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Antiphagocytic activity of streptococcal M protein: Selective binding of complement control protein factor H

(*Streptococcus pyogenes*/microbial surface protein/alternative complement pathway)

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ABSTRACT **Isolated complement components were used to study the regulation of the alternative complement pathway C3 convertase (EC 3.4.21.47), also called C3b,Bb, on M protein-carrying (M^+) and M protein-lacking (M^-) streptococci. Neither M^- nor M^+ streptococci directly affected the formation or dissociation of the surface-bound C3b,Bb or the inactivation of surface-bound C3b by factor I. However, the activity of the serum control protein of the alternative complement pathway, factor H, in controlling streptococcus-bound C3b and C3b,Bb was 6–8 times stronger on M^+ organisms than on M^- organisms. Furthermore, M^+ streptococci of different serotypes and purified streptococcal M6 protein were shown to selectively bind factor H, the dissociation constants ranging from 4.5×10^{-6} M to 6×10^{-7} M. We conclude that the antiphagocytic activity of streptococcal M protein may be due to complement inhibition mediated by the binding of factor H. Binding of a regulatory protein appears to be a previously unrecognized route by which a pathogen is able to evade alternative pathway activation.**

The alternative complement pathway is considered an important part of the nonspecific defense system against infection (1). Activators set in motion the complement cascade in the absence of specific antibodies, thereby eliciting a variety of inflammatory and defense responses. One of the major effects of complement activation is opsonization—i.e., deposition of large numbers of molecules of complement fragment C3b on the surface of activators, which thereby become accessible to uptake by phagocytic cells carrying complement component C3 receptors (2). Deposition of C3b on the surface of particles is achieved by an amplification system driven by particle-bound alternative-complement-pathway C3 convertase (EC 3.4.21.47), also called C3b,Bb, which is a labile enzyme complex that activates C3 (3). In the fluid phase and on nonactivators such as autologous cells, this process is inhibited by the control of C3b through factors dissociating the C3b,Bb complex and rendering C3b accessible to inactivation by factor I. The membrane components decay-accelerating factor and C3b receptor, present on several mammalian cell types, possess such activities (4, 5). The soluble control protein factor H, a 150-kDa β-globulin, is freely available in serum (500 μg/ml) to control fluid-phase C3b (6, 7). Moreover, the affinity of surface-bound C3b for factor H on autologous cells and other nonactivators is 10-fold higher than on activators, indicating that factor H also contributes to the regulation of surface-bound C3b (8, 9).

More recently it has been shown that pathogens may prevent activation of the alternative pathway by mimicking surface structures of host cells. Sindbis virus (10), certain strains of *Escherichia coli* (11), and group B streptococci (12) may exhibit carbohydrate moieties that, like those on host cells, allow the binding of factor H to surface-bound C3b. Herpes simplex virus (13), *Trypanosoma cruzi* (14), and *Candida* species (15) appear to express surface proteins that may function like the decay-accelerating factor or the C3b receptor of the host cell membrane.

It is well known that strains of group A streptococci possess a surface fibrillar molecule, the M protein (16, 17), which is the major virulence factor for the organism by protecting it from attack by human phagocytic cells (16). Resistance to group A streptococcal infection is achieved through the production of type-specific antibodies to the M molecule, making the bacterium susceptible to phagocytosis (18, 19). To date, however, >80 serologically distinct variants of the M molecules are known. Current data indicate that the M molecule is an α-helical coiled-coil structure extending about 60 nm from the cell surface (17, 20) with the N-terminal half (distal from the cell surface) variable and the C-terminal half conserved among different M serotypes (21, 22).

Evidence has accumulated suggesting that the mechanism by which the M protein exerts its antiphagocytic effect may be to prevent complete opsonization of the streptococcus by interfering with the activation of the alternative complement pathway on the bacterial surface (23–25); however, the exact mechanism has been elusive. Herein we report that streptococci of different M serotypes as well as purified M6 protein selectively bind factor H, the serum control protein of the alternative complement pathway. This interaction may inhibit the effective opsonization of the streptococcus, resulting in the inhibition of phagocytosis (25). Thus, the binding of factor H may be the mechanism by which M protein achieves its antiphagocytic effect.

