly susceptible to phagocytosis, on the other hand, produced platelet-leukocyte clumps similar to those produced by purified M protein.

The clumping effects of various concentrations of purified M protein mixed with fresh, cell-rich plasma samples were observed by phase-contrast microscopy (Fig. 2). Platelet aggregation occurred within 5 min after mixing with M protein at concentrations between 0.1 and 100 μg/ml. In the range of approximately 0.1–1.0 μg/ml, the platelets were loosely aggregated and most, whether aggregated or free, were unchanged except for occasional vacuolation (Fig. 2 b and 2 c). At these low concentrations, PMN remained motile and morpho-

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2 Kindly supplied by Dr. Roger M. Cole, National Institute of Allergy and Infectious Diseases, Bethesda, Md.
logically intact. The degree of platelet clumping paralleled M concentration. Complete fusion and lysis were noted at 10 μg/ml or greater (Fig. 2d). At these higher concentrations PMN migrated toward the fused platelet clumps and a few PMN ingested deformed platelets at the periphery of the aggregates. The phagocytes then rapidly became vacuolated and lost motility.

The Effect of M Protein on the Migration of Blood PMN in Glass Capillary Tubes.—The sensitive method of the inhibition of leukocyte migration in glass capillary tubes was employed to estimate the effect of M protein on PMN motility semiquantitatively. The results of a typical migration experiment with serial dilutions of M 24 are depicted in Fig. 3. Purified M 24 at concentrations equal to, or greater than, 6.25 μg/ml completely inhibited PMN migration. At the higher concentrations (6.25 μg/ml or greater), precipitation of fibrinogen by M protein was observed, an effect which has been previously described (22, 23).

The same morphological and functional changes at comparable concentrations of M protein were observed in the bloods of six different normal adults and in umbilical cord blood from two normal infants. None of the above bloods contained homologous M antibody by opsonic (bactericidal) tests. Purified

![Graph showing comparison of PMN migration inhibitory capacities of three different M6 preparations.](image)

**Fig. 4.** Comparison of the PMN migration-inhibitory capacities of three different M 6 preparations. The M titers varied as follows: Lot-PB, 256; Lot-EB, 128; and crude HCl extract, 32 (See also Table I).
fractions of types 5, 6, 12, 30, and 56 M protein produced similar effects to those shown for M 24. The degree of toxicity varied with the M titer (see Materials and Methods) of each preparation.

The Relation of “Purity” of M Protein to Inhibition of PMN Migration.—The migration-inhibitory capacities of three different type 6 M protein fractions of varying degrees of purity were compared. The M titer of each fraction was determined by precipitin tests with the same lot of hyperimmune type-specific M 6 rabbit antiserum. The purity or specific activity of M protein preparations was defined in terms of the ratio of the reciprocal of the M titer to the protein concentration of the sample, expressed as a function of ultraviolet light absorption at 280 nm.

The degree of inhibition of PMN migration was directly proportional to the purity of the M protein preparation (Fig. 4 and Table I). Thus, the ratio of the M titer to the toxicity of each preparation of M 6 was constant (Table I).

Type-Specific Absorption of the PMN Migration-Inhibitory Effect of Purified M Protein.—To determine whether cytotoxicity was due to the type-specific M protein moiety itself, or to a toxic moiety such as mucoprotein which might have been co-purified along with the M determinant, we attempted type-specific absorption with homologous antibody. Purified types 6, 24, and 56 M protein were incubated for 30 min at 37°C with unabsorbed homologous or heterologous, hyperimmune rabbit antisera to Group A streptococci. Precipitates were removed by centrifugation and the supernatant solutions were tested in migration experiments. A typical migration experiment with antibody treated M 24 and M 6 is illustrated in Fig. 5. The capacity to inhibit PMN migration, as well as the capacity to precipitate plasma fibrinogen, were removed from the solution of each serotype of M protein treated with homologous, but not heterologous, antiserum (Table II). The neutralizing effect of antiserum could be demonstrated only after removing the type-specific precipitates from the M protein solutions. When left in suspension, the immune precipitates exhibited cytotoxic effects similar to those of untreated M protein.

