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Type-Specific Inhibition of Preopsonization Versus Immunoprecipitation by Streptococcal M Proteins

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Purified M protein of group A streptococci inhibits in vitro phagocytosis of bacteria nonspecifically when it is mixed directly with the test blood. We devised a method of preopsonization inhibition that avoids direct contact between the test blood and the opsonic inhibitory agent. Each of the M proteins tested by this method inhibited opsonization and phagocytosis of only homologous-type streptococci. Titration of the M antigen in various purified preparations demonstrated a clear dissociation between titers of type-specific precipitating M antigen and type-specific opsonic inhibitory M antigen. Since opsonization reflects protective antibody, assays of M protein based on reaction with opsonic antibody should reflect more accurately the presence of the type-specific M determinant involved in protective immunity against streptococcal infections.

The potency of streptococcal M protein vaccines is usually estimated by the capacity of purified preparations to (i) produce type-specific precipitates with M antisera or (ii) elicit type-specific opsonins upon injection into laboratory animals (10). The first method may be inaccurate, however, because type-specific immunoprecipitation may not necessarily reflect the presence of the antigen which is responsible for eliciting protective antibody; occasionally M antisera which contain high titers of type-specific precipitating antibody fail to opsonize homologous type streptococci (11). Conversely, a few antisera contain high titers of type-specific opsonizing antibody but fail to precipitate homologous M protein extracts (E. H. Beachey et al., Bacteriol. Proc., p. 76, 1970).

Although the second method (immunization of laboratory animals to elicit opsonic antibody) is more accurate, the procedure is cumbersome and time consuming and does not lend itself readily to studies of a large number of purified M protein fractions (10).

In our M protein purification studies we have sought, therefore, an additional, relatively simple, in vitro marker of the M determinant to insure the protective power of our purified M protein vaccines. This report describes a method of inhibition of streptococcal opsonization which we have devised to assay M protein preparations. It has enabled us not only to detect, but also to quantitate, the moiety of M protein which is responsible for eliciting type-specific opsonic (and presumably protective) antibodies against homologous streptococci. We believe this should facilitate more accurate in vitro assays of M protein preparations for their protective power as vaccines.

MATERIALS AND METHODS

Streptococci. The strains of group A streptococci used in this study included M types 6(S43) obtained from Rebecca Lancefield, The Rockefeller University, N.Y., an M24 strain isolated from a patient with acute rheumatic fever, and an M56 strain from a patient with acute glomerulonephritis (6). Stock cultures were stored lyophilized or frozen (−70 C) in sheep blood or in Todd-Hewitt broth (THB) supplemented with 20% normal rabbit serum.

Extraction and purification of M protein. Streptococci were grown in 30- to 50-liter batches in THB, sedimented by continuous-flow centrifugation, washed in phosphate-buffered 0.9% NaCl (PBS), extracted with hot HCl, and purified by ribonuclease digestion, gel filtration, and column chromatography as previously described (1). In addition, M protein was also prepared by extraction of streptococcal cell walls with M hydroxylamine by the method described by Fox and Wittner (5). The purified M protein preparations were stored lyophilized under desiccation at 4 C. Purified electrophoretic fractions were also eluted from polyacrylamide gel.

Titration of type-specific precipitating M antigen. Titration was performed on the type-specific precipitating antigen by testing serial twofold dilutions of M protein made in PBS with type-specific antisera obtained from the Communicable Disease Center, Atlanta, Ga., as previously described (2, 15). The immunochemical purity of M preparations was also checked, as previously described (1), by double diffusion tests in agar gel with homologous-type unabsorbed antisera. Streptococcal antisera were produced in rabbits by immunization with whole heat-killed group A streptococci by the method of Lancefield (9).

