Human Plasma Fibronectin Inhibits Adherence of *Streptococcus pyogenes* to Hexadecane

H. S. COURTNEY,¹ I. OFEK,² W. A. SIMPSON,¹ E. WHITNACK,¹ AND E. H. BEACHEY^{1*}

Veterans Administration Medical Center and the University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104,¹ and Department of Human Microbiology, Tel-Aviv University, Tel-Aviv, Israel²

Received 16 July 1984/Accepted 16 October 1984

The effect of human plasma fibronectin on the adherence of *Streptococcus pyogenes* to hexadecane droplets was investigated. Fibronectin blocked the adherence of streptococci to hexadecane in a dose-dependent manner. The inhibitory effect resulted from the binding of fibronectin to the streptococcal cells; radiolabeled fibronectin failed to bind to the hexadecane but bound readily to untreated streptococci. Chemical treatments of streptococci that decreased streptococcal binding of fibronectin also decreased their binding to hexadecane. Pretreatment of fibronectin with lipoteichoic acid blocked the binding of fibronectin to streptococci and abolished its ability to inhibit streptococcal adherence to hexadecane in a dose-related manner. In contrast, wheat germ agglutinin, which binds to *N*-acetylglucosamine on the surface of *S. pyogenes* cells, failed to alter hexadecane adherence. The data suggest that fibronectin binds to lipoteichoic acid on the surface of the streptococci, thereby preventing lipoteichoic acid from interacting with the hexadecane phase.

Fibronectin, a high-molecular-weight glycoprotein found in various body fluids and on cell surfaces (1, 6, 17), binds to a variety of bacterial species (2, 8, 16). Modulation of bacterial adherence to mucosal cells (1, 24) and enhancement of phagocytosis (9, 16, 19) are some of the properties attributed to fibronectin-bacteria interactions in mammalian hosts. The identity of fibronectin receptors on bacterial surfaces, therefore, is important for a better understanding of the influence of fibronectin on the natural course of bacterial infections.

Group A streptococci were shown by a number of investigators to bind fibronectin (1, 2, 18, 21, 22). The lipid moiety of lipoteichoic acid (LTA) was suggested as a fibronectin receptor because it binds to fibronectin in solution and it blocks binding of fibronectin to streptococci (4). Recently, we and others have shown that the hydrophobic properties of the streptococcal surface can be measured by partitioning in a two-phase polymer system (10, 11) or by adherence to hexadecane droplets (14). Since the hydrophobicity of group A streptococci is due to surface LTA (11), we undertook the present investigation to determine the effect of various treatments of bacteria on fibronectin binding and adherence to hexadecane. We present data to show that fibronectin inhibits the adherence of streptococci to hexadecane and that fibronectin binding to these organisms is dependent on exposure of the lipophilic residues of LTA.

Human plasma fibronectin was purified by gelatin-agarose chromatography, using the method of Engvall and Ruoslahti (5) and was judged to be pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18). Fibronectin was iodinated by the lactose-peroxidase and glucose-oxidase technique (New England Nuclear Corp., Boston, Mass.), using the manufacturer's protocol. The ¹²⁵I-labeled fibronectin was then repurified over a gelatin-Sepharose column and dialyzed against phosphate-buffered saline (PBS; 0.02 M PO₄, 0.15 M NaCl [pH 7.2]). LTA was prepared by growing *Streptococcus pyogenes* M type 5 overnight in Todd-Hewitt broth, washing with distilled water, and extracting the pellet

with cold 50% phenol. The phenol extract was exhaustively dialyzed against distilled water, further extracted with chloroform-methanol (2:1), and purified over a Sepharose 6B column (12). The eluted LTA was chemically analyzed and judged to be pure as previously described (20). The *S. pyogenes* M type 5 used in all assays was grown overnight in Todd-Hewitt broth, washed, and resuspended in PBS.

