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Interaction of Soluble Fibronectin with Group B Streptococci

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Fibronectin binds to a variety of bacterial species, and we hypothesized that differential fibronectin binding might influence the invasive potential of group B streptococci (GBS). Human plasma fibronectin purified by a standard two-step chromatographic procedure was radiolabeled with ^3H. Fifty GBS strains (invasive, colonizing, or bovine) representing serotypes Ia (10 strains), Ib (6 strains), Ia/c (6 strains), II (10 strains), III (11 strains), IV (1 strain), and nontypable (6 strains) were tested. No source or serotype variability was detected among GBS strains, and binding was uniformly less than 1.5% of available fibronectin. Lack of detectable binding occurred at both the log and stationary growth phases and persisted despite treatment with trypsin or neuraminidase or opsonization with immunoglobulin G containing high levels (>40 μg/ml) of antibody specific for the Ia, II, or III GBS capsular polysaccharides. Incubation with GBS did not inhibit fibronectin binding to the Cowan 1 strain of *Staphylococcus aureus*. Strain COH 31-15, an isogenic, type III, capsule-deficient mutant of COH 31r/s, also failed to bind fibronectin. In contrast to other streptococci, GBS do not have readily detectable receptors for soluble fibronectin as part of their surface structures. If present, binding sites for soluble fibronectin are deep to surface structures, obscured from host defense systems, or require the presence of other factors to facilitate their recognition of fibronectin. The uniform ability of GBS to resist binding to soluble fibronectin could be a significant virulence factor in the pathogenesis of invasive infections of infants.

Fibronectin (Fn) is a major protein of blood and tissue that occurs in both soluble (plasma) and insoluble (tissue-associated) forms. This high-molecular-weight glycoprotein, with its many binding sites, is uniquely suited to function as a link between substances. Since its ability to bind staphylococci was first reported in 1978 (15), many bacteria have been shown to interact with Fn (3, 9, 17, 18, 23–25). The interaction between Fn and bacteria is postulated to modulate mucosal surface colonization (1, 21) and may promote phagocytosis and reticuloendothelial clearance of invading organisms (11, 12, 22). Since neonates show time-limited susceptibility to group B streptococcal (GBS) disease and also have physiologically low Fn levels (6, 10), we hypothesized that differential Fn binding could influence the invasive potential of GBS.

A previous study of the interaction of Fn with GBS (17), while suggesting a potential role for Fn in the pathogenesis of GBS, did not address the issue of strain or serotype variability among isolates. A disparity among GBS serotypes or strains within a given serotype in Fn binding might further elucidate the predilection of particular GBS strains to evade neonatal host defenses and cause invasive disease. In this study we examined the interaction of soluble Fn with a variety of GBS strains categorized by source and serotype and possible cell surface structures mediating binding.

MATERIALS AND METHODS

Fn purification and labeling. Fn was purified by the method of Engvall and Ruoslahti (8) as described by Verbrugh et al. (26). All chemicals were purchased from Fisher Scientific Co. (Pittsburgh, Pa.) unless otherwise specified. Fresh citrated human plasma containing 0.2% sodium azide and 10^{-4} M phenylmethylsulfonyl fluoride (Eastman Kodak Co., Rochester, N.Y.) was chromatographed on a gelatin-agarose (Bio-Rad Laboratories, Richmond, Calif.) affinity column in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.15 M NaCl) with 1 mM EDTA and 0.1% bovine serum albumin (BSA; Armour Pharmaceutical Co., Kankakee, Ill.), pH 8. Bound Fn was eluted with 20 mM sodium acetate–1 M sodium bromide–0.1% BSA, pH 5. Phenylmethylsulfonyl fluoride at a final concentration of 10^{-4} M was added to all buffers to inhibit protein degradation. Eluted Fn was dialyzed against 10 mM sodium phosphate–50 mM sodium chloride (pH 8.5), chromatographed on a DEAE ion-exchange column equilibrated with 10 mM phosphate–50 mM NaCl (pH 8.5), and eluted with a linear gradient of sodium chloride (0.05 to 0.7 M NaCl). Purified Fn was concentrated by precipitation with 50% ammonium sulfate. The yield, determined turbidimetrically (Fibronectin, Opsonic Protein Turbidimetric Immunoassay; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), was 40 to 50% of the starting material.

