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Biochemical and Biological Properties of the Binding of Human Fibrinogen to M Protein in Group A Streptococci

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Fibrinogen is known to bind to group A streptococci and precipitate with extracts containing streptococcal M protein. We have previously shown that the binding of fibrinogen to M-positive streptococci prevents opsonization by complement and protects the organism from phagocytosis in nonimmune blood. In the present study, we used ^{3}H-labeled fibrinogen, a highly purified peptide fragment of type 24 M protein (pep M24), and anti-pep M sera to show that (i) fibrinogen binds to M-positive streptococci with high affinity (dissociation constants, 1 to 5 nM); (ii) occupation of the high-affinity binding sites suffices to protect the organism from phagocytosis; (iii) proteolytic treatments that remove M protein from streptococcal cells abolish binding; (iv) binding is competitively inhibited by anti-pep M sera; (v) pep M24 precipitates fibrinogen; and (vi) binding to type 24 cells is inhibited by pep M24. We conclude that M protein is the cell surface structure principally responsible for binding fibrinogen on the surface of M-positive streptococci and that this binding contributes to the known antiopsonic property of M proteins.

The binding of fibrinogen to hemolytic streptococci was first noticed some 50 years ago when Tillet and Garner (31) reported that certain strains were agglutinated by plasma and by partially purified fibrinogen. More recently, binding of ^{125}I-labeled fibrinogen to 92 of 100 strains of group A streptococci was described by Kronvall and his colleagues (15, 16). Runehagen et al. in the same laboratory showed that fibrinogen degradation products containing one or both of the outer domains of the trinodular fibrinogen dimer (fragments X, Y, and D) bound to group A streptococci, whereas the central domain (fragment E) did not (26). Whitnack et al. have reported that the binding of fibrinogen to virulent group A streptococci of types 5, 6, 19, and 24 protects the organisms from opsonization by complement in nonimmune serum (33–35). Thus, the interaction of fibrinogen with group A streptococci has biological significance.

In 1959, Kantor and Cole (14) discovered that acid extracts of cells of many streptococcal M types would precipitate fibrinogen obtained from a number of mammalian species. Kantor subsequently determined by immunological techniques that the precipitates contained M protein (13), the molecule associated with resistance to opsonization and phagocytosis and with virulence (19). M protein is therefore the obvious candidate for a fibrinogen binding site on cells of virulent streptococci. In our previous studies, circumstantial evidence favored this hypothesis in that fibrinogen bound to the surface fibrillae of the streptococcal cells, known to be the location of the M protein (29), and it interfered with opsonization of streptococci by antisera to purified M proteins (34, 35). However, M protein may not be the only streptococcal structure that interacts with fibrinogen. ^{125}I-labeled fibrinogen has been reported to bind to strains presumed to be M negative (6). The binding of ^{125}I-labeled fibrinogen to one M-positive strain was trypsin resistant (16), precluding M protein as the receptor in the trypsin-treated cells of this strain. In addition, fibrinogen has been reported to bind to isolated T proteins of several serotypes (21, 27). These investigations therefore left the specificity of binding of fibrinogen to group A streptococci in some question; in fact, it has not been directly shown that M protein is the receptor on intact cells. Accordingly, we sought to characterize the binding of human fibrinogen to M protein-rich streptococci highly resistant to phagocytosis in vitro. In this report, we show that fibrinogen binds to a purified 33.5-kilodalton (kDa) fragment of type 24 M protein and present evidence that M protein is a high-affinity receptor for fibrinogen, occupation of which is sufficient to protect the organism from phagocytosis. The results support the hypothesis that the binding of fibrinogen contributes to the known antiopsonic function of streptococcal M protein.

MATERIALS AND METHODS

Streptococci. Group A streptococci were M and T typed by standard capillary precipitation and slide agglutination tests (20, 30). Strains of M serotypes 5 (Manfredo, T type 5/27/44) and 24 (Vaughn, T nontypable) were blood passed to a high level of resistance to phagocytosis. Cultures were stored at −80°C and subsequently in some cases on blood agar plates at 4°C. Organisms were subcultured to early log phase in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% normal rabbit serum (GIBCO Laboratories, Grand Island, N.Y.), harvested by centrifugation, exposed to UV light for 3 min, washed in phosphate-buffered saline (PBS; 0.15 M NaCl–0.02 M phosphate [pH 7.4]), and suspended in PBS to the desired optical density (33, 35). Neither strain was agglutinated by fibrinogen in concentrations ranging from 1.0 nM to 10 μM.

Streptococcal M proteins and antistreptococcal sera. A polypeptide fragment of type 24 M protein was extracted by limited peptic digestion of whole organisms and purified by ion exchange and gel filtration chromatography. The product, designated pep M24, was judged to be pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4). Preimmune rabbit sera and rabbit antisera to pep M proteins (4), purified group A streptococcal lipoteichoic acid (1), and whole streptococci of strain D58X, an M-negative derivative of type M3 (12), were obtained from laboratory files, as was an anti-pep M24 serum from a human volunteer

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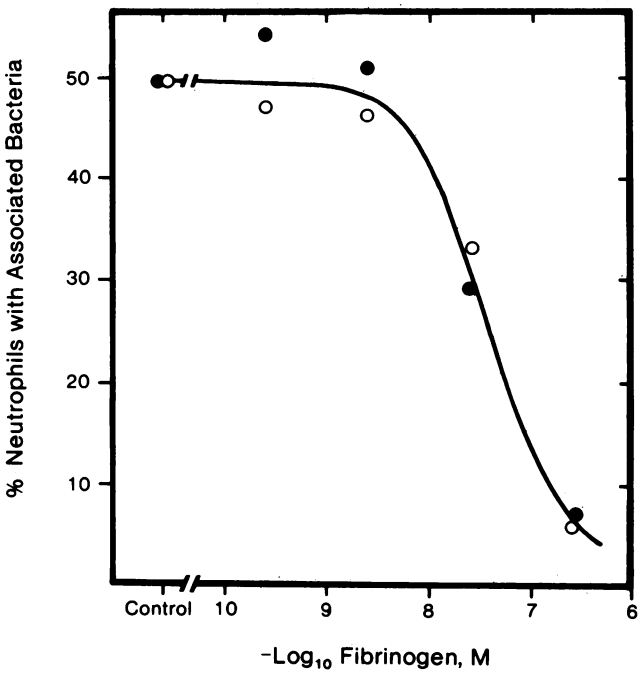

FIG. 1. Inhibition of phagocytosis of type 24 streptococci by unlabeled (●) and ^{3}H-labeled (○) fibrinogen. Phagocytosis was assessed by light microscopic scoring of neutrophils in the test mixtures.