Treatment of streptococci with agents that abolished their adhesion to hexadecane droplets (hydrophobicity) also reduced the amount of fibronectin bound (Table 1). Treatment of streptococci with pepsin at pH 5.8, which removes a large polypeptide fragment from the outer ends of the M protein molecules (13), did not alter streptococcal hydrophobicity or fibronectin binding but decreased fibrinogen binding by 86%. The latter molecule has been shown to interact with intact surface M protein (23) as well as with pepsin-extracted fragments of this molecule in a stoichiometric manner (E. Whitnack and E. H. Beachey, unpublished data). These data suggest that the NH₂-terminal fragment of M protein which binds fibrinogen does not bind fibronectin, nor does it influence the hydrophobicity of the organisms. Thus, the binding site on the surface of the streptococci for fibronectin is clearly different from that for fibrinogen. These findings are in agreement with work by Miorner et al. (10), who found a lack of correlation between the content of M protein and hydrophobicity.

An assessment of the role of fibronectin in streptococcal adherence to hexadecane was made by mixing various concentrations of fibronectin in 1 ml of a streptococcal suspension (*A*₅₃₀ = 0.4), vortexing for 1 min with 100 µl of hexadecane, and measuring the absorbance of the aqueous phase at 530 nm. Fibronectin inhibited streptococcal adherence in a dose-dependent manner, with >90% inhibition at a concentration of less than 20 nM (Fig. 1). Bovine serum albumin also inhibited adherence to hexadecane, which is consistent with our previous findings that bovine serum albumin binds to both hexadecane (14) and streptococcal LTA (20). In contrast, a 500 nM solution of wheat germ agglutinin, which binds to *N*-acetylglucosamine on the streptococcal surface (15), did not inhibit the adherence of this organism to hexadecane (Fig. 1). The inhibition of adherence

* Corresponding author.

TABLE 1. Hexadecane and protein binding activities of *S. pyogenes* after various treatments

Treatment ^a	Adherence ^b to hexadecane (%)	Fibronectin ^c binding (% of control)	Fibrinogen ^c binding (% of control)
None (PBS)	98	100	100
Pepsin (pH 5.8)	94	142	14
Trypsin	0	13	1
Hot HCl (0.1 N, 100°C, 30 min)	0	22	ND ^d
Heat (100°C, 20 min)	96	96	ND

^a *S. pyogenes* M type 5 was suspended in PBS ($A_{530} = 1.0$), treated as described, washed, and resuspended in PBS ($A_{530} = 0.4$) for the adherence and binding assays. Pepsin and trypsin were used at 1 mg/ml.

^b Hexadecane (100 μ l) was added to 1 ml of treated streptococci and vortexed for 1 min. Adherence values were calculated as follows: % adherence = $1 - (A_{530} \text{ aqueous phase after mixing} / A_{530} \text{ aqueous phase before mixing}) \times 100$.

^c Iodinated fibronectin (1 μ g; 10,000 cpm/ μ g) and iodinated human fibrinogen (1 μ g; 32,000 cpm/ μ g) (KabiDiagnostica, Stockholm, Sweden) were added to 1 ml of streptococci, incubated for 30 min at 37°C, washed with PBS, and counted. Percent binding = (counts per minute bound by treated organisms / counts per minute bound by untreated organisms) $\times 100$.

^d ND, Not done.

to hexadecane by albumin and fibronectin is particularly relevant when one considers that both albumin (20) and fibronectin (4) contain fatty acid binding sites and that LTA can bind to these sites. Experiments in which aqueous solutions of fibronectin were vortexed with hexadecane revealed that less than 5% bound to hexadecane at any concentration tested. These results suggest that the fibronectin-mediated inhibition of streptococcal adherence to hexadecane is due to fibronectin-bacteria rather than fibronectin-hexadecane interactions.

To determine whether LTA plays a role in the fibronectin-mediated inhibition of streptococcal adherence to hexadecane, increasing concentrations of LTA were mixed with 10 μ g of fibronectin and incubated for 1 h at 37°C. A

FIG. 1. Effect of fibronectin on adherence of *S. pyogenes* to hexadecane. Increasing concentrations of protein were added to M type 5 *S. pyogenes* (final volume, 1 ml; $A_{530} = 0.4$). Hexadecane (100 μ l) was then added, and the mixture was vortexed for 1 min. After phase separation was completed, the absorbance of the aqueous phase was measured on a Coleman Jr. II spectrophotometer. Abbreviations: Fn, Fibronectin; BSA, bovine serum albumin; WGA, wheat germ agglutinin.

streptococcal suspension was then added, and the mixture was vortexed with 100 μ l of hexadecane. The absorbance (A_{530}) of the aqueous phase was then measured. LTA abolished the inhibitory activity of fibronectin in a concentration-dependent manner (Fig. 2). A fibronectin-binding study performed concomitantly revealed that LTA also inhibited the binding of fibronectin to streptococci (Fig. 2). When LTA was added alone to the streptococcal suspension at the highest concentration tested (50 μ g/ml), it had no effect on adherence to hexadecane (data not shown).