Purified Fn was radiolabeled with [^3H]formaldehyde (specific activity, 75 mCi/mmol; New England Nuclear Corp., Boston, Mass.) by reductive alkylation (19). The specific activity of the labeled protein was ≈2,000 to 2,500 cpm/μg. Functionally active [^3H]Fn was isolated by repeat affinity chromatography, concentrated by precipitation with 50% ammonium sulfate, suspended in PBS-BSA (pH 7.4), dialyzed against the same, filtered (Millex-GV 0.22-μm-pore-size filter unit; Millipore Corp., Bedford, Mass.), and assayed for Fn. The final product was stored at −20°C until used in binding assays.

Preparation of IgG. Human sera containing a high level of antibody, as determined by a radioactive antigen binding assay (4, 14), to the capsular polysaccharide of type Ia, II, or III GBS were chromatographed on a QAE-Sephadex A-50 column (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.048 M ethylene diamine–0.073 M acetic acid buffer, pH 7 (13). Immunoglobulin G (IgG) was eluted with EDTA buffer, concentrated with an ultrafiltration membrane (Diaflo PM30; Amicon Corp., Danvers, Mass.), and dialyzed against PBS, pH 7.4. The preparations used contained IgG

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antibody to the capsular polysaccharides of serotype Ia, II, or III GBS at concentrations of 630, 46, and 1,310 μg/ml, respectively.

Bacterial strains and growth conditions. Fifty strains of GBS (37 isolates from patients with invasive disease, 11 colonizing strains, and 2 of bovine origin) representing serotypes Ia (10 strains), Ib (6 strains), Ia/c (6 strains), II (10 strains), III (11 strains), IV (1 strain), and nontypable (6 strains) were tested. These strains, available in our laboratory, had been maintained with limited passage in Todd-Hewitt Broth (THB; Difco Laboratories, Detroit, Mich.) at −70°C. Fresh isolates were tested when feasible. Two additional GBS strains, designated COH 31r/s and COH 31-15, generously provided by Craig Rubens (University of Washington School of Medicine, Seattle) were included. COH 31r/s is a derivative of a type III GBS clinical isolate, COH 31, made rifampin and streptomycin resistant as described previously (5). COH 31-15 is an isogenic, type III, capsule-deficient mutant of COH 31r/s derived by Tn*916* transposon mutagenesis which does not produce the type III capsule but still expresses the common group B polysaccharide (C. E. Rubens, M. R. Wessels, L. M. Heggen, and D. L. Kasper, Proc. Natl. Acad. Sci. USA, in press). *Staphylococcus aureus* Cowan 1 was provided by Edward O. Mason (Baylor College of Medicine, Houston, Tex.). Two group A streptococcal isolates representing a pharyngeal and a blood culture isolate were obtained from the Microbiology Laboratory, Texas Children's Hospital, Houston.

For each experiment, isolates were subcultured onto blood agar plates, incubated at 37°C overnight, grown in THB at 37°C, and harvested at the log (2 to 4 h in unbuffered medium) or stationary (16 to 20 h) phase. Bacteria were collected by centrifugation (IEC Centra-FR; International Equipment Company, Needham Heights, Mass.) at 550 × *g* for 15 min at 4°C, washed once in 0.15 M NaCl and once in PBS containing 0.1% BSA, (pH 7.4), and resuspended in PBS-BSA to give concentrations of 0.5×10^9 to 1.5×10^9 CFU/ml. For selected experiments, bacteria were grown in the presence of either trypsin (2 mg/ml of THB; Sigma Chemical Co., St. Louis, Mo.) or neuraminidase type V (0.43 U/ml of THB; Sigma).