(5). Anti-pep M sera were tested in an enzyme-linked immunosorbent assay for reaction with pep M proteins with a semiautomated enzyme immunoassay system (PR-50; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (2). Group A antisera (rabbit) were obtained from BBL Microbiology Systems, Cockeysville, Md. Anti-T sera were obtained from the Centers for Disease Control, Atlanta, Ga.

^{3}H-labeled and unlabeled fibrinogen. Human fibrinogen (grade L, 90% clottable; KABI Diagnostica, Stockholm, Sweden) was dissolved in PBS at a concentration of 3.4 mg of clottable protein per ml (10 μM), dialyzed against PBS to remove salts, and stored at −20°C. For tritium labeling, fibrinogen was dissolved in 0.2 M sodium borate (pH 9.2) at a concentration of 10 μM, dialyzed against the same buffer, and subjected to reductive N-methylation at 4°C with 0.1 volume of a 1% aqueous solution of [^{3}H]formaldehyde (85 mCi/mmol; New England Nuclear Corp., Boston, Mass.), followed by 0.3 volume of sodium borohydride (12.8 mg/ml) by the method of Rice and Means (25) as modified by Grinnell (10). After dialysis against PBS, the product was stored at −20°C. Specific activity was 1.6 Ci/mmol, indicating an average of 19 N-^{3}H-methylated sites per molecule.

Binding of [^{3}H]fibrinogen to streptococcal cells and precipitation with pep M24. Incubation mixtures (200 μl) consisted of 2×10^8 to 3×10^8 CFU of streptococci, test substances diluted in PBS, and Tween 20 at a final concentration of 0.1% (vol/vol), which in preliminary experiments reduced losses of labeled fibrinogen to undetectable levels without affecting binding to the streptococcal cells. For binding experiments employing the full range (up to 5 μM) of [^{3}H]fibrinogen concentrations, mixtures were incubated at ambient temperature for 45 min in 1.5-ml polypropylene microcentrifuge tubes and then centrifuged at 15,600 × *g* for 2 min. One-half of each supernatant (100 μl) was transferred to a counting vial containing 0.5 ml of PBS–0.1% Tween 20. Pellets were washed three times in 1.25 ml of PBS–0.1% Tween 20, suspended in 250 μl of the same buffer, and transferred to counting vials with two 250-μl rinses. Pronase (1 mg, protease type XIV; Sigma Chemical Co., St. Louis, Mo.) was added to each vial. The samples were incubated for 1 h at 37°C, mixed with 10 ml of Hydrofluor (National Diagnostics, Somerville, N.J.), and counted in a Packard Tricarb 460C liquid scintillation counter (Packard Instrument Company, Inc., Rockville, Md.) within 2 h and again the following day. A decrease of less than 10% in the sample counts confirmed adequate digestion of the samples. In preliminary experiments, the binding of [^{3}H]fibrinogen at a concentration (5 μM) such that <1% of the counts were bound was directly proportional to the number of CFU in the test mixture, with no binding detected when streptococcal cells were omitted. In all linear plots of the data (Lineweaver-Burk and Scatchard), the position of the straight lines was determined by sight.

In experiments in which the binding of [^{3}H]fibrinogen was tested at a single concentration under various conditions, the total concentration was 20 nM, at which approximately 90% of the counts were bound at pH 7.4 in the absence of inhibitors. Test mixtures and supernatants (80 μl each) were counted to obtain the percentage of counts bound. In these binding assay mixtures, the concentration of fibrinogen binding sites exceeded the dissociation constant by approximately sixfold, so that the concentration of free fibrinogen was not necessarily equal to the total concentration. This

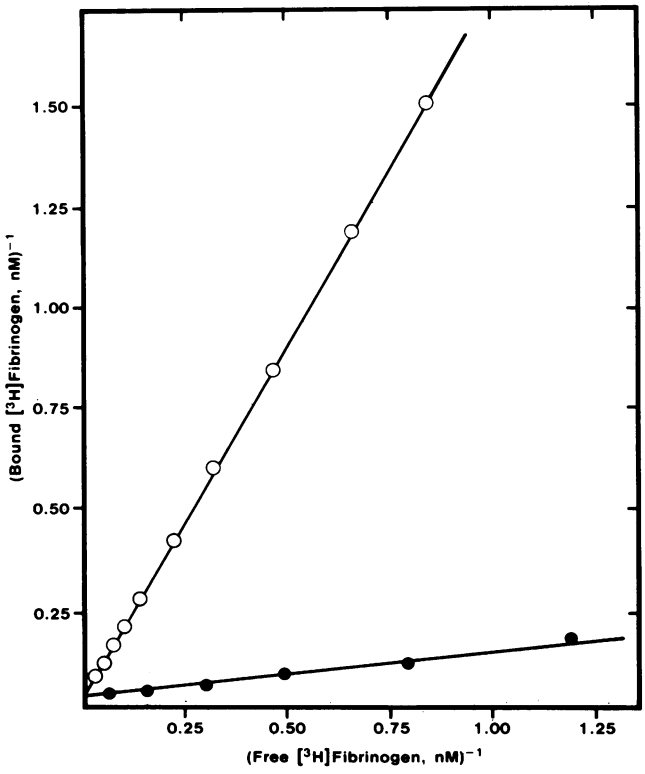

FIG. 2. Lineweaver-Burk plot of binding of [^{3}H]fibrinogen to streptococcal cells in the presence (○) or absence (●) of 100 nM unlabeled fibrinogen. The dissociation constant of the labeled molecule is 5 nM. The dissociation constant of the unlabeled molecule, calculated for better accuracy from the parallel shift in the binding isotherm (not shown), is 3.3 nM.

fact was taken into account in estimating the inhibition constant of unlabeled fibrinogen.