MATERIALS AND METHODS

Bacteria. *Streptococcus pyogenes* strains D471 (M6 protein), B788 (M5 protein), 1GL205 (M19 protein), 22RS72 (M24 protein), T28/150A/5 (M28 protein), and T28/51/4-4 [an M protein-negative (M^-) isogenic variant of strain T28/150A/5 and devoid of the M protein gene (26)] were from the Rockefeller University collection. Bacteria were grown in Columbia or Todd–Hewett broth containing 0.2% yeast extract and were killed by overnight incubation in the presence of mitomycin C at 50 μg/ml (Serva, Heidelberg) (27). Bacterial counts were assessed by the pellet volumes after centrifugation at 8400 × *g* for 8 min in an hematocrit centrifuge. The pellet volumes were standardized by using calibrated latex particles measuring 1.1 μm in diameter, which were counted in parallel in an electronic particle counter using an orifice tube of 30-μm diameter (Coulter model ZF).

Proteins. M6 protein was isolated from the periplasm of *E. coli* cloned to contain the streptococcal gene for M6 protein (28) and was purified as described (29). The complement components C3 (30), factor B (31), factor D (32), and factor H (33) were isolated as described. Purified factor I was

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generously provided by Hans J. Müller-Eberhard (Research Institute of Scripps Clinic, La Jolla, CA) and Michael K. Pangburn (University of Texas Health Center, Tyler). Proteins were radiolabeled with ^{125}I (New England Nuclear) by the Iodo-Gen technique (Serva). Unbound iodide was removed by centrifugation of the sample through Bio-Gel P6 (Bio-Rad). The radioiodide was then 94–98% precipitable in 10% (wt/vol) trichloroacetic acid. The specific activities obtained ranged from 0.3 to 1.0 μCi/μg for all proteins labeled (1 Ci = 37 GBq). Antisera to isolated proteins were raised in rabbits.

Buffers. Experiments with isolated complement components were performed in veronal-buffered saline (VBS; 4 mM veronal/150 mM NaCl/0.02% NaN_3, pH 7.3); in VBS containing 5 mM $MgCl_2$ or 1 mM $NiCl_2$ or 0.1% gelatin (Sigma); and in VBS/0.1% gelatin containing 5 mM $MgCl_2$ or 1 mM $NiCl_2$ or 10 mM EDTA.

Deposition of C3b on Particles. Zymosan particles (10^9) or streptococci (2×10^{12}) bearing C3b were prepared as described (34) by using Ni^{2+}-stabilized C3b,Bb enzyme (35). The numbers of bound C3b were estimated by measuring bound radioactivity after having used trace-labeled C3 for C3b depositions. After the final incubations, the numbers of C3b molecules bound were verified by Scatchard analysis of factor B binding in the presence of 1 mM Ni^{2+} (34).

Dissociation of Particle-Bound C3b,Bb. The decay of ^{125}I-labeled Bb from particle-bound C3b was measured by using 7×10^7 zymosan particles or 1.4×10^{10} streptococci as described (36).

Cleavage of Particle-Bound C3b. A modification of the method developed by Carlo *et al.* (37) was used (36). To verify the fragmentation of C3b to the inactive form iC3b, the chain structure of the particle-bound C3 fragments before and after treatment with factors H and I was analyzed. Particle-bound C3 fragments were released by the method of Law and Levine (38). After reduction, the supernatants were submitted to $NaDodSO_4$ electrophoresis in a 9% polyacrylamide slab gel and subsequent autoradiography.

Protein-Binding Studies. The binding of factor H to streptococci was assessed as described for the binding of proteins to cell-bound C3b (34). Streptococci (2×10^9) were incubated with a mixture of labeled and unlabeled factor H at room temperature for 30 min, at which time the binding was shown to be at equilibrium. Specific binding to M^+ streptococci was calculated by subtracting background values determined with M^- streptococci. Scatchard plots were determined by regression analysis. Binding studies of proteins out of serum were performed similarly except that trace amounts of radiolabeled proteins were added only.