Opsonization of bacteria. Bacteria were incubated with purified IgG in a shaking water bath (Precision Scientific Group, Chicago, Ill.) for 45 min at 37°C. After centrifugation at 13,000 × *g* for 5 min (Fisher microcentrifuge model 235 B), the supernatant was discarded and the bacterial pellet was suspended in PBS-BSA for use in binding experiments. This method has previously been shown to effect efficient opsonization of bacteria as determined by a luminol-enhanced chemiluminescent and phagocytic assay (2). Selected isolates treated with either neuraminidase or trypsin were opsonized in the same manner and also tested.

Binding experiments. Binding experiments were performed in 1.5-ml polypropylene microcentrifuge tubes (West Coast Scientific, Inc., Emeryville, Calif.) which had been incubated with PBS–0.1% BSA (pH 7.4) overnight to minimize nonspecific Fn binding to the tubes. A 250-μl volume of bacterial suspension was incubated with various concentrations of [^{3}H]Fn (2.5 to 110 μg/ml) in a total volume of 500 μl in a shaking water bath at 37°C for 30 min. In selected experiments, a 500-μl volume of bacteria was used. Bacteria were pelleted by centrifugation at 13,000 × *g* for 5 min, washed three times with PBS-BSA, pelleted, and allowed to dry. The pellet was dissolved in 1 ml of Protosol (New England Nuclear Corp.); 10 ml of Liquifluor (New England Nuclear Corp.)-toluene (Fisher) was added; and the radioactivity was counted in a liquid scintillation counter (Tri-Carb liquid scintillation spectrometer, model 2650; Packard Instrument Co., Inc., Laguna Hill, Calif.). Results were expressed as the percentage of available Fn which was bound by the bacterial pellet.

TABLE 1. Precipitation of tritiated Fn with polyclonal rabbit anti-human Fn antibodies

[^{3}H]Fn/anti-Fn ratio	% Radioactivity precipitated by:	
	Antibody lot 8431	Antibody lot 8432
1:100	94	95
1:10	92	78
1:1	21	6
10:1	8	1
100:1	4	4

As the specific binding of Fn to *S. aureus* has been shown to be pH dependent (17), binding of soluble Fn to GBS also was studied under conditions of varying pH with PBS-BSA as the buffer for experiments at pH 6, 7.4, and 8 and citrate-phosphate buffer at pH 4.

RESULTS

Confirmation of purity and specificity. Ouchterlony gel diffusion with rabbit to human Fn antiserum (kindly supplied by Robert Baughn, Baylor College of Medicine, Houston, Tex.) revealed a line of identity between the Fn preparation and commercially purified Fn (Boehringer Mannheim Biochemicals). Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8 to 20% gradient), we found a band corresponding to a molecular weight of 440,000 and a minor band at 68,000 molecular weight representing albumin, which was added to inhibit nonspecific binding. The specificity of radiolabeled Fn was assessed by precipitation with rabbit antiserum to human Fn. Each of two anti-Fn preparations precipitated more than 90% of the available radioactivity after incubation for 24 h at antibody excess (Table 1). Competitive inhibition with unlabeled commercially obtained Fn reduced the precipitation of radiolabeled Fn by 41%.