Assays of precipitation of [^{3}H]fibrinogen by pep M24 were performed similarly to the binding assays except that pep M24 was substituted for streptococcal cells. The percentage of counts precipitated was calculated from counts of test mixtures and supernatants. In both binding and precipitation assays, blanks lacking streptococci or pep M24 were included to rule out precipitation of [^{3}H]fibrinogen by other components of the test mixtures; such precipitation was never observed in the blank mixtures.

SDS-polyacrylamide gel electrophoresis of pep M24 and fibrinogen. Samples containing equivalent amounts of uncentrifuged pep M-fibrinogen mixture, precipitate, and supernatant were heated at 100°C for 2 min in reducing sample buffer (0.06 M Tris [pH 6.8] containing 12% glycerol, 1.2% SDS, 0.001% bromphenol blue, and 6% mercaptoethanol). Each sample contained the amount of material (precipitate, supernatant, or both) present in 30 μl of mixture. The reduced samples were subjected to electrophoresis on 10% polyacrylamide gels by the method of Laemmli (17). Gels were stained with Coomassie brilliant blue G. Molecular weight standards were purchased from Bio-Rad Laboratories, Richmond, Calif.

Proteolytic treatment of streptococci. For trypsin treatment, UV-treated streptococcal cells were suspended in PBS to which 0.1 mg of trypsin per ml was added (tolylsulfonyl phenylalanyl chloromethyl ketone treated and three times crystallized; Worthington Diagnostics, Freehold, N.J.) and incubated for 30 min at 37°C, after which the reaction was stopped by the addition of soybean trypsin inhibitor (Worthington) at 0.1 mg/ml (1). For gentle peptic digestion under suboptimal conditions, suspensions of organisms in 67 mM phosphate (pH 5.8) were exposed to pepsin (Worthington) at 20 μg/ml for 20 min at 37°C; the reaction was stopped by raising the pH to 7.5 with 1 N NaOH (1, 4). Treated suspensions were washed twice in PBS and resuspended to the desired optical density.

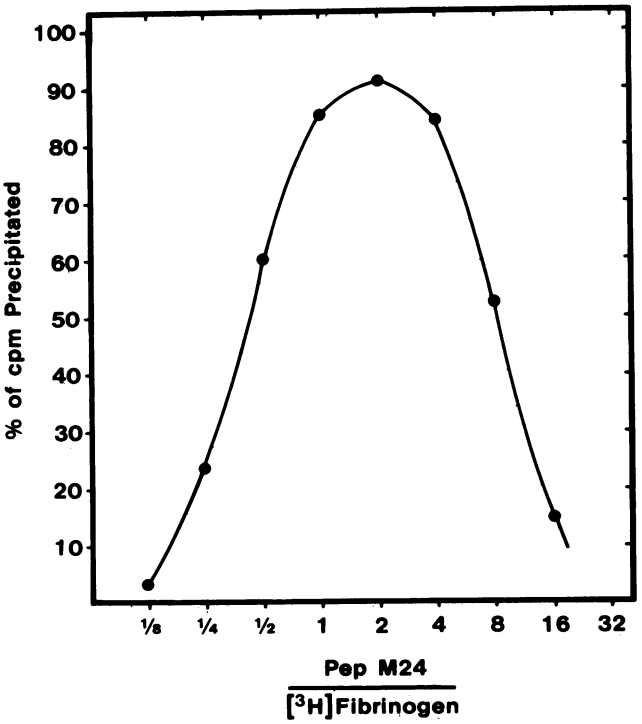

FIG. 3. Precipitation of [^{3}H]fibrinogen by pep M24. Equal volumes of 10 μM [^{3}H]fibrinogen in PBS and pep M24 in PBS at concentrations yielding the molar ratios shown were mixed at room temperature. The percentage of counts precipitated was calculated from counts of mixtures and supernatants.

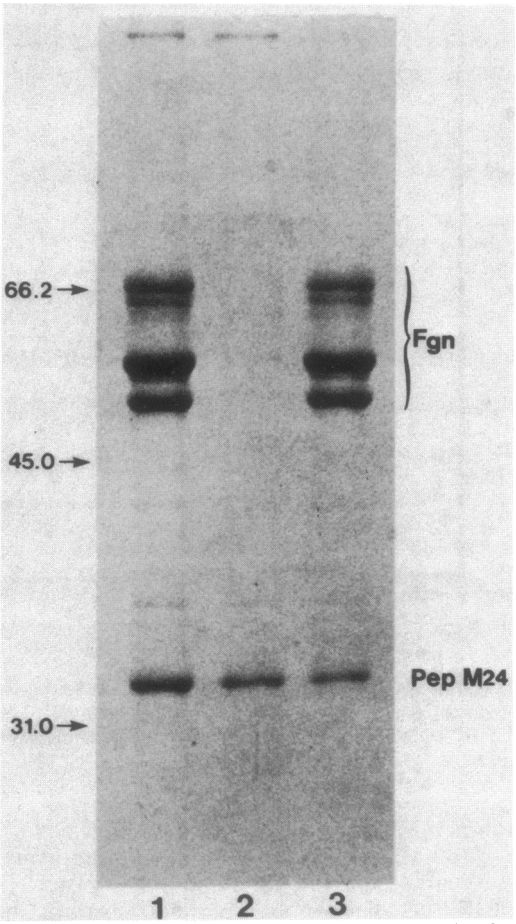

FIG. 4. SDS-10% polyacrylamide gel electrophoresis of reduced samples of an uncentrifuged mixture of 5 μM fibrinogen and 10 μM pep M24 (lane 1), the supernatant (lane 2), and the pellet (lane 3). The position of the molecular weight standards is shown (in thousands) at the left. The fibrinogen bands are the Aα, Bβ, and γ chains. Fgn, Fibrinogen.