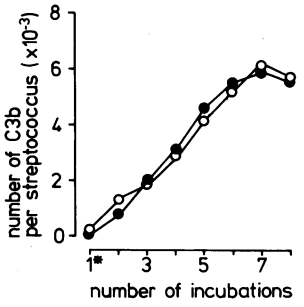

FIG. 1. Deposition of C3b on M^- streptococci (strain T28/51/4-4) (○) and M^+ streptococci (strain D471) (●) by the isolated complement components C3, factor B, and factor D in the absence of complement control proteins. Fluid-phase (*) (for the first incubation) and streptococcus-bound (for subsequent incubations) Ni^{2+}-stabilized C3b,Bb enzymes were used with trace-radiolabeled C3 as the substrate. The numbers of particle-bound C3b were calculated from the bound cpm. Data points represent the mean of duplicate experiments.

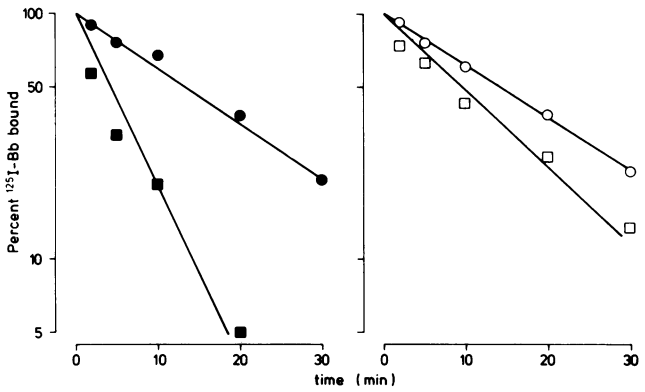

FIG. 2. Spontaneous dissociation of the alternative-complement-pathway C3 convertase, C3b,Bb, bound to M^- streptococci (○) and to M^+ streptococci (●), and the effect of the complement control protein factor H (□, ■). To generate streptococcus-bound C3b,Bb, streptococci carrying C3b were incubated with factor D and radiolabeled factor B in the presence of 5 mM Mg^{2+}. The decay of radiolabeled Bb was measured after addition of EDTA. In the absence of factor H, the dissociation of the complexes was similar on both types of bacteria, the half-lives at 20°C being 14.5 min on M^+ streptococci (strain D471) and 13.5 min on M^- streptococci (strain T28/51/4-4). In the presence of factor H at 1 μg/ml, the half-life of the streptococcus-bound C3b,Bb complex was 4 min on M^+ bacteria (■) and 9 min on M^- bacteria (□). Data points represent the mean of duplicate experiments.

Enzyme-Linked Immunosorbent Assay. The standard procedure of Engvall and Perlmann (39) was carried out by using swine anti-rabbit IgG-peroxidase conjugate with *o*-phenylenediamine as substrate. M protein or ovalbumin, diluted in carbonate buffer (pH 9.6), was adsorbed to the microtiter plates at a concentration of 5 μg per well.

RESULTS

Deposition of C3b on M^- and M^+ Streptococci. The process of opsonization of M^- and M^+ streptococci was mimicked by using the purified complement components C3, factor B, and factor D in the absence of complement control proteins. To deposit initial molecules of C3b on the streptococci, preformed Ni^{2+}-stabilized fluid-phase C3b,Bb com-

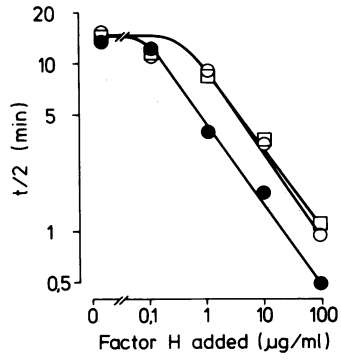

FIG. 3. Activity of increasing concentrations of factor H in accelerating the dissociation of C3b,Bb bound to M^- streptococci (○), M^+ streptococci (●), and zymosan (□). Experimental conditions were as described for Fig. 2. The effect of factor H on M^- streptococcus-bound C3b,Bb and zymosan-bound C3b,Bb was similar: the factor H concentrations required to reduce the half-life (t/2) of the complex to 50% were 3 μg/ml and 4 μg/ml, respectively. For C3b,Bb bound to M^+ streptococci, the corresponding concentration of factor H was 0.5 μg/ml. Data points represent the mean of duplicate experiments.

