Antiopsonic Activity of Fibrinogen Bound to M Protein on the Surface of Group A Streptococci

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ABSTRACT When virulent group A streptococci of M type 24 were incubated in fresh heparinized whole blood or in blood reconstituted from cellular elements and plasma, little uptake by neutrophils occurred as determined by light microscopy. When fresh human serum (with or without added heparin) was substituted for plasma, uptake occurred promptly. Uptake in serum could be prevented by adding either plasma or purified human fibrinogen to the incubation mixtures, or by pretreating the organisms with plasma or fibrinogen. Fibrinogen solutions absorbed with purified homologous M protein and centrifuged to remove precipitates lost their inhibitory activity. Uptake in serum depended on heat-labile factors. Immunofluorescent staining of bacteria using fluorescein-labeled antibody to the third component of complement showed that streptococci incubated in fresh serum bound complement evenly over the entire cell surface, whereas streptococci incubated in fresh plasma or in serum plus fibrinogen fluoresced only at some of the cross-walls between adjacent daughter cocci and at occasional terminal cocci. In electron micrographs, the surface fibrillar layer of streptococci treated with plasma or fibrinogen lost its hairlike appearance and became a dark band that stained heavily with ferritin-labeled antifibrinogen. We conclude that the known antiopsonic effect of M protein derives in part from binding of fibrinogen.

INTRODUCTION

Streptococcal M protein, which is a constituent of the surface fibrillae of group A streptococci (1), is the principal virulence factor of these organisms. Its presence is required for resistance to opsonization and phagocytosis in human blood in vitro and for infectivity in laboratory animals and, by inference, in man (2). Recently Bisno (3) and Peterson et al. (4) have shown that streptococci lacking M protein (either phenotypically or by proteolytic treatment) activate complement via the alternate pathway, which must be intact for opsonization and phagocytosis of these avirulent organisms. Streptococci that possess M protein do not activate complement efficiently, nor are they opsonized by antisera to heterologous strains. Only antibody to homologous M protein overcomes its antiphagocytic effect and confers protective immunity (2).

Clumping of streptococci by plasma and fibrinogen was first described almost 50 years ago (5). In 1959, Kantor and Cole (6) found that fibrinogen could be precipitated by acid extracts of 70 out of 131 group A strains of 33 different M types. Subsequently Kantor (7) showed that M protein was the component in the extracts responsible for the reaction. In a recent study, all of 20 group A strains tested bound ^{125}I-fibrinogen on their surfaces (8).

In this report, we present evidence that fibrinogen enhances the resistance of type 24 streptococci to opsonization and phagocytosis by binding to surface M protein.

METHODS

Streptococci. Early log-phase cultures of an M-protein rich, encapsulated strain of type 24 streptococci (Vaughn) were washed, exposed to UV light, and suspended in phosphate-buffered saline (0.02 M phosphate—0.15 M NaCl, pH 7.4) (PBS)[1] as previously described (9).

M protein. Type 24 M protein was extracted from the streptococci by limited peptic digestion and purified to homogeneity (10).

Trypsin treatment of streptococci. For removal of the surface fibrillae and M protein, UV-killed organisms were treated with trypsin-TPCK (Worthington Biochemical Cor-

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[1] *Abbreviations used in this paper*: C3, third component of complement; cfu, colony-forming units; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes.

poration, Freehold, NJ), 1,000 U/ml in PBS for 30 min at 37°C. The reaction was terminated with soybean trypsin inhibitor 0.1 mg/ml (T-9003, Sigma Chemical Co., St. Louis, MO). Control organisms were treated with PBS or with premixed trypsin and trypsin inhibitor. Washing was omitted to ensure equal numbers of organisms in each suspension. Complete removal of functional M protein was confirmed as described (9). Trypsin treatment did not remove the capsule.

Fibrinogen. Purified human fibrinogen (grade L, Kabi Group, Inc., Greenwich, CT) was dissolved in PBS, dialyzed overnight in the cold to remove citrate, and used within 2 d.

Blood and blood components. Blood was drawn from healthy laboratory personnel, none of whom had antibody to type 24 M protein as determined by opsonophagocytic tests (see below). Plasma was separated from blood containing heparin 10 U/ml (16–17 U/ml plasma). Heparin was added to serum at 17 U/ml except as noted. Blood cells were isolated from heparinized blood (20 U/ml) mixed with 0.2 vol 6% Dextran-75 in saline (Abbot Laboratories, North Chicago, IL). The dextran-sedimented erythrocytes were washed two times in 10 vol Hanks' balanced salt solution. The polymorphonuclear leukocytes (PMN) were separated from the leukocyte-rich plasma by density gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) according to the manufacturer's instructions. The pellet consisting of >97% PMN (>90% neutrophils) was washed two times in Hanks' balanced salt solution, counted in a hemocytometer, and resuspended in plasma or serum at 10^7 cells/ml. Blood was reconstituted by mixing 4 vol PMN suspension with 3 vol packed erythrocytes.