Opsonophagocytic tests. Normal volunteers were selected who lacked antibody to streptococci of the M serotypes employed, as determined by enzyme-linked immunosorbent assay (reciprocal titers, <200) and opsonophagocytic tests with whole blood and blood reconstituted from serum and washed cells. Serum, plasma, and blood cells were obtained as described previously (35). Opsonophagocytic test mixtures (470 μl) consisted of 200 μl of serum or plasma, 1.6 U of heparin, 150 μl of washed blood cells (erythrocytes and buffy coat), 100 μl of test fluid, and finally 20 μl of streptococcal suspension containing 10^7 CFU. Mixtures were incubated with rotation at 37°C. Small samples (20 μl) were removed at 15-min intervals, smeared on glass slides, stained with Wright stain, and examined under oil immersion. One hundred neutrophils were counted, and the percentage with associated bacteria was noted. Whitnack et al. have previ-

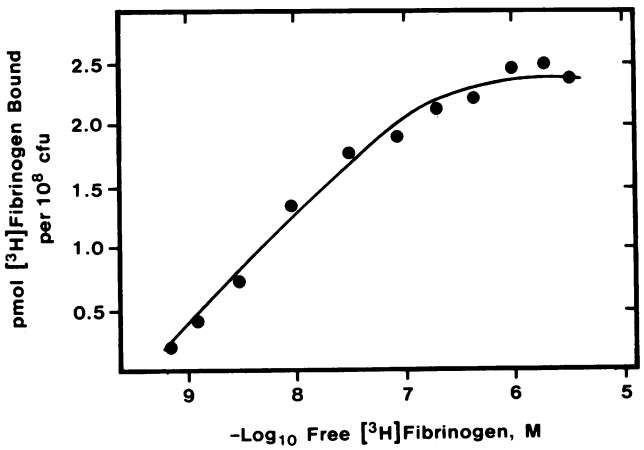

FIG. 5. Isotherm of the binding of [^{3}H]fibrinogen to type 24 streptococcal cells. Mixtures contained 2.6 × 10^8 CFU of streptococci in a total volume of 226 μl. Incubations were carried out for 45 min at ambient temperature.

ously shown that inhibition of bacterial uptake by neutrophils in this test correlates with decreased killing in bactericidal tests (35).

Electron microscopy. Electron microscopy was performed to assess the effect of proteolytic treatment on the surface fibrillae of the streptococcal cells. Streptococci were fixed in 2.5% glutaraldehyde–0.1% sodium cacodylate at pH 7.2, postfixed in osmium tetroxide, dehydrated in alcohol, and embedded in Spurr medium for transmission electron microscopy (1).

RESULTS

In previous studies we showed that group A streptococci bind fibrinogen to the surface fibrillae (as shown by electron microscopy) and are thereby protected from opsonization by complement (third component). After trypsin treatment, which removes the fibrillae, the organisms are readily opsonized by complement regardless of the presence of fibrinogen (33). Based on these observations, we undertook the following studies of the characteristics of the binding of human fibrinogen to intact group A streptococci and a purified fragment of M protein.

^{3}H-labeled fibrinogen. To carry out these studies, fibrinogen was radiolabeled by reductive N-methylation with [^{3}H]formaldehyde. The labeled product was readily clotted by thrombin (91% of the counts, compared with 90% by specifications of the manufacturer) and inhibited uptake of streptococci by neutrophils in a dose-dependent manner identical to that of the parent compound (Fig. 1). It retained the binding properties of the parent molecule, as shown by competitive inhibition experiments (Fig. 2).

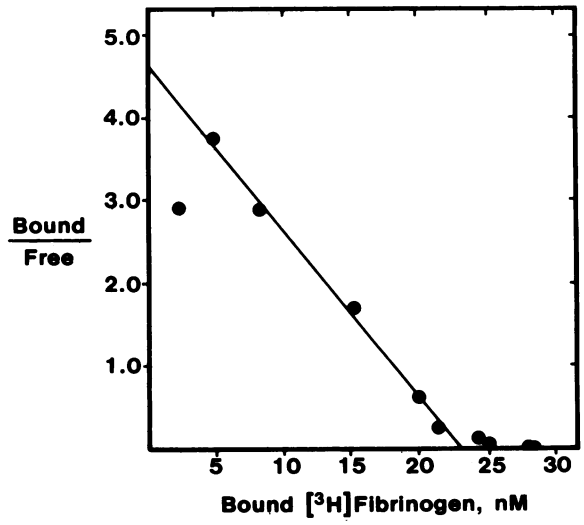

FIG. 6. Scatchard plot of the data shown in Fig. 5. The negative inverse slope of the straight line is 5 nM.