plexes were added together with C3 as the substrate. For further depositions of C3b, the bacteria were washed, and streptococcus-bound C3b,Bb enzymes were generated by adding factor B and factor D in the presence of Ni^{2+}. The substrate C3 was added with EDTA to prevent the formation of fluid-phase C3b,Bb complexes. By using this procedure, similar numbers of C3b were deposited on M^- streptococci (strain T28/51/4-4) and on M^+ streptococci (strain D471), indicating that the presence of the M6 protein on strain D471 did not influence the overall activity of the streptococcus-bound C3b,Bb enzyme (Fig. 1).

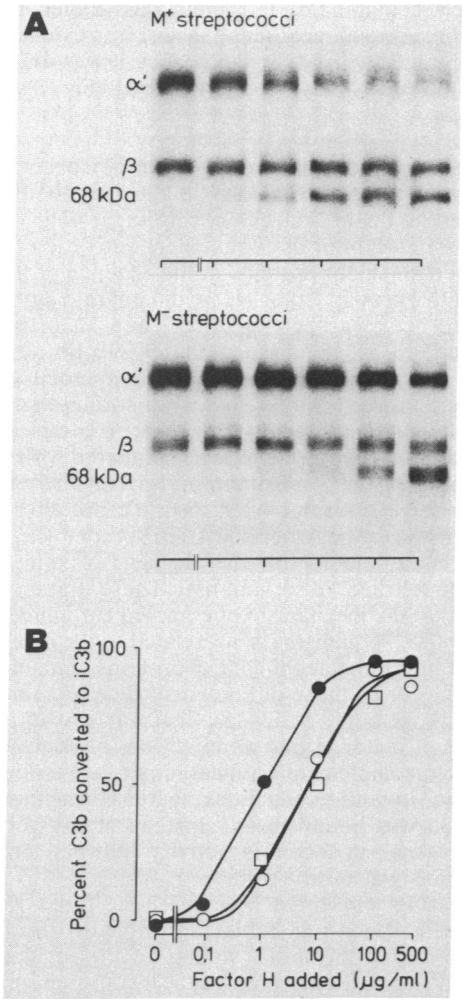

FIG. 4. Activity of factor H as a cofactor for factor I in the cleavage of C3b bound to M^- streptococci (○), M^+ streptococci (●), and zymosan (□). Particles carrying radiolabeled C3b were incubated for 15 min at 37°C with factor I at 1 μg/ml and various concentrations of factor H. (*A*) Particle-bound C3 fragments were released by treatment with hydroxylamine, reduced, and analyzed by autoradiography after $NaDodSO_4$/PAGE. The conversion of particle-bound C3b to iC3b is demonstrated by the cleavage of the α' chain into fragments of 68 and 46 kDa, the latter of which is not visible because of its low radioiodine incorporation by the method used here. The lanes correspond to experiments with increasing concentrations of factor H as indicated on the abscissa of *B*. (*B*) For quantifications, release of particle-bound radioactivity was measured after continuing the incubation for 10 min in the presence of trypsin at 2 μg/ml to cleave iC3b to C3d, releasing C3c. In the absence of factor H, no cleavage of C3b bound to any particle was observed. To cleave 50% of zymosan-bound C3b, a concentration of 9 μg/ml of factor H was required. A similar result, 8 μg/ml, was obtained with C3b bound to M^- streptococci, whereas a concentration of only 1 μg/ml of factor H was needed to cleave 50% of C3b bound to M^+ bacteria. Data points represent the mean of duplicate experiments.