Standardization of assay system. The Fn binding assay was standardized with *S. aureus* Cowan 1, an organism with known affinity for Fn. Binding of Fn to *S. aureus* Cowan 1 was readily demonstrated at both the log and stationary growth phases. In the absence of bacteria, Fn binding to the tubes was negligible. The saturability of binding was demonstrated by using a standard 2.5×10^8 CFU inoculum of *S. aureus* and increasing concentrations of Fn over the range of 3 to 110 μg/ml (Fig. 1). The specificity of this binding was confirmed by its inhibition by the addition of excess unlabeled Fn (data not shown). Detection of changes in binding capacity was maximal when lower concentrations (10 to 20 μg/ml) of Fn were added to the fixed number of bacteria. At high concentrations, substantial Fn binding was obscured by the presence of excess unbound Fn. To confirm the suitability of the assay system for detecting Fn binding to streptococci, we selected two group A streptococcal isolates for testing. As with *S. aureus* Cowan 1, binding of soluble Fn to group A streptococci was readily detected over a wide range of concentrations (Fig. 1). Again, binding was saturable, and conditions were most favorable for the detection of Fn binding when lower concentrations of Fn were used and thus the percentage of available radioactivity bound was greater.

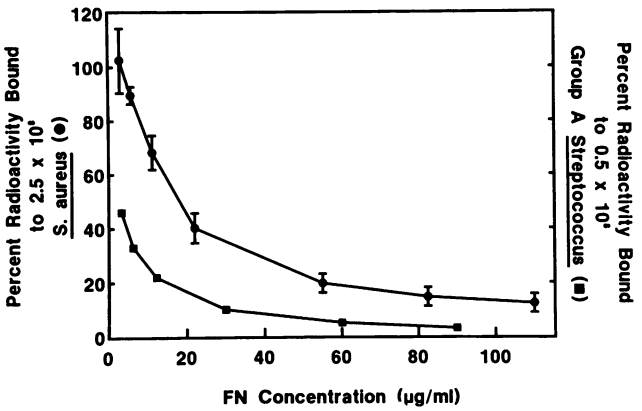

FIG. 1. Percentage of isotope-labeled Fn bound to 2.5×10^8 *S. aureus* cells or 0.5×10^8 group A streptococci at the Fn concentrations indicated. The results represent a mean (± standard deviation) of three determinations (●) or duplicate determinations (■).

S. aureus Cowan 1 demonstrated reproducible binding within this assay system. In 24 experiments performed with 12 μg of Fn per ml, *S. aureus* bound a mean of 68 ± 9.9% of available Fn. Two group A streptococcal strains similarly tested bound a mean of 28% of available Fn when harvested at log phase.

Fn binding to GBS. Initially, GBS isolates were studied for evidence of binding at Fn concentrations ranging from 2.5 to 300 μg/ml. To screen large numbers of isolates, we selected a single concentration permitting maximal discrimination of binding to *S. aureus* Cowan 1 and group A streptococci. Each of the 50 GBS strains was tested in duplicate with Fn at a concentration of 12 μg/ml. In each experiment, *S. aureus* Cowan 1 was included as a positive control. In contrast to *S. aureus*, GBS regardless of source, strain, or serotype consistently bound less than 1.5% of available counts (Table 2).

A randomly selected group of GBS isolates were incubated with 300 μg of Fn per ml, a concentration chosen to approximate the adult human level. Under these conditions of Fn excess, *S. aureus* bound 4.4% of available radioactivity. Again, no significant binding to GBS could be demonstrated (Table 2).

Manipulation of assay conditions. We attempted to enhance Fn binding by manipulation of GBS assay conditions. In two experiments, each run in duplicate, 500 μl of bacteria was incubated with a very low concentration of Fn (1.2 μg/ml), and the bacteria bound a mean of 3.7 and 4.1% of the available radioactivity, respectively. As previously described (17), the Fn binding to *S. aureus* exhibited pH dependency. Binding was maximum at pH 6 (88% of available radioactivity bound). At pH 6, GBS bound 3% of the available radioactivity, higher than that seen at pH 4 (1.7%), 7.4 (0.7%), or 8 (0.5%).