Phagocytosis inhibition tests. Indirect phagocytosis tests for type-specific M antibody were performed as previously described (2). In brief, phagocytosis test mixtures consisted of fresh heparinized (10 units/ml) human blood (0.4 ml), a standardized suspension of phagocytosis-resistant streptococci (0.05 ml; approximately 10 streptococcal units/leukocyte), and rabbit antiserum (0.05 ml) 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 rotating apparatus at 8 rpm. The percentage of polymorphonuclear leukocytes (PMN) containing ingested streptococci (percentage of phagocytosis) was estimated by microscopy examination of stained (Wrights-Giemsa) smears prepared from a drop of the mixtures at 15, 30, and 60 min.

The inhibitory effect of M protein fractions on opsonization and phagocytosis was studied in two ways. In the first method, anti-M antiserum was incubated with various concentrations of homologous or heterologous serotypes of purified M protein fractions for 30 min at 37 C. Any precipitates which formed were removed by centrifugation at 10,000 $\times$ *g* for 10 min. The supernatant antiserum was then added to the test mixture containing homologous type streptococci as described above. In the second method, log-phase phagocytosis-resistant streptococci in THB were adjusted to an optical density of 0.05 at 530 nm in a Coleman Jr. spectrophotometer. A sample (1 ml) of the adjusted suspension was centrifuged at 10,000 $\times$ *g* for 10 min, and the sedimented organisms were suspended in diluted anti-M antiserum, either unabsorbed or absorbed with protein as in the first method. After incubation for 15 min at 37 C and 15 min in an ice-water bath, the organisms were again sedimented and washed 3 times by centrifugation with ice-cold THB or PBS. A sample (0.05 ml) of this suspension was then added to 0.4 ml of fresh heparinized human blood, and the mixture was incubated in the rotating apparatus at 37 C. Smears were prepared at 15 and 30 min as described above. Hereafter the first method will be called direct opsonization inhibition and the second method will be called preopsonization inhibition.

Long-chaining inhibition tests. Streptococcal long-chain tests for anti-M antibody were performed by the method of Stollerman and Ekstedt (14). For inhibition studies, the highest dilution of anti-M antiserum that produced an optimal long-chaining effect of homologous M type streptococci was preincubated for 30 min at 37 C with various M protein preparations. To 0.2 ml of the absorbed antiserum was added 0.1 ml of a standardized suspension of M-rich streptococci, homologous with respect to antiserum type. The mixture was incubated at 37 C for 2 h. The mean length of 50 streptococcal chains was expressed as numbers of cocci per chain estimated by microscopy as previously described (14). The long-chain index was calculated by dividing the mean chain length of streptococci grown in unabsorbed or absorbed antiserum by the mean chain length in the presence of normal rabbit serum.

RESULTS

In preliminary experiments it was shown that the rate of phagocytosis of preopsonized streptococci in unsupplemented fresh human blood was similar to that of untreated streptococci added to fresh blood supplemented with homologous type M antiserum (Fig. 1). Normal rabbit serum and heterologous M antisera had no effect on phagocytosis, neither by the preopsonization nor the direct opsonization methods.

Type-specificity of the inhibition of pre- and direct opsonization by M protein. When M protein-absorbed antiserum was added directly to test mixtures containing homologous-type streptococci and fresh human blood, phagocytosis was not inhibited type-specifically (Fig. 2). The presence of excess heterologous M6 or M56 in the absorbed M24 antiserum produced inhibition equal to that of the homologous M24. The addition of these M proteins to fresh blood also inhibited phagocytosis of avirulent group A streptococci, *Bacillus subtilis* and *Escherichia coli* (data not shown). However, when the same absorbed antiserum was used to preopsonize type 24 streptococci, only the homologous type M protein inhibited opsonization and phagocytosis (Fig. 3). Similar type-specific inhibition of phagocytosis was observed in preopsonization inhibition tests with types 6 and 56 antisera and streptococci, respectively (Table 1). It should be noted also that preincubation of *B. subtilis*, *E. coli*, and M protein-negative streptococci with these M preparations did not inhibit their phagocytosis, suggesting that purified M protein does not enhance virulence of heterologous organisms as was suggested previously (8).