The Dissociation of the C3b,Bb Complex Bound to M^- and M^+ Streptococci. To elucidate the regulation of the C3b,Bb complex on the surface of streptococci in more detail, the enzyme complex was generated by using radiolabeled factor B, and the time course of the decay of Bb from the surface-bound subunit C3b was measured. The spontaneous dissociation of the complex was similar on streptococci of both M^+ and M^- strains and, indeed, appeared unaffected by the presence of M6 protein on the M^+ cells (Fig. 2). However, the activity of factor H in accelerating the decay of Bb was substantially stronger when C3b was deposited on M^+ instead of M^- bacteria. Further analysis by a dose–response experiment revealed that the difference in activities was approximately 6-fold (Fig. 3). For comparison, we measured the dissociation of the C3b,Bb complex bound to zymosan, the experimental standard activator of the alternative complement pathway. The spontaneous decay of Bb was similar to that found for bacteria of either M^+ or M^- strain of streptococci. The dose-dependent effect of factor H on the dissociation of zymosan-bound C3b,Bb corresponded to the results obtained with M^- streptococci. Together these results indicate that, with respect to the regulation of surface-bound C3 convertase, M^- streptococci behave like activators of the alternative complement pathway, whereas M^+ streptococci have surface properties facilitating the control of alternative-pathway activation by the regulatory protein factor H.

The Inactivation of C3b Bound to M^- and M^+ Streptococci. Besides the dissociation of C3b,Bb, the other function of factor H in controlling alternative-pathway activity is rendering C3b accessible to inactivation by factor I. The cleavage activity of factor I was assessed by using the release of radioactivity from surface-bound radiolabeled C3b. In addition, the chain structure of the particle-bound C3 fragments was analyzed by $NaDodSO_4$/PAGE (Fig. 4*A*). No cleavage of C3b bound to streptococci of either strain (T28/51/4-4 and D471) was observed in the absence of factor H. Again, a difference in the activity of factor H was seen by comparing C3b bound to M^- streptococci and to M^+ streptococci. The cofactor activity of factor H for the cleavage of C3b by factor I was ≈8 times higher when C3b was bound to the M^+ streptococci instead of M^- streptococci (Fig. 4*B*). Also in this regard, the surface properties of M^- streptococci corresponded to those of zymosan. This finding again indicates that streptococci lacking the M protein behave like activators of the alternative complement pathway, allowing relatively slow cleavage of bound C3b by the control proteins, whereas the presence of the M6 protein

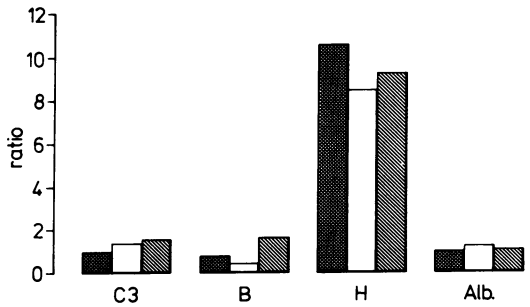

FIG. 5. Selective binding of factor H by M^+ streptococci. Bacteria of M^+ streptococcal strain D471 or M^- strain T28/51/4-4 were incubated with trace-radiolabeled factor H, C3, factor B, and albumin either as isolated proteins at serum concentrations (▨) or in whole serum in the presence (□) or absence (▨) of EDTA. Results are presented as the ratio of radioactivity bound to M^+ streptococci to radioactivity bound to M^- streptococci after a 30-min incubation at 20°C. Each bar represents the mean of duplicate experiments.

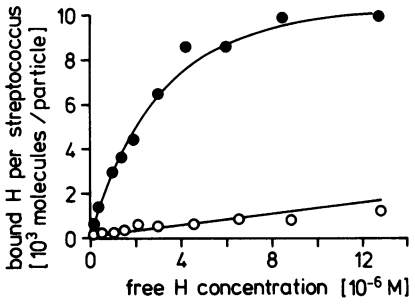

FIG. 6. Binding of purified factor H to M^- (○) and M^+ (●) streptococci. Trace-radiolabeled factor H at 0.04 to 2 mg/ml was incubated with 2×10^9 bacteria of strains T28/51/4-4 (M^-) and D471 (M^+), respectively. After a 30-min incubation at 20°C, streptococcus-bound radioactivity was determined. Data points represent the mean of triplicate experiments.

was found to enhance the rate of inactivation of streptococcus-bound C3b.