TABLE 2. Fn binding to GBS

GBS serotype	No. of strains tested at:		% (SD) of available Fn bound at:	
	12 μg of Fn/ml	300 μg of Fn/ml	12 μg/ml[a]	300 μg/ml[b]
Ia	10	6	0.4 (0.1)	0.4 (0.1)
Ib	6	1	0.3 (0.1)	0.4
Ia/c	6	3	0.5 (0.3)	0.5 (0.2)
II	10	1	0.3 (0.1)	0.3
III	11	4	0.4 (0.2)	0.3 (0.1)
IV	1	0	0.7 (0.4)	ND[c]
NT[d]	6	2	0.2 (0.1)	0.3 (0.2)

[a] Results represent the mean of duplicate determinations for each isolate tested.
[b] Mean ± standard deviation of percent binding for *S. aureus* Cowan 1 was 68 ± 9.9% at 12 μg/ml and 4.4% at 300 μg/ml.
[c] ND, Not tested.
[d] NT, Nontypable.

TABLE 3. Influence of enzyme treatment or opsonization with type-specific antibody upon Fn binding to GBS

Treatment	% (SD) of available Fn bound by GBS serotype (no. of strains tested):			
	Ia	II ± C protein	III	NT[a]
Trypsin[b]		0.1 (2)		0.2 (2)
Neuraminidase[c]	0.6 (1)		0.4 (1)	
Opsonization with type-specific IgG antibody[d]	0.3 (3)	0.2 (4)	0.2 (5)	
None	0.3 (3)	0.4 (4)	0.4 (5)	0.2 (2)

[a] NT, Nontypable.
[b] 2 mg/ml of THB.
[c] 0.42 U/ml of THB.
[d] Type-specific IgG antibody concentration was ≥40 μg/ml in reaction mixture.

Modification of surface antigenic components. Growth in the presence of trypsin (2 mg/ml of THB) of a nontypable strain and two type II strains known to possess the trypsin-sensitive component of the c protein did not affect binding (Table 3). Growth of type Ia and III strains in the presence of neuraminidase (0.43 U/ml) to cleave a portion of sialic acid residues (6) also did not alter binding. To examine the possible influence of serotype-specific IgG antibody, we tested preopsonized GBS. No enhancement of binding was found (Table 3). To exclude the possibility that the uniformly low binding to GBS could represent an inhibitory phenomenon by the GBS, we incubated Fn with either *S. aureus* or GBS type Ia or III. Binding to the pellet was determined and found to be 47, 0.4, and 0.4%, respectively. Subsequently, each supernatant was incubated with *S. aureus* which bound 52, 56, and 68% of the available radioactivity, respectively. The newly added *S. aureus* bound Fn with the expected avidity regardless of its initial exposure to either *S. aureus* or GBS. This mitigates against an inhibitory effect by GBS on Fn or at least on the receptor for *S. aureus*.

Role of capsule in Fn binding. To further delineate the potential role of the GBS type-specific polysaccharide capsule in inhibiting binding with Fn, we tested the COH 31-15 mutant, lacking the type-specific capsule, and its parent strain, COH 31r/s. Both failed to bind Fn, each binding a mean of 0.4% of the available Fn in contrast to the 78% binding exhibited by *S. aureus* in the same experiment.

DISCUSSION

The preceding experiments indicate that regardless of source, serotype, or strain, GBS uniformly resist binding substantially to Fn. Such uniformity is unusual among gram-positive organisms, in which strain-to-strain variability appears to be common (17, 23). For example, although Fn binds to most staphylococci and promotes their association with neutrophils, there was no single species among the 10 examined by Switalski et al. (23) in which strains were uniformly positive or negative in Fn binding. Myhre and Kuusela (17) presented evidence for a similar heterogeneity

of binding among strains representing group A, C, and G streptococci. These investigators also detected minimal Fn binding to 16 strains of GBS, but neither a description of the isolates by source or serotype nor the growth conditions were specified. In view of the known strain-to-strain variability in binding among isolates of other species, we hypothesized that similar inconstancy might be seen among GBS and account for differences in virulence.