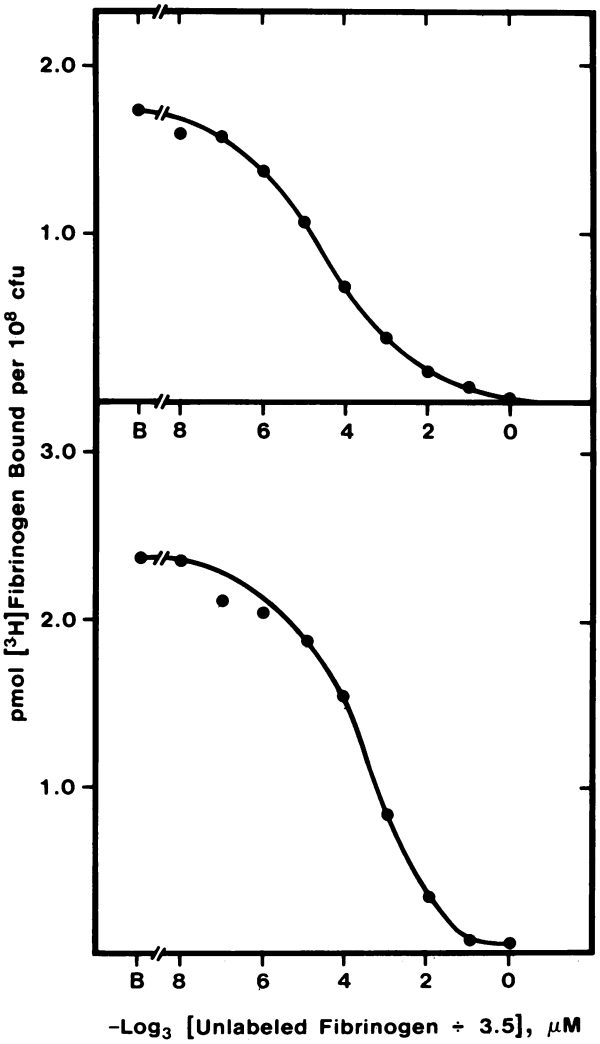

FIG. 7. Concentration-dependent inhibition of binding of [^{3}H]fibrinogen to streptococcal cells by unlabeled fibrinogen. The total concentrations of the labeled molecule (upper panel, 33 nM; lower panel, 100 nM) were chosen from the middle and upper range of the high-affinity binding region depicted in Fig. 6. No significant nonspecific binding is apparent at these concentrations. The dissociation constant of the unlabeled fibrinogen was estimated to be 2.4 nM, with data from the upper panel, or 3.1 nM, with data from the lower panel. Control binding in the absence of unlabeled fibrinogen is designated B (buffer).

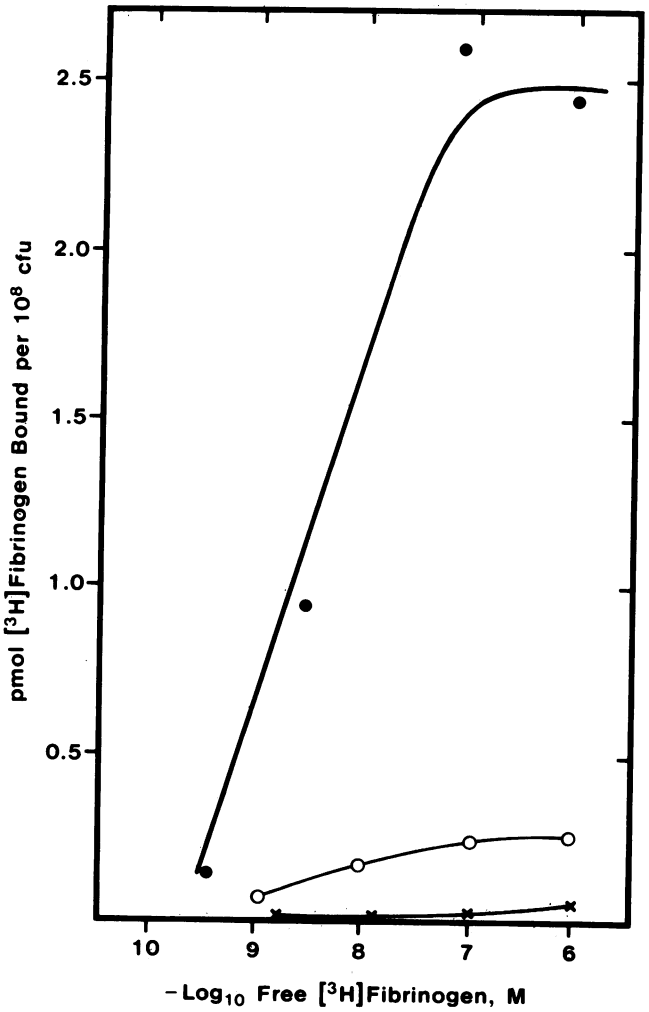

FIG. 8. Effect of proteolytic enzyme treatment of streptococcal cells on binding of [^{3}H]fibrinogen (same cells as in Table 1). Treatments (all at 37°C) included PBS for 1 h (●), 20 μg of pepsin per ml at pH 5.8 for 20 min (○), and 0.1 mg of trypsin per ml at pH 7.4 for 1 h (×).

Precipitation of pep M24 and fibrinogen. In the original study of Kantor and Cole, an extract of the one type 24 strain tested did not precipitate fibrinogen (14). In our studies, however, pep M24 precipitated fibrinogen both in heparinized plasma and in PBS. To examine the stoichiometry of the M protein-fibrinogen interaction, a precipitation curve was constructed by mixing pep M24 with 10 μM [^{3}H]fibrinogen at various molar ratios (Fig. 3). Maximum precipitation was obtained at a pep M-to-fibrinogen molar ratio of 2:1, at which 91% of the counts were precipitated, as compared with 91% clotted by thrombin. To determine the ratio of fibrinogen to pep M24 in the precipitate, samples containing equivalent amounts of uncentrifuged 2:1 mixture, precipitate, and supernatant were subjected to SDS-polyacrylamide gel electrophoresis. pep M24 appeared to be equally distributed between supernatant and precipitate (Fig. 4). The ratio of pep M24 to fibrinogen in the precipitate was therefore approximately 1:1, suggesting an equal number of binding sites on each molecule. Fibrinogen is a covalent dimer that binds to group A streptococci via the terminal D domains (26). pep M24 contains several internal sequence repeats (3)

TABLE 1. Phagocytosis in serum or plasma of streptococci treated with proteolytic enzymes[a]

Treatment of bacteria	% Neutrophils with associated bacteria after incubation in:		
	Serum + NRS	Plasma + NRS	Serum + anti-pep M24
PBS (control)	54	1	79
Pepsin (pH 5.8)	58	50	57
Trypsin	72	61	69

[a] UV-treated streptococci (3×10^7 CFU per test) were treated with trypsin, dilute pepsin at suboptimal pH (pH 5.8), or PBS, washed, and incubated for 45 min at 37°C with serum or plasma and washed blood cells plus normal rabbit serum (NRS) or anti-pep M24. Wright-stained smears were scored for neutrophils with associated bacteria.

and may have more than one binding site on that basis. Like pep M6 (24), pep M24 (33.5 kDa) probably exists in solution as a noncovalent dimer, since it elutes with bovine serum albumin (66.2 kDa) from molecular sieving columns (E. H. Beachey, unpublished observation). The precipitate obtained at the optimum ratio may therefore consist of fibrinogen-pep M dimers at a ratio of 2:1, with two pep M binding sites per (dimeric) fibrinogen molecule and four fibrinogen binding sites per pep M dimer.