Binding of Factor H to M^- and M^+ Streptococci. Since the activity of factor H on streptococcus-bound C3b was consistently higher when C3b was bound to M^+ instead of M^- organisms, the direct interaction between factor H and the bacteria was studied. Streptococci of M6 strain D471 were found to bind ≈10 times more factor H than did organisms of M^- streptococcal strain T28/51/4-4 (Fig. 5). Factor H was bound in its isolated form as well as in the presence of whole serum. The binding in the presence of serum was independent of complement activation as indicated by the similarity of the results of experiments carried out in the absence or presence of EDTA. Compared to the binding of C3, factor B, and albumin, the difference in binding between bacteria of the two strains was found to be selective for factor H.

The binding reaction of factor H to M^+ streptococci was studied in greater detail. In contrast to the binding to M^- organisms, the binding of factor H to streptococci carrying the M6 protein was saturable (Fig. 6) and reversible (not shown). The dissociation constant was determined to be 4.3×10^{-6} M, with the average number of binding sites per streptococcus being ≈14,000 (Fig. 7, panel M6). The binding of factor H was not an exclusive property of bacteria of M6-carrying strain D471. Experiments were performed with M^+ streptococci of four additional strains carrying M5, M19, M24, and M28 proteins, respectively. These experiments also resulted in saturable binding of factor H. However, while the binding affinity to streptococci carrying the M6, M19, and M24 proteins was rather similar, organisms of strain B788 carrying the M5 protein and organisms of strain T28/150A/5 carrying the M28 protein exhibited a substantially higher affinity for factor H (Fig. 7). Under the culture and treatment conditions used, the average number of binding sites for factor H per streptococcus was in the range of 10,000–14,000 for the M6, M5, and M19 strains, whereas the M24 and M28 organisms expressed an average of only 2000 and 1000 binding sites, respectively. For the above calculations, homogeneity of binding was assumed, although the results of some of the Scatchard plots might indicate heterogeneous binding behavior.

Binding of Factor H to Purified M6 Protein. The difference between M^+ and M^- streptococci in binding factor H suggested that the streptococcal surface molecule responsible for this effect was the M protein. The binding of factor H to purified recombinant M6 protein was studied by using an enzyme-linked immunosorbent assay. Corresponding to the results obtained with M^+ streptococci, it was found that the isolated M6 protein also bound factor H (Fig. 8).

DISCUSSION

Incubated in whole serum, M^- streptococci have been shown to bind approximately double the number of C3b molecules as do M^+ streptococci, suggesting that the M protein is an inhibitor of complement activation (25). Using purified complement proteins in the absence of the complement control protein factor H, we did not find any significant difference between bacteria of M^+ and M^- strains in the deposition of C3b, the dissociation of C3b,Bb, or the cofactor function for the cleavage of C3b by factor I. In fact, no such function was exhibited by either strain of streptococci, and the spontaneous decay of the C3b,Bb complex appeared to be unaffected by the bacteria as compared with its half-life on zymosan particles. Furthermore, purified recombinant M protein added in a solubilized form did not alter the dissociation of the zymosan-bound C3b,Bb complex, nor did it function as a cofactor for the cleavage of zymosan-bound C3b (unpublished observations). These findings strongly suggest that the mechanism of complement inhibition by the surface of M^+ organisms is not related to the mechanisms of action of the complement control proteins of mammalian cell membranes or to those of known surface proteins found on other pathogens (4, 5, 13–15). Instead, it was only in the presence of factor H that an effect on the regulation of the alternative complement pathway could be attributed to the M protein. In both its functions, factor H was more efficient when C3b was bound to M^+ instead of M^- streptococci. The difference in factor H activity between organisms of both strains was 6- to 8-fold.

The results presented here resemble those findings obtained with sheep and rabbit erythrocytes used as model particles during the initial studies of the regulation of the alternative complement pathway. The different properties of "nonactivating" and "activating" particles were found to be reflected by a critical difference in the efficiency of factor H