Speziale et al. (21) suggested that a streptococcal protein serves as a receptor for fibronectin, based on inhibition of fibronectin binding to streptococci by proteinaceous material. The surface exposure of this putative inhibitor, however, was not established. The difference between the results of Speziale et al. and those found in our study with respect to the fibronectin receptor may be due to the difference in organisms used, intact organisms in our experiments versus heat-treated organisms in theirs. Moreover, caution should be exercised in defining a fibronectin receptor based solely on the inhibition of binding of this molecule to streptococci, because fibronectin has an array of binding sites for a variety of ligands (17); the binding of any one ligand may modify the binding of other ligands to the molecule (7). Clearly, more than one criterion should be used to define a streptococcal surface component as a fibronectin receptor. Since our previous studies (4, 19) suggested that the lipid moiety of LTA on the streptococcal surface is a receptor for fibronectin, we took advantage of an assay that measures surface hydrophobicity, i.e., adherence of streptococci to hexadecane, to establish the role of lipophilic residues in binding fibronectin.

FIG. 2. Effect of LTA on the fibronectin-mediated inhibition of the adherence of *S. pyogenes* to hexadecane. Increasing concentrations of LTA were added to a constant amount of fibronectin (10 μ g) and incubated for 1 h at 37°C. A streptococcal suspension was then added (final volume, 1 ml; $A_{530} = 0.4$), and the mixture was vortexed for 1 min. After phase separation was completed the absorbance of the aqueous phase was determined (■). Concomitant with the above, LTA was tested for its effect on binding of fibronectin to streptococci. The same concentrations of LTA, fibronectin, and streptococci, as indicated in the legend to Fig. 1, were used. LTA and 125 I-labeled fibronectin were incubated at 37°C for 1 h. Streptococci were then added, and the mixture was rotated for 30 min at 37°C. Free 125 I-labeled fibronectin was removed by sequential washings in PBS. The pellet was suspended in 100 μ l of water and counted in a gamma scintillation counter (●).

Our results suggest that fibronectin interacts with hydroaliphobic moieties on the streptococcal surface since (i) fibronectin blocks adherence of bacteria to hexadecane, (ii) treatments that abolish surface hydrophobicity also substantially reduced binding of fibronectin, and (iii) treatment which did not affect hydrophobicity did not reduce fibronectin binding. This relationship was specific for fibronectin since fibrinogen, which binds to M protein (23), was not bound by pepsin-treated bacteria that retained their hydrophobic properties. Furthermore, wheat germ agglutinin, a lectin which binds to surface sugars on the streptococci (15), did not inhibit adherence to hexadecane. Since LTA is the major surface component responsible for the hydrophobicity of streptococci (11), we conclude that fibronectin binds to LTA on the surface of the organisms and prevents LTA from interacting with the hexadecane droplets. This conclusion is strengthened by the results showing that preincubation of fibronectin with LTA abolishes the ability of fibronectin to inhibit adherence to hexadecane and those from previous studies demonstrating that LTA binds to fibronectin via its fatty acid moiety (4).

These studies were supported by research funds from the U.S. Veterans Administration and by research grants AI13550 and AI10085. H.S.C. is the recipient of National Institutes of Health predoctoral traineeship award AI07238. E.W. is the recipient of a Research Associate Award from the Veterans Administration.

We thank T. Poirier for critical review of this manuscript.