Characteristics of the binding of [^{3}H]fibrinogen to intact streptococcal cells. The binding of [^{3}H]fibrinogen to type 24 streptococcal cells was rapid, reaching a maximum by 10 min of incubation at ambient temperature and remaining constant over 60 min. Binding approached saturation at concentrations above 5 μM (Fig. 5). Bound fibrinogen was recovered intact from coated organisms by boiling in reducing sample buffer, as determined by SDS-polyacrylamide gel electrophoresis, and binding was unaffected by the inclusion of 0.14 mg of soybean trypsin inhibitor per ml and 0.01 M

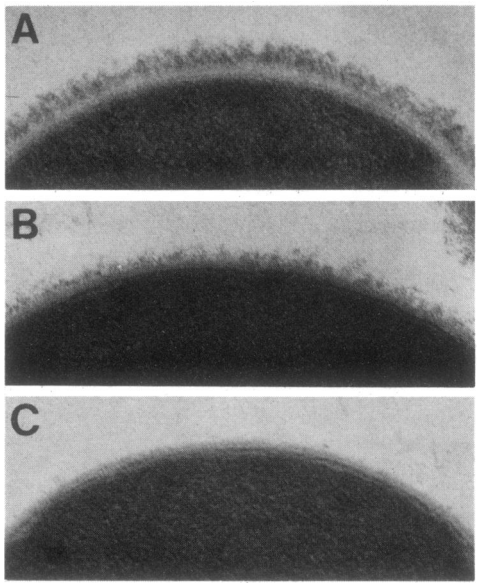

FIG. 9. Transmission electron micrographs of the streptococcal cells used for the experiment shown in Fig. 8 and Table 1. Organisms were treated with PBS (A), dilute pepsin at suboptimal pH (B), and trypsin (C). Magnification, ×60,000.

TABLE 2. Inhibition of binding of [^{3}H]fibrinogen to type 24 streptococci by antistreptococcal sera[a]

Serum	Immunizing agent	ELISA titer vs. pep M24[b]	% Inhibition of binding
	PBS (control)		0
EW (human)	Nonimmune	<1:200	8
8225-8	Nonimmune (4 sera)	ND[c]	2–5
NRS[d]	Nonimmune pool	ND	2
0281	pep M24	1:51,000	100
RF (human)	pep M24	1:3,200	57
7908	pep M5	<1:200	6
8104	pep M19	<1:200	5
G2H	Group A	1:400	42
8016	Lipoteichoic acid	ND	7
7629	M-negative strain D58X	ND	4
7630	M-negative strain D58X	ND	5

[a] Suspensions of streptococci (3×10^8 CFU) in PBS were incubated for 1 h in mixtures containing 50% serum, 20 nM [^{3}H]fibrinogen, and 0.1% Tween 20. All sera were raised in rabbits except as noted. Binding was determined from counts of test mixtures and supernatants; control binding (two experiments) was 86 and 92% of added counts.
[b] ELISA, Enzyme-linked immunosorbent assay.
[c] ND, Not done.
[d] NRS, Normal rabbit serum.

N-ethylmaleimide in the incubation mixtures (data not shown). Analysis by means of Lineweaver-Burk (Fig. 2) and Scatchard (Fig. 6, a different experiment) plots showed that at fibrinogen concentrations below 0.2 μM (bound plus free), the data points could be fitted to a straight line, suggesting a single population of high-affinity binding sites with a dissociation constant of 5 nM; this analysis may slightly underestimate the affinity and overestimate the capacity of binding (23). The binding of [^{3}H]fibrinogen in the high-affinity region (33 and 100 nM bound plus free) was determined in the presence of increasing concentrations of unlabeled fibrinogen (Fig. 7). Inhibition was ≥98%, showing that there is little or no binding to high-capacity, low-affinity sites at these ligand concentrations. Binding isotherms were determined for several different stocks of the type 24 strain and for one strain each of types 5 and 6, with similar results to those shown in Fig. 5 and 6; dissociation constants lay between 1 and 5 nM. The binding capacity of the streptococcal cells at saturation was approximately 10^4 molecules per CFU, which is of the same order as the number of M protein molecules per CFU, as estimated from the yield of pep M protein (1 mg [30 nmol] per g [wet weight] [10^{12} CFU] of streptococci). Comparison of the data in Fig. 1, 5, and 6 shows that the threshold of inhibition of phagocytosis by fibrinogen occurs at approximately 50% occupancy of the high-affinity binding sites, with complete inhibition at 100% occupancy.