Type-specific M-anti-M reactions have also been demonstrated to mediate the formation of long chains which occurs when group A streptococci are grown in the presence of homologous M antisera (14). In agreement with preopsonization inhibition tests, the long-chaining effect of each M antiserum we tested was abolished by absorption with homologous, but not heterologous, serotypes of M protein (Table 2).

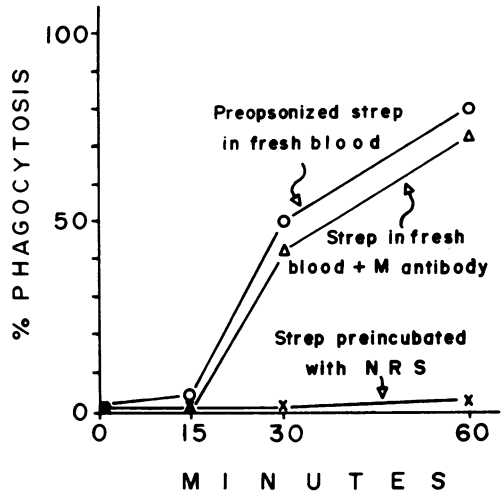

FIG. 1. *Comparison of rate of phagocytosis of type 24 streptococci in preopsonization and direct opsonization tests (see text).*

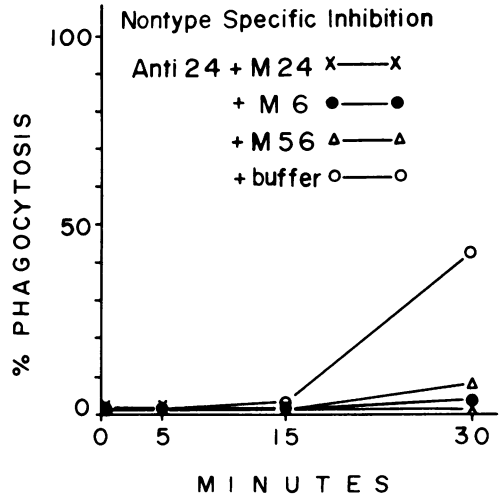

FIG. 2. *Non-type-specific inhibition of direct opsonization tests of type 24 streptococci by purified M proteins.*

Comparison of type-specific precipitating and opsonic inhibitory effects of M protein fractions. Four pooled fractions of type 24 M protein eluted from Sephadex G-75 were examined for type-specific reactivity in capillary precipitin tests with homologous, type-specific M24 antiserum. M precipitinogens were present in relatively high titers in the first two fractions (1:1024 and 1:128, respectively) and were absent in fractions III and IV (Table 3). In contrast, inhibitory activity (1:512 titer) was present only in the first of these four fractions, demonstrating a partial dissociation between type-specific M precipitinogens and opsonogens. A similar dissociation was observed in disk electrophoretic fractions of M protein eluted from polyacrylamide gel (Table 4). The first three of four polyacrylamide gel column segments contained relatively high titers (1:256, 1:1024, and 1:128, respectively) of M24 precipitinogens. However, only the second fraction, segment B, contained opsonic inhibitory activity.

Furthermore, type 24 M protein prepared by extraction at pH 10.0 with M hydroxylamine

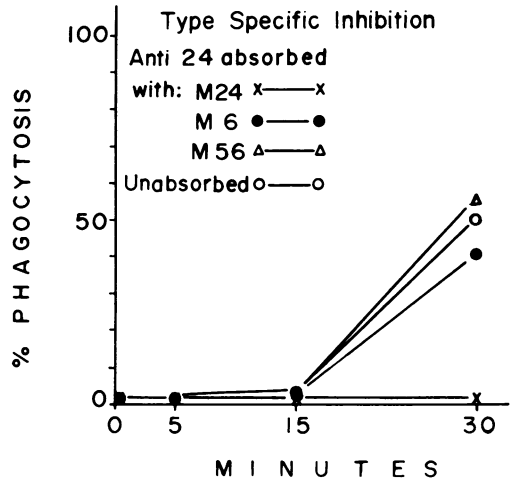

FIG. 3. *Type-specific inhibition by M24 of preopsonization of type 24 streptococci by type 24 M antiserum.*

TABLE 1. *Type-specificity of preopsonization inhibition by streptococcal M proteins*

Streptococci	Rabbit serum[b]	% Phagocytosis[a] in presence of:		
		M6	M24	M56
Type 6	Anti-6	*3*	55	66
Type 24	Anti-24	42	*1*	57
Type 56	Anti-56	52	50	*9*

[a] Expressed as number of neutrophiles per 100 counted cells containing one or more cocci.