LITERATURE CITED

1. Abraham, S. N., E. H. Beachey, and W. A. Simpson. 1983. Adherence of *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* to fibronectin-coated and uncoated epithelial cells. *Infect. Immun.* **41**:1261-1268.
2. Babu, J. P., W. A. Simpson, H. S. Courtney, and E. H. Beachey. 1983. Interaction of human plasma fibronectin with cariogenic and non-cariogenic oral streptococci. *Infect. Immun.* **41**:162-168.
3. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M-protein. *J. Exp. Med.* **143**:759-771.
4. Courtney, H. S., W. A. Simpson, and E. H. Beachey. 1983. Binding of streptococcal lipoteichoic acid to fatty acid binding sites of human plasma fibronectin. *J. Bacteriol.* **153**:763-770.
5. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer* **20**:1-5.
6. Grinnel, F. 1980. Fibroblast receptor for cell-substratum adhesion: studies on the interaction of baby hamster kidney cells with latex beads coated with cold insoluble globulin (plasma fibronectin). *J. Cell Biol.* **86**:104-112.
7. Hormann, H., V. Jelinic, and H. Richter. 1983. Generation of fibronectin receptors on macrophages by wheat germ lectin. *Hoppe-Seyler's Z. Physiol. Chem.* **364**:s1011-1018.
8. Kuusela, P. 1978. Fibronectin binds to *Staphylococcus aureus*. *Nature (London)* **267**:680-685.
9. Lanser, M. E., and T. M. Saba. 1981. Fibronectin as a cofactor necessary for optimal granulocyte phagocytosis of *Staphylococcus aureus*. *RES J. Reticuloendothel. Soc.* **30**:415-424.
10. Miorner, H., J. Havlicek, and G. Kronvall. 1984. Surface characteristics of group A streptococci with and without M-protein. *Acta Pathol. Microbiol. Scand. Sect. B* **92**:23-30.
11. Miorner, H., G. Johansson, and G. Kronvall. 1983. Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. *Infect. Immun.* **39**:336-343.
12. Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell. 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. *J. Exp. Med.* **141**:990-1003.
13. Ofek, I., W. A. Simpson, and E. H. Beachey. 1982. Formation of molecular complexes between a structurally defined M-protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. *J. Bacteriol.* **149**:426-433.
14. Ofek, I., E. Whitnack, and E. H. Beachey. 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets. *J. Bacteriol.* **154**:139-145.
15. Pistole, P. G. 1981. Interaction of bacteria and fungi with lectins and lectin-like substances. *Annu. Rev. Microbiol.* **38**:85-112.
16. Pommier, C. G., S. Inada, L. F. Fries, T. Takahashi, M. M. Franks, and E. J. Brown. 1983. Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. Exp. Med.* **157**:1844-1854.
17. Ruoslahti, E. 1981. Fibronectin. *J. Oral Pathol.* **10**:3-13.
18. Simpson, W. A., and E. H. Beachey. 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. *Infect. Immun.* **39**:275-279.
19. Simpson, W. A., D. L. Hasty, J. M. Mason, and E. H. Beachey. 1982. Fibronectin-mediated binding of group A streptococci to human polymorphonuclear leukocytes. *Infect. Immun.* **37**:805-810.
20. Simpson, W. A., I. Ofek, and E. H. Beachey. 1980. Binding of streptococcal lipoteichoic acid to the fatty acid binding sites on serum albumin. *J. Biol. Chem.* **255**:6092-6097.
21. Speziale, P., M. Hook, L. Switalski, and T. Wadstrom. 1984. Fibronectin binding to a *Streptococcus pyogenes* strain. *J. Bacteriol.* **157**:420-427.
22. Switalski, L. M., A. Ljungh, C. Ryden, K. Rubin, M. Hook, and T. Wadstrom. 1982. Binding of fibronectin to the surface of group A, C, and G streptococci isolated from human infections. *Eur. J. Clin. Microbiol.* **1**:381-387.
23. Whitnack, E., and E. H. Beachey. 1982. Antiopsonic activity of fibrinogen bound to M protein on the surface of group A streptococci. *J. Clin. Invest.* **69**:1042-1045.
24. Woods, D. E., D. C. Straus, W. G. Johnson, and J. A. Bass. 1981. Role of fibronectin in the prevention of adherence of *Pseudomonas aeruginosa* to buccal epithelial cells. *J. Infect. Dis.* **143**:784-790.