Effect of proteolytic treatment of streptococci on inhibition of opsonization by fibrinogen and on binding of [^{3}H]fibrinogen to streptococcal cells. The surface M protein of group A streptococci renders the organisms resistant to opsonization and phagocytosis (19). Optimal resistance to phagocytosis requires the presence of fibrinogen. Accordingly, we determined the effect of proteolytic enzymes on the binding of fibrinogen in relation to phagocytosis of the treated streptococci in the presence or absence of fibrinogen or antibody to a purified fragment of pep M24. Untreated organisms bound [^{3}H]fibrinogen in a concentration-dependent manner (Fig. 8) and resisted phagocytosis when suspended in plasma (Table 1). A second suspension of organisms was treated with dilute pepsin at suboptimal pH (pH 5.8). This gentle proteolysis, used to extract pep M protein (see Materials and Methods), leaves shortened surface fibrillae that retain their epithelial cell-adhesive (1), fibronectin-binding (7), and hydrophobic (22) properties, which are attributed to lipoteichoic acid (1, 7, 8, 22, 28). In the present study, pepsin-treated organisms retained their fibrillae (Fig. 9), lost resistance to phagocytosis, and were no longer opsonized by antibody to pep M24. Fibrinogen binding was reduced by more than 90%, which was accompanied by a loss of inhibition of phagocytosis by fibrinogen (Table 1). Trypsin treatment removed the fibrillae (Fig. 9) and completely eliminated fibrinogen binding (Fig. 8). Trypsin-treated type 24 organisms were highly susceptible to phagocytosis and, like the pepsin-treated organisms, could not be opsonized by anti-M antibody or protected from phagocytosis by fibrinogen (Table 1). Similarly, the binding of [^{3}H]fibrinogen to pepsin- and trypsin-treated type 5 organisms was reduced by 89 and 100%, respectively.

Specificity of binding. To investigate the specificity of binding of fibrinogen to various cell surface structures, the binding of [^{3}H]fibrinogen to type 5 and 24 streptococcal cells was determined in the presence of rabbit antistreptococcal sera raised against whole M-negative streptococci of strain D58X, lipoteichoic acid (with specificity for the polyglycerol phosphate backbone), group A carbohydrate, homologous pep M proteins, cross-reacting and non-cross-reacting heterologous pep M proteins, and T proteins. PBS and nonimmune sera served as controls. In the case of the type 24 organisms, only the anti-pep M24 and the anti-group A sera gave greater than 8% inhibition (Table 2). The partial inhibition produced by the anti-group A serum proved to be the maximum obtainable in that it was not reduced by twofold dilutions of the serum up to 1:24. Similarly, the binding of [^{3}H]fibrinogen to type 5 streptococci was inhibited by anti-pep M5 serum, two cross-reacting sera (anti-pep M6 and anti-pep M19), and anti-group A serum but not the non-cross-reacting anti-pep M24 or other antistreptococcal sera, including anti-T of the types present on this strain (Table 3). Two of the sera, the homologous anti-pep M24 and the cross-reacting anti-pep M6, were tested with a range of [^{3}H]fibrinogen concentrations; in each case, inhibition proved to be competitive (Fig. 10).

TABLE 3. Inhibition of binding of [^{3}H]fibrinogen to type 5 streptococci by antistreptococcal sera[a]

Serum	Immunizing antigen	ELISA titer vs. pep M5[b]	% Inhibition of binding
	PBS (control)		0
NRS[c]	Nonimmune pool	ND[d]	0
7908	pep M5	1:102,400	83
7633	pep M6	1:25,600	32
8104	pep M19	1:6,400	59
0281	pep M24	<1:200	4
111	T type 5	ND	0
123	T type 27	ND	0
112	T pool W (including type 44)	ND	0
8016	Lipoteichoic acid	ND	0
7629	M-negative strain D58X	ND	0
7630	M-negative strain D58X	ND	0
G2H	Group A	1:200	42

[a] Binding was determined as described in footnote *a* to Table 2. Control binding was 81% of added counts.
[b] ELISA, Enzyme-linked immunosorbent assay.
[c] NRS, Normal rabbit serum.
[d] ND, Not done.