[b] Partially purified M proteins at concentrations of 100 mg/ml were preincubated for 30 min at 37 C with antisera at a concentration of 100 mg/ml. Log-phase cultures of streptococci were treated with the absorbed antisera for 30 min at 37 C and 15 min at room temperature, washed twice with ice-cold Todd-Hewitt broth, and resuspended to a concentration of approximately 10^8 streptococcal units per ml. These suspensions (0.05 ml) were mixed with 0.45 ml of fresh heparinized (10 units/ml) human blood and rotated at 8 rpm at 37 C for 30 min. Stained (Wright-Giemsa) smears were examined by microscopy with oil immersion lens to estimate percentage of phagocytosis.

TABLE 2. *Type-specificity of long-chain inhibition by streptococcal M proteins*

Streptococci	Rabbit serum	Long-chain index[a] after absorption of antiserum with:			
		M6[b]	M24	M56	Unabsorbed
Type 6 (S43)	Anti-6	*1*	4	4	4
Type 24 (Vaughn)	Anti-24	5	*1*	5	5
Type 56 (VW)	Anti-56	16	13	*2*	16

[a] Long-chain index = Mean chain length of streptococci grown in antiserum/mean chain length of streptococci grown in normal rabbit serum.

[b] Partially purified M protein (100 μg/ml) preincubated with diluted antiserum for 30 min at 37 C.

TABLE 3. *Comparison of type-specific precipitating and opsonic inhibitory capacities of Sephadex G-75 fractions of type 24 M protein*

Fraction eluted from Sephadex G-75	Type-specific M antigen titer	
	Immunoprecipitation[a]	Opsonic inhibition[b]
I	1,024	512
II	128	2
III	2	2
IV	2	2

[a] Reciprocal of highest dilution of a solution (1 mg/ml) of protein producing a precipitate with absorbed, homologous-type antiserum.

[b] Reciprocal of highest dilution of a solution (1 mg/ml) of protein inhibiting the opsonic effect of unabsorbed type 24 antiserum diluted 1:64 with normal rabbit serum.

showed a relatively low precipiting antigen titer (1:16 as compared to 1:128 for an acid-extracted M24), but showed a high opsonic inhibition titer (1:64 as compared to 1:4 for the acid preparation; Table 5).

Reproducibility of preopsonization inhibition tests. The results of the averages of five opsonization inhibition tests performed on 5 different days with serial dilutions of partially purified hydroxylamine extract of M24 are depicted in Fig. 4. The half-maximal inhibitory dose obtained from this sigmoid dose-response curve is approximately 20 mg/ml. A two-way analysis of variance without replication of the normalized data of these five inhibition tests was performed as described by Sokol and Rohlf (13). There was no significant difference between the degrees of opsonization inhibition obtained at any given dilution of M protein tested on different days with different bloods. However, the differences in degree of phagocytosis with increasing concentrations on any given day were significant at the 0.01 level ($F = 3.71$, $Fs = 6.87$).

DISCUSSION

The data reported here demonstrate that M protein inhibits phagocytosis of group A streptococci non-type-specifically in direct opsonization tests, whereas it inhibits type-specifically in preopsonization tests.

In 1945 Rothbard (12) reported attempts to inhibit streptococcal opsonization by mixing M protein extracts with blood samples from patients who were convalescing from infections due to known serotypes of streptococci. He demonstrated that heterologous serotypes of M protein often inhibited phagocytosis to the same degree as did the homologous type. This non-type-specific inhibition was attributed to cross-reactive antibody that was present in the patients' sera and directed against non-type-specific substances of the M protein extracts.