Understanding the interaction of Fn and GBS is potentially important. GBS rarely cause invasive disease in adults, although vaginal colonization is present in up to 30% of adult women. It is attractive to postulate that the physiologically low levels of Fn occurring in neonates is one factor contributing to their susceptibility to invasive GBS disease. While there is no definite correlation between ability to bind Fn and bacterial virulence, significant alterations in Fn concentrations in plasma have been noted in a variety of conditions including sepsis. Enhancement of reticuloendothelial function in septic patients after the infusion of Fn-rich products has been reported (20). The study of Hill and associates (11) was the first to show that Fn promoted the interaction of GBS with neutrophils and antibodies. In their rat model of GBS disease, Fn increased the protective effect of monoclonal and polyclonal IgG preparations against GBS (11). Both this and a study by Jacobs et al. (12), demonstrating that Fn enhanced phagocytosis of GBS by monocytes preopsonized by a commercial preparation of immunoglobulin for intravenous use, suggest a potential role for Fn in the pathogenesis of GBS disease.

Contrary to expectations, our studies indicated that GBS do not bind to soluble Fn in this in vitro system. Since previous studies of the Fn-binding sites for streptococci and staphylococci indicate that such sites are at least closely related, if not identical (16), it is reasonable to expect that an assay system capable of binding to both *S. aureus* Cowan 1 and group A streptococci would detect Fn binding to GBS. To exclude the possibility that long-term storage at −70°C could have adversely affected the isolates, inhibiting Fn binding, we tested fresh isolates, and no binding was detected.

Having failed to detect binding under conditions approximating the physiological state, we manipulated growth conditions to induce binding. Under standard assay conditions, Fn binding to GBS was uniformly less than 1.5%. Manipulation of pH did appear to augment binding minimally, and 3% binding was detected. This contrasted with the 87% bound to *S. aureus* under similar conditions. It is questionable whether this minimal binding has clinical significance. Similarly, alterations of the bacteria-to-Fn ratio allowed an apparent increase in Fn binding (4% Fn bound to GBS), but the total Fn available was significantly lower in this experiment; thus, absolute binding remained unchanged. Treatment with trypsin or neuraminidase failed to alter results. The work of both Hill et al. (11) and Jacobs et al. (12) suggested a role for IgG in enhancing the interaction of Fn with the reticuloendothelial system. We examined the interaction of Fn with GBS isolates which had been opsonized with type-specific IgG antibody to the GBS polysaccharides. The failure of this procedure to enhance binding suggests that any change in bacterial surface charge or configuration promoted by type-specific antibody was not sufficient to expose Fn-binding sites (7). As noted by Hill et al. (11), a direct effect of Fn upon bacteria may require the presence of host cells.

Studies of *Streptococcus pyogenes* and selected staphylococci suggest that Fn receptors are part of the surface structure of these bacteria. In demonstrating its binding to staphylococci, Verbrugh et al. (26) found that Fn would not bind the encapsulated M strain of *S. aureus* and suggested that the capsule was a barrier preventing binding to the cell wall proteins beneath the capsule. While this is not the case for all organisms, the GBS capsule could inhibit binding. To explore this, we tested COH 31-15, a mutant which is deficient in the type III-specific capsule but does express the common group B polysaccharide in the cell wall. Fn binding was negligible. Failure of GBS to bind soluble Fn in this system does not exclude the possibility that alteration of Fn structure or conformation, as might be seen with insoluble Fn, could allow direct interaction with the bacteria. Fn, a complex molecule with many additional binding sites (27), is undoubtedly sensitive to the presence of additional host factors.

In conclusion, unlike most other tested gram-positive organisms, GBS uniformly resist binding to soluble Fn. Binding sites, if present, either require the presence of factors facilitating their interaction with Fn or lie deep to surface structures. Lack of detectable binding sites was a consistent feature among all GBS strains tested regardless of source or serotype. The remarkably uniform capacity of GBS to evade binding to this "molecular glue" could be a feature increasing their invasive potential in neonates, although it is unlikely to account for the interstrain variability in invasiveness seen among GBS.

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