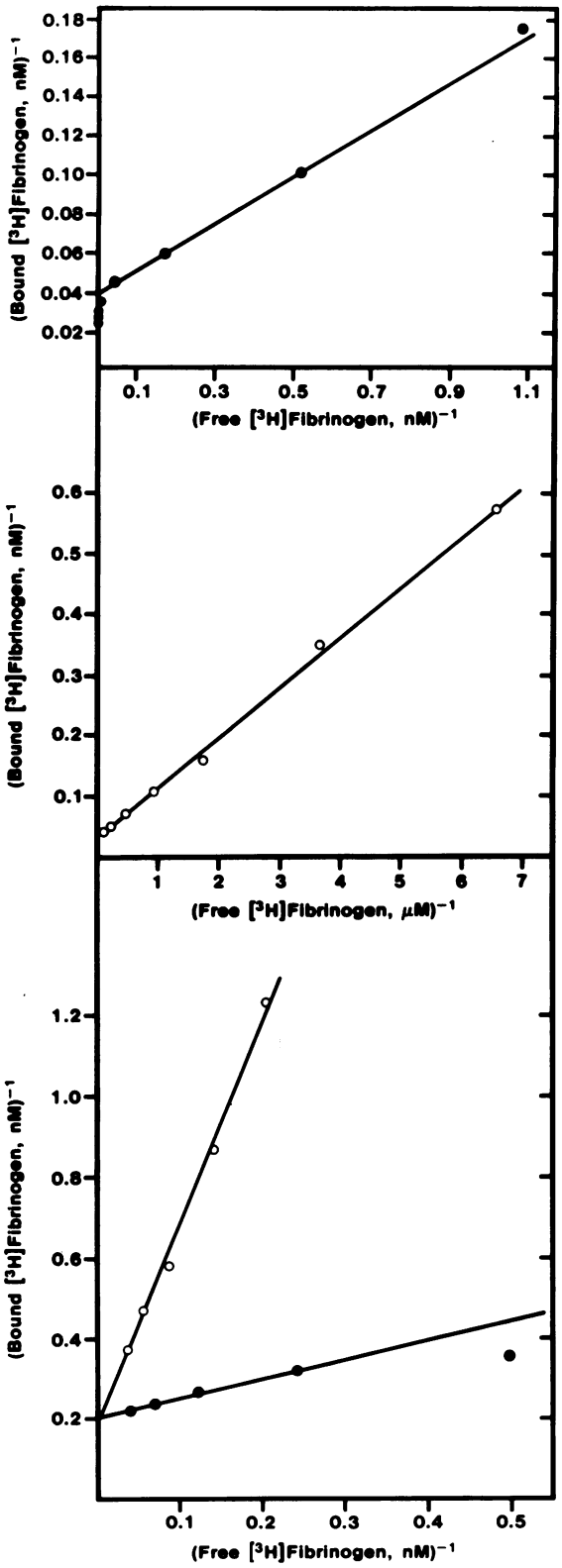

FIG. 10. Inhibition of the binding of [^{3}H]fibrinogen to streptococcal cells by homologous and heterologous anti-pep M sera. Upper panel, Binding of [^{3}H]fibrinogen to type 24 streptococcal cells in the presence of 50% normal rabbit serum. The y-intercept is 0.039/nM. Middle panel, Binding of [^{3}H]fibrinogen to type 24 streptococcal cells in the presence of 50% anti-pep M24 serum, shown separately because of the change of scale on both axes. The y-intercept is 0.032/nM. Lower panel, Binding of [^{3}H]fibrinogen to type 5 streptococcal cells in the presence of 50% normal rabbit serum (●) or cross-reactive anti-pep M6 serum (○).

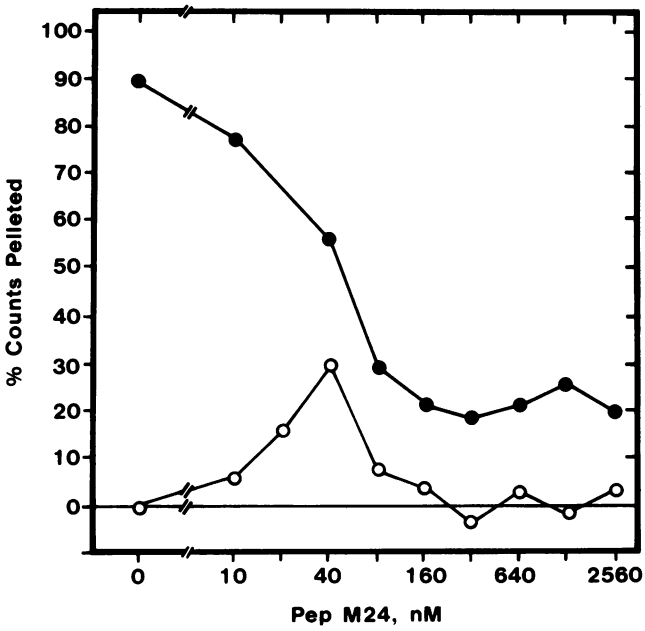

FIG. 11. Inhibition of binding of [^{3}H]fibrinogen to type 24 streptococcal cells by pep M24. The total concentration of [^{3}H]fibrinogen was 20 nM. The percentage of counts pelleted was determined in the presence (●) or absence (○) of streptococcal cells to take into account precipitation of [^{3}H]fibrinogen by the pep M24.

Inhibition of binding of [^{3}H]fibrinogen to streptococcal cells by pep M24. If the pep M moiety of M protein were a fibrinogen binding site, one would expect this molecule to inhibit binding of fibrinogen to the cell surface. We therefore ascertained the binding of [^{3}H]fibrinogen to type 24 streptococcal cells in the presence of increasing concentrations of pep M24. Control mixtures lacking streptococci were assayed to ensure that concentrations of pep M24 exceeded the zone of precipitation (Fig. 3). The results (Fig. 11) showed that pep M24 inhibited a maximum of 78% of the binding of 20 nM [^{3}H]fibrinogen.

DISCUSSION

This study was undertaken to examine the qualitative and quantitative aspects of the binding of fibrinogen to group A streptococci. The results indicate that fibrinogen binds to M-positive organisms with high affinity (dissociation constants in the nanomolar range) and that, in the strain studied in detail, occupation of the high-affinity binding sites suffices to protect the organism from opsonization and phagocytosis in nonimmune blood. The evidence from this and previous studies that M protein is the high-affinity binding site in M protein-rich streptococci may be summarized as follows: (i) fibrinogen binds to partially purified M proteins of most serotypes (14) and to a highly purified peptide of type 24 M protein; (ii) fibrinogen inhibits the binding to streptococcal cells of antibodies raised against pep M proteins, both type specific and cross-reactive (34, 35); (iii) binding is abolished

by proteolytic treatments that remove type-specific M protein from the cell surface, including treatments sufficiently mild to leave the proximal portion of the fibrillae intact; (iv) binding is competitively inhibited by antisera raised against purified pep M proteins; and (v) binding to type 24 cells is inhibited by an excess of pep M24. The data support the conclusion that M protein is the cell surface structure principally responsible for the high-affinity binding and that, at least in the case of type 24 organisms, the pep M moiety is part of the binding site.

The partial inhibition of binding by a group A antiserum is unexplained, given the low level of reactivity of the serum with pep M proteins. Fibrinogen does not appear to bind to cell surface carbohydrate, because binding was prevented by pretreatment of the cells with trypsin, which does not remove carbohydrate (11). Group A carbohydrate is exposed and available for interaction with anticarbohydrate antibody over the entire surface of M-positive streptococcal cells (11), raising the possibility of steric inhibition of fibrinogen binding by the anticarbohydrate antibodies.

Our results differ from those of Kronvall and his colleagues (16) in that we found binding to be sensitive to proteolytic enzymes that destroy M proteins. Possible explanations for the discrepant results of the two studies include different binding characteristics of ^{125}I-labeled as opposed to ^{3}H-labeled fibrinogen and loss of M protein from the strain studied by Kronvall et al., due either to repeated culture passage (32) or to digestion by bacterial proteases in broth culture (9). The structures responsible for binding fibrinogen in M-negative streptococci are unknown but might include T protein, which is more stable on culture passage than M protein (20) and requires prolonged digestion with trypsin for removal (18, 20). Extracts of T proteins of several serotypes have been shown to interact with fibrinogen (16, 21). In the present study, however, binding to a T-positive strain was trypsin sensitive and was not blocked by anti-T sera. Whether binding of fibrinogen to structures other than M protein protects the cells from opsonization and phagocytosis in immune and nonimmune blood is an interesting question that remains to be investigated.

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