Opsonophagocytic tests. Test mixtures consisted of 350 μl whole or reconstituted blood, 20 μl streptococcal suspension, and 100 μl test solution. The mixtures contained 2 × 10^6 PMN and 10^6 to 3 × 10^7 colony-forming units (cfu) streptococci in 330 μl extracellular fluid. After incubation at 37°C with end-over-end rotation at 10 rpm for the desired time intervals, small samples were smeared on glass slides, stained, and examined by light microscopy. The percentage of PMN associated with streptococcal chains (expressed as "uptake" in the text) was determined without attempting to distinguish attached from ingested bacteria (9).

Immunofluorescence microscopy. To detect the third component of complement (C3) bound on the surface of streptococci after treatment with plasma or serum, organisms were incubated in dilutions of fluorescein-labeled goat antihuman C3 (Cappel Laboratories, Inc., Cochranville, PA) for 15–30 minutes at room temperature, washed four times in PBS, air-dried on glass slides, embedded in 20% polyvinyl alcohol (Gelvatol, Monsanto, Springfield, MA) in 0.01M phosphate at pH 7.2, and examined under oil immersion with a fluorescence microscope.

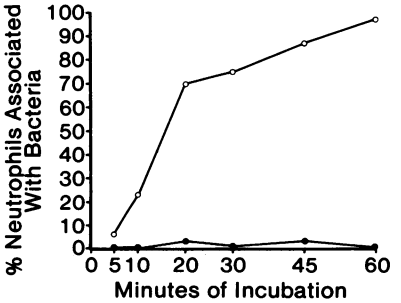

FIGURE 1 Uptake of type 24 streptococci by neutrophils in blood reconstituted from plasma (●) or heparinized serum (○). Streptococcal concentration, 3 × 10^7 cfu/ml.

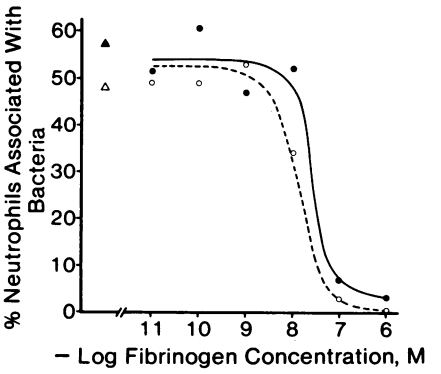

FIGURE 2 Inhibition of bacterial uptake upon addition of plasma (●) or fibrinogen (○) to blood reconstituted from heparinized serum, assuming a plasma fibrinogen concentration of 10 μM. Controls: fibrinogen dialysis buffer (△); PBS (▲). Incubation time, 30 min; streptococci, 3 × 10^7 cfu/ml.

Electron microscopy and immunoelectron microscopy. Streptococci (10^7–10^8 cfu) were treated with 1 ml plasma, fibrinogen 4 mg/ml in PBS, or PBS alone for 15 min at room temperature, washed, fixed, embedded, and sectioned for electron microscopy (9). For immunoelectron microscopy the organisms were treated additionally with 100 μl ferritin-labeled goat antihuman fibrinogen (Cappel Laboratories, Inc.) for 30 min at room temperature before fixation.

RESULTS

When M-protein-rich type 24 streptococci were incubated in fresh heparinized blood or in blood reconstituted from erythrocytes, PMN, and plasma, little uptake of bacteria by the PMN occurred in 1 h of incubation. When serum (with or without heparin) was substituted for plasma, uptake occurred promptly (Fig. 1). Uptake in serum could be abolished by adding either plasma or purified fibrinogen to the test mixture (Fig. 2), or by pretreating the bacteria for 5 min at ambient temperature with plasma or fibrinogen 4 mg/ml and then washing them.

Binding of fibrinogen to the surface of the streptococci was apparent in electron micrographs. The surface fibrillar layer of organisms treated with either plasma or fibrinogen lost its hairlike appearance and became a dark band that stained heavily with ferritin-labeled antifibrinogen. Given the location of M protein on the surface fibrillae (1) and the known precipitation reaction between M proteins and fibrinogen, it seemed likely that M protein was the surface structure responsible for binding fibrinogen. Although in the original observations of Kantor and Cole (6) extracts of a type 24 strain did not precipitate fibrinogen, our prep-

aration of purified M 24 protein precipitated it readily. Plasma or fibrinogen solutions absorbed with equimolar M protein and centrifuged to remove precipitates no longer inhibited uptake of bacteria by PMN. Removal of M protein from the bacterial surface with trypsin increased uptake in both serum and plasma and abolished the difference between them (Fig. 3). Similarly, an avirulent, M-negative strain (D58X) was ingested equally well in serum, plasma, and serum containing added fibrinogen 4 mg/ml; the percentage of PMN associated with bacteria in these three tests differed by no more than five percentage points at any concentration of streptococci tested.