### TABLE I
Comparison of PMN Migration Inhibition and Type-Specific Precipitating Capacities of Crude and Purified M 6

| Preparation of M 6 | Reciprocal of highest dilution producing | Type-specific precipitating (M titer) | 50% inhibition of migration (toxicity) | Ratio A:B |
|--------------------|-----------------------------------------|-------------------------------------|---------------------------------------|-----------|
| Crude acid extract | 32                                      | 16                                  | 2:1                                   |           |
| Purified (Lot-EB)  | 128                                     | 64                                  | 2:1                                   |           |
| Purified (Lot-PB)  | 256                                     | 128                                 | 2:1                                   |           |
The Effect of Enzymatic Digestion with Lysozyme or Trypsin on the Cytoxicity of M Protein.—Because of the recently reported cytotoxicity of the mucopeptide of streptococcal cell walls (13), the question arose whether or not a mucopeptide impurity might have been co-precipitated with the M protein in the above neutralization experiments. The cytotoxicity of streptococcal mucopeptide is abolished by digestion with lysozyme, but not trypsin (13). M protein, on the other hand, is very sensitive to trypsin but not to lysozyme. Samples of type 24 M protein were digested, therefore, with either lysozyme or trypsin and the digests were then tested for inhibition of leukocyte migration. Lysozyme digestion showed no effect whereas trypsin virtually abolished the PMN migration-inhibitory effect of purified M protein (Table III).

Turbidimetric Studies of the Platelet-Aggregating Effects of M Protein and
Cell-Wall Mucopeptide.—Mucopeptide isolated from Type 6 streptococci was sonicated with a Branson sonifier at 8 kc to achieve a stable suspension. The mucopeptide sonicate was then compared with purified M 6 in platelet-aggregation tests by the turbidimetric method. M protein and mucopeptide were tested at a final concentration of 50 μg/ml and compared with the aggregating effect of 2 μM/ml of adenosine diphosphate (ADP). ADP has recently been shown to be intimately related to the phenomenon of platelet aggregation (20, 24). ADP aggregated platelets immediately after mixing. M6 produced a similar final degree of aggregation after an initial delay of approximately 2 min. In contrast, mucopeptide produced no aggregation after 10 min (Fig. 6).

Preliminary studies suggest that the cytotoxic effects of M protein are mediated by serum factors. Fresh blood cells were washed twice in Tyrode's buffer and resuspended in (a) Tyrode's buffer, (b) fresh serum, or (c) heated serum. Each suspension was treated with M protein (100 μg/ml) for 30 min at 37°C. The cells were again washed in Tyrode's buffer and finally resuspended.

| TABLE II |
| — Type-Specific Absorption of Migration-Inhibiting Capacity of Purified M Protein with Streptococcal Antiserum |

| M protein | % inhibition of PMN migration after absorption with |
|-----------|---------------------------------------------------|
|           | Anti-24  | Anti-6  | Anti-56  | NRS* |
| Type 24   | 14       | 100     | 100      | 100  |
| Type 6    | 100      | 3       | 100      | 100  |
| Type 56   | 66       | 49      | 4        | 55   |

* NRS, normal rabbit serum.
† Concentration of M protein added to each absorbing serum.

| TABLE III |
| — The Effect of Trypsin or Lysozyme Digestion on the Migration-Inhibitory Capacity and Type-Specific Precipitin Reactivity of M 24 |

| M protein (1 mg/ml) treated with | M 24 precipitin titer* | % inhibition of PMN migration |
|---------------------------------|------------------------|------------------------------|
| Not treated                     | 256                    | 100                          |
| Trypsin (1%)                    | 0†                     | 7                            |
| Lysozyme                        | 256                    | 100                          |

* See Materials and Methods.
† Precipitin test with homologous antiserum, negative at 1 mg/ml of M protein after trypsin digestion.

3 Kindly supplied by Dr. Richard M. Krause, The Rockefeller University, New York.
in fresh plasma. Control suspensions not treated with M protein were included in each experiment.

PMN suspensions treated with M protein in Tyrode's buffer appeared unharmed and migrated normally when washed and resuspended in fresh plasma. PMN exposed to M protein in heated serum (56°C for 30 min) appeared normal morphologically before resuspension in fresh plasma. After resuspension in fresh plasma, however, they failed to migrate and stained smears revealed the characteristic morphologic changes already described.

![Graph showing aggregation produced in platelet-rich plasma by purified type 6 M protein, type 6 cell-wall mucoprotein, and ADP.](image)

**Fig. 6.** Aggregation produced in platelet-rich plasma by purified type 6 M protein, type 6 cell-wall mucoprotein, and ADP. The final concentration of M protein and mucoprotein was 50 µg/ml and that of ADP, 2 µM/ml.

**DISCUSSION**

The work reported here demonstrates the presence of a cytotoxic factor(s) which we were unable to separate from the type-specific M determinant by the purification methods employed. The toxic factor(s) could be removed from solutions of purified M protein by absorption with homologous type antiserum, only.