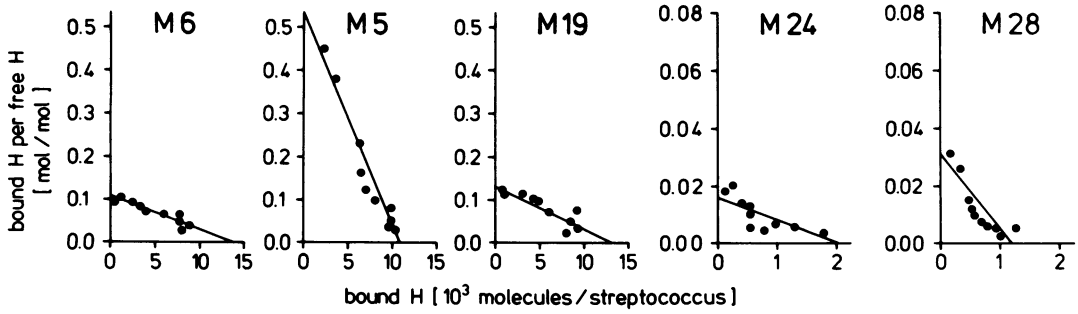

FIG. 7. Scatchard analyses of the binding of factor H to bacteria of the M^+ streptococcal strains D471 (panel M6), B788 (panel M5), 1GL205 (panel M19), 22RS72 (panel M24), and T28/150A/5 (panel M28). Uptake by organisms of strain T28/51/4-4, which is the isogenic M^- variant of the $M28^+$ strain T28/150A/5, was considered background, and the corresponding values were subtracted. Data points represent the mean of triplicate experiments. Assuming homogeneous binding, the calculated dissociation constants, K_d, were: 4.3×10^{-6} M (D471, M6), 6×10^{-7} M (B788, M5), 3.4×10^{-6} M (1GL205, M19), 4.5×10^{-6} M (22RS72, M24), and 1.1×10^{-6} M (T28/150A/5, M28).

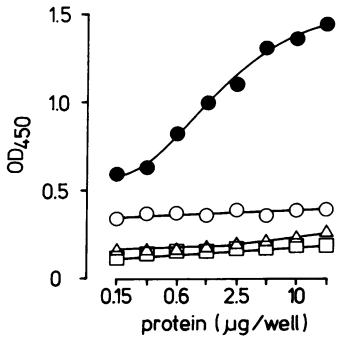

FIG. 8. Binding of purified factor H to purified recombinant M6 protein in an enzyme-linked immunosorbent assay (●). M6 protein was adsorbed to microtiter plates at 5 μg/200 μl per well. Incremental concentrations of factor H were tested for binding, which was determined with polyvalent rabbit anti-factor H antiserum and swine anti-rabbit IgG. For controls, M protein was replaced by ovalbumin (△), factor H by C3 (○), or anti-factor H antibody by anti-C3 antibody (□). Data points represent the mean of duplicate measurements.

to control particle-bound C3b (8, 9). This resulted from an ≈10-fold difference in binding affinity between factor H and particle-bound C3b (9, 34, 40), which was dependent on the structure of the carbohydrate residues exposed on the respective particle (40, 41). Indeed, several human pathogens have been found to be covered by sialic acid moieties, which may support (41) or not impede (9) the binding between human C3b and factor H and, thereby, may prevent alternative-pathway activation upon contact with human serum (10–12). In none of these studies, however, was the direct binding of factor H to a microbial surface observed.

In contrast, our results indicate that M^+ streptococci directly bind factor H to their surface through the M protein. Binding was found to occur in the absence of bound C3b or complement activation. All five streptococcal strains studied carrying different M proteins were found to bind factor H. This underlines the functional significance of the binding reaction. For the calculations presented here, we assumed a homogeneous binding behavior. Whether binding heterogeneity occurs between factor H (34) and M protein of certain M types is currently under investigation.

As known so far, binding of factor H to a microbial surface is a unique property of M^+ streptococci. It appears to be a previously unrecognized route by which a pathogen is able to evade alternative-pathway activation. Based on these findings, we propose the following concept to explain the antiphagocytic surface properties of M^+ streptococci. Upon contact with serum, the M protein selectively binds factor H to the surface of the organism. Any molecule of C3b fixed in the vicinity of this complex is efficiently controlled by factor H, which is present to inhibit or reverse formation of the C3 convertase (6, 7) and to serve as cofactor for the inactivation of C3b by factor I (33). Furthermore, any molecule of C3b bound to an immune complex in the vicinity of the factor H binding site might likewise be inactivated. The lack of C3b deposition on surface areas of the bacteria covered by the M protein will prohibit phagocytosis that is dependent on the binding of phagocyte C3 receptors.

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