Uptake of bacteria in serum was unaffected by preabsorption of the serum at 0°C with streptococci 10^{12} cfu/ml, but was abolished by heating the serum at 56°C for 30 min, suggesting that in unheated serum, added fibrinogen interferes with opsonization by heat-labile factors, e.g., complement. Streptococci were incubated in plasma or in fresh heparinized serum with or without 10 μM fibrinogen for 30 min at 37°C and stained with fluorescein-labeled antihuman C3. As shown in Fig. 4, organisms treated with serum stained evenly over their entire surface; organisms treated with plasma stained only at occasional cross-walls between daughter cocci and, rarely, at terminal cocci of the chains. An identical pattern was produced by incubating the streptococci in heparinized serum containing fibrinogen 4 mg/ml. To see whether fibrinogen prevented binding of C3 to the cocci or binding of the fluorescent antibody to surface C3, organisms were treated sequentially with serum and fibrinogen; such organisms stained identically to those treated with serum alone. In other control experiments, organisms treated with heat-inactivated serum did not bind fluorescent anti-C3; organisms treated sequentially with trypsin and plasma stained uniformly, as in Fig. 4a.

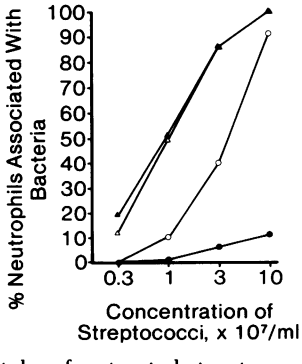

FIGURE 3 Uptake of untreated streptococci in plasma (●) and heparinized serum (○) compared with that of trypsin-treated organisms in plasma (▲) and serum (△). Incubation time, 30 min.

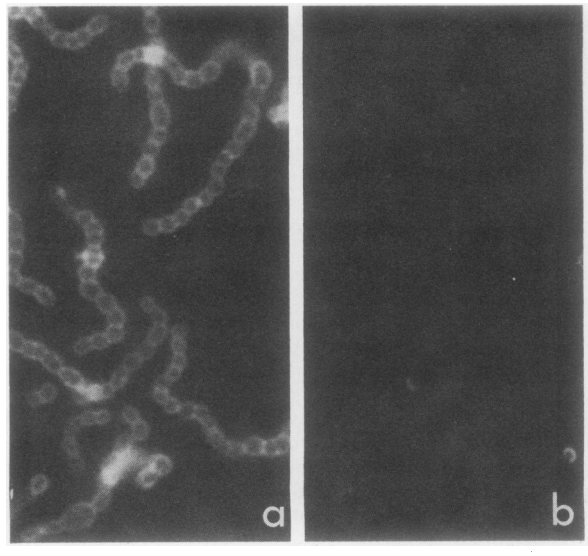

FIGURE 4 Immunofluorescent staining of streptococci treated sequentially with serum (A) or plasma (B) and fluorescein-labeled anti-C3. In B, some of the cross-walls fluoresce dimly; two cocci (both at the ends of their respective chains) fluoresce around part of their circumference.

DISCUSSION

A number of blood proteins other than specific antibody and complement are known to bind to the surface of gram-positive cocci (8). Despite speculation about the pathogenic significance of these reactions (7, 8), none has been definitively shown to benefit either the host or the parasite. In one study of a type 12 strain of group A streptococci, the acquisition of binding sites for the Fc end of IgG correlated with an increase in virulence during mouse passage (11), but binding of IgG to the Fc receptor was not shown to be responsible for the increased virulence. In another study, the antiphagocytic effect of IgG bound to staphylococcal protein A was counterbalanced by the ability of this protein to fix complement (12). The data presented in this report, which indicate that fibrinogen binds to the surface of a group A streptococcus and prevents opsonization by complement, show that a gram-positive bacterium can take on a host protein to protect itself against host defenses.

The similarity of the inhibition curves for plasma and purified fibrinogen (Fig. 2) suggest that this protein is the substance principally responsible for inhibiting opsonization. It would not be surprising if fibrinogen catabolites contribute to the effect, and we cannot exclude this possibility. That M protein is the streptococcal structure responsible for binding fibrinogen is indicated by the following findings: fibrinogen reacted with purified homologous M protein; it bound

to the surface fibrillae, known to be the location of M protein; and it did not inhibit the phagocytosis of M-negative organisms. Our data do not indicate a mechanism for the antiopsonic effect of M protein-fibrinogen, but the most straightforward possibility is that bound fibrinogen impedes access of complement proteins to cell-wall structures. Fibrinogen might even cross-link the fibrillae.

The surface fibrillae afforded considerable protection against phagocytosis even in the absence of fibrinogen, as shown in Fig. 3. Accordingly, our data do not necessarily conflict with results of various other investigations (including [3] and [4] cited above) demonstrating resistance to opsonization and phagocytosis in serum. Also, the findings reported here may not apply to phagocytosis by monocytes, which we did not study.

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