It is of interest that the M-anti-M complexes had to be removed by centrifugation before the cytotoxic effects could be prevented. The suspended M-anti-M precipitates remained toxic to platelets and PMN. The homologous M antibody, therefore, did not actually neutralize, but rather absorbed and removed, the toxic moiety along with the M determinant.

Because of this observation, the possibility was considered that the toxic moiety could have been a co-precipitated contaminant with potent inflammatory properties such as mucoprotein, even though we have consistently
found only minute traces of the carbohydrate cell-wall constituents in our purified preparations (2). Because trypsin digestion abolished the cytotoxic activity of our M preparations, and treatment with lysozyme had no effect, the probability that mucoprotein was involved in the reactions to purified M proteins seems unlikely. Jones and Schwab (13) demonstrated that the cytotoxic properties of streptococcal cell-wall mucoprotein was abolished by lysozyme but not by trypsin.

Despite the close association of the toxic moiety with the M determinant in purified M protein preparations, the intact surface of the M-rich streptococcus had no apparent effect on platelets and PMN in fresh blood samples. The extraction of M protein and the purification process may uncover common toxic determinants, perhaps buried in the M-associated, cell-wall fimbriae recently described by Swanson et al. (25). Antibodies to the uncovered antigens may be universally present in human blood but not previously demonstrated because of the emphasis upon type-specific immunity.

The ready recognition by blood phagocytes of so-called "M-negative" Group A streptococci may actually be due to antibodies to such hypothetical, common antigenic determinants which remain exposed in the absence of a fully formed M protein structure. The clumping of platelets and PMN produced by suspension in blood of the M-negative strain we employed suggests that the surface of such avirulent organisms contains substances with affinities similar to that of the toxic M moiety. In fact, previous studies (26) have demonstrated that even the bloods of colostrum-deprived piglets and germfree mice react readily with M-negative Group A streptococci to produce rapid phagocytosis and that thermostable and thermolabile serum factors are involved. The surface of avirulent strains of Group A streptococci, therefore, appear to be reactive with serum components that are present "naturally." The close association of such moieties with purified M protein may account for its reactivity with blood elements when removed from its "protected" state in the intact surface structure of the virulent organism.

The platelet-PMN reactions we have described present an additional manifestation of cytotoxicity to that previously observed in studies of delayed allergy to purified M protein in guinea pigs and man (2–6). Sensitized lymphocytes do not appear to be involved in the platelet-PMN reactions observed in whole blood in the present studies because (a) unbilical cord blood contains lymphocytes which, theoretically, are not yet sensitized to bacterial antigens and (b) infants have shown relative tolerance to intradermal injections of M protein (5). Reactions to purified M protein in cord blood were similar in intensity to those in adult blood.

Some investigators (27, 28) have presented evidence that components closely associated with the type-specific M determinant are involved in cross-reactions with host tissue antigens. Others have demonstrated an M-associated capacity
of purified preparations to precipitate plasma fibrinogen (22, 23) and to opacify horse serum (29). It appears, therefore, that the M determinant is associated with a complex protein or family of proteins which possess not only multiple antigenic determinants but varied biological activities.

It is noteworthy that some of the toxic properties of M protein are similar to those described for staphylococcal protein A (30, 31). Protein A has the unique capacity to interact with immunoglobulin G (IgG) by binding to the Fc portion of the molecule rather than to the antigen binding site (32). The complex of protein A and IgG can fix complement. Recently it has been reported that blood platelets are injured during the interaction of protein A in blood samples (31). The possibility that extracted M protein behaves similarly to protein A should be explored. Avid phagocytosis of M-negative streptococci, like that of staphylococci, might relate to surface proteins with such avidities for immunoglobulins.

SUMMARY

Purified M protein isolated from Group A streptococci produced cytotoxic reactions in normal human blood in vitro. In the presence of M antigen, platelets aggregated, fused, and lysed. Polymorphonuclear leukocytes (PMN) surrounded the platelet aggregates, then became highly vacuolated and lysed. In addition, PMN progressively lost their capacity to phagocytose unrelated bacteria and to migrate in glass capillary pipettes.

Platelet-PMN reactions were directly proportional to the type-specific precipitin reactivity of each M preparation and could be removed with homologous M antibody, only. Moreover, the reactivity of M protein was abolished by enzymatic digestion with trypsin, but not with lysozyme, strongly suggesting that cell-wall mucopeptide was not involved.

Preliminary studies showed that platelet-PMN reactions require heat-stable and heat-labile serum factors, presumably antibody and complement. It is suggested that cytotoxic determinants are uncovered by the extraction and purification process and are intimately associated with the type-specific M determinant, possibly in a molecular complex.

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