TABLE 4. *Comparison of type-specific precipitation and opsonic inhibitory capacities of polyacrylamide disk electrophoretic fractions of type 24 M protein*

Elution fraction[a]	Type-specific M antigen titer	
	Immunoprecipitation[b]	Opsonic inhibition[b]
A	256	1
B	1,024	512
C	128	1
D	1	1

[a] Polyacrylamide gel column was cut into four equal segments immediately after electrophoresis. Segment A includes the origin. The fractions were eluted by incubating the segments for 18 h at 4 C in PBS.

[b] See footnotes, Table 3.

TABLE 5. *Comparison of opsonization inhibition and immunoprecipitation titers of acid and alkaline M 24*

M protein preparation	Type-specific M antigen titer	
	Immunoprecipitation[a]	Opsonic inhibition[a]
Hydroxylamine-M 24	16	64
HCl-M 24	128	4

[a] See footnotes, Table 3.

Vacuolization and smudging of PMN suggested that phagocytosis of such non-type-specific antigen-antibody complexes produced PMN injury, thereby blocking phagocytosis of both homologous and heterologous streptococci.

More recently, it was reported that streptococcal M protein extracts enhance the virulence not only of group A streptococci (7), but also of heterologous serogroups and other species of microorganisms (8). The latter investigators mixed their M protein extracts directly with fresh blood-streptococcus mixtures, as did Rothbard (12). They did not report, however, whether or not M protein produced toxic effects upon PMN in their test mixtures.

We have recently described toxic effects of purified M protein upon human blood platelets and PMN (2, 3; E. H. Beachey and G. H. Stollerman, in press). These effects appear to be mediated by antibody, widely distributed in normal human sera, which acts against a non-type-specific moiety of the purified M protein molecule (3; E. H. Beachey and G. H. Stollerman, in press). The presence of such antibodies in fresh human blood samples used in phagocytosis tests apparently accounts for non-type-specific inhibition of phagocytosis due to PMN injury when M protein is mixed directly with the blood. Therefore, PMN injury, rather than virulence enhancement, is the most likely explanation for the decreased phagocytosis of heterologous bacterial species observed in the present study.

The preopsonization inhibition test for type-specific M antigen described here avoids direct contact between M protein and fresh human blood. Streptococci preopsonized with M antibody were phagocytosed at the same rate as were streptococci added to fresh blood mixed with homologous type M antibody. Because excess M protein and M antibody are removed from the preopsonized streptococci before they are suspended in fresh blood, reduced phagocytosis reflects the effects of M protein on opsonic M antibody only.

A comparison of the opsonic inhibitory and type-specific immunoprecipitating activities of purified M preparations against the same M antiserum clearly shows dissociation between these two type-specific reactivities. Low titers of TSM opsonogens might account for the poor "protective" immunogenicity of some M protein preparations, even though they contain high titers of type-specific M precipitinogens (11).

The preopsonization inhibition test we have described should provide a more accurate tool for measuring, in purified vaccine preparations, the amounts of the type-specific antigenic determinant involved in protective immunity against infections with group A streptococci.

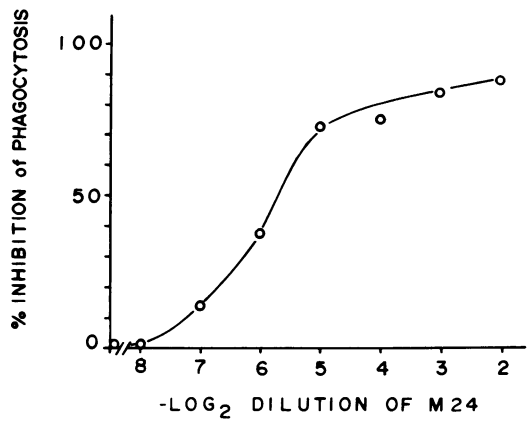

FIG. 4. *Titration of opsonic inhibitory activity of a purified hydroxylamine extract of type 24 M protein. The undiluted solution contained 1 mg of M protein per ml.*

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