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Differential Effects of the Streptococcal Fibronectin-Binding Protein, FBP54, on Adhesion of Group A Streptococci to Human Buccal Cells and HEp-2 Tissue Culture Cells

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We have previously demonstrated that fibronectin mediates streptococcal adhesion to host cells and that streptococci interact primarily with the N-terminal domain of fibronectin. FBP54 is a 54-kDa protein from group A streptococci that binds fibronectin. In this report, we show that the N-terminal domain of fibronectin reacts with FBP54 and preferentially blocks streptococcal adhesion to buccal epithelial cells. FBP54 blocked adhesion to human buccal epithelial cells by 80% in a dose-related fashion. In contrast, FBP54 had little effect on adhesion of group A streptococci to HEp-2 tissue culture cells. The fibronectin-binding domain of FBP54 has been localized to the first 89 N-terminal residues of the protein. Experiments using affinity-purified antibodies to this region indicated that the N terminus of FBP54 is exposed on the surface of streptococci in a manner that can interact with immobilized receptors. Analysis of sera from patients with post-streptococcal glomerulonephritis and acute rheumatic fever indicated that FBP54 is expressed in vivo and is immunogenic in the human host. These data indicate that FBP54 is a streptococcal adhesin that is expressed in the human host and that preferentially mediates adhesion to certain types of human cells.

Group A streptococci are responsible for a number of clinical syndromes, including pharyngitis, impetigo, pneumonia, puerperal sepsis, myositis, and the sequelae of such infections, acute rheumatic fever (ARF) and acute glomerulonephritis (AGN). A dramatic increase in the incidence of severe, life-threatening streptococcal infections has recently been noted (34). Antibiotic therapy is not always effective or available, and other forms of treatment are being pursued. These efforts include the development of vaccines that protect the host against colonization and invasion by streptococci, development of adhesin or receptor analogs that block adhesion, and replacement therapy using a commensal organism that competes with group A streptococci for available sites in the host (4, 12–15, 29). In order to develop effective new therapies, a detailed knowledge of the molecular mechanisms utilized by group A streptococci to adhere to host cells is required.

To date, approximately 10 surface components of group A streptococci have been proposed to act as adhesins (2, 3, 5–9, 11, 12, 17–22, 25–28, 30, 35–39). We recently cloned and sequenced the gene for one such component, termed FBP54, a fibronectin-binding protein with a calculated molecular mass of 54 kDa (7). Although FBP54 was expressed on the streptococcal surface, it did not contain the typical LPXTG anchoring motif commonly found in surface proteins of gram-positive bacteria (16). Thus, it was not clear if the N-terminal domain of FBP54 was exposed on the surface of group A streptococci. In this report, we investigated the orientation of FBP54 on the streptococcal surface and the effects of FBP54 on streptococcal adhesion to two types of human epithelial cells. Furthermore, we examined the sera of patients suffering from AGN or ARF for the presence of antibodies to FBP54.

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MATERIALS AND METHODS

Growth of streptococci. M type 5 *Streptococcus pyogenes* Manfredo was incubated for 16 h at 37°C in Todd-Hewitt broth supplemented with 1.5% yeast extract. The bacteria were washed in 0.05 M Tris-HCl–0.15 M NaCl (pH 7.4) (Tris-saline) and suspended in Tris-saline to the indicated concentration.

Purification of fragments of fibronectin. Fibronectin was purified from human plasma by gelatin affinity chromatography. The fibronectin was digested with thermolysin, and fragments were purified by hydroxyapatite chromatography by the method of Zardi et al. (41). Details concerning the generation, purification, and characterization of these fragments have been previously described (33). Figure 1 shows a model of fibronectin and locations of the various domains used in this study.

Purification of FBP54 and its truncated fusion products. Recombinant FBP54 (rFBP54) was purified from extracts of *Escherichia coli* expressing FBP54 by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (7). The construction and purification of the truncated form of FBP54 consisting of the N-terminal first 89 residues (Fp 1-89) fused to a polyhistidine leader sequence have been described elsewhere (7).

Adhesion assays. HEp-2 cells (ATCC CCL23) were obtained from the American Type Tissue Culture Collection, Rockville, Md. Adhesion of group A streptococci to HEp-2 tissue culture cells has been described elsewhere (11). To test effects of rFBP54 on adhesion, the indicated concentrations of rFBP54 were mixed with the streptococci and added to subconfluent monolayers of HEp-2 cells. All reagents were suspended or dissolved in Dulbecco's modified minimal essential medium. After incubation at 37°C for 30 min, the chambers were washed, fixed with 2.5% glutaraldehyde, and stained with Giemsa. The numbers of streptococci attached to at least 50 cells were enumerated. All assays were done in triplicate.

Adhesion of group A streptococci to human buccal epithelial cells has been previously described (11). Essentially, M type 5 *S. pyogenes* cells were mixed with the indicated concentrations of rFBP54 in Tris-saline and added to $\sim 10^4$ buccal epithelial cells. The mixtures were rotated for 30 min and subjected to differential centrifugation three times. The washed buccal cells with adherent bacteria were heat fixed to glass slides and stained with crystal violet, and the numbers of streptococci attached to at least 50 buccal cells were determined. All assays were done in triplicate.

To determine the effects of purified domains of fibronectin on adhesion to buccal cells, serial 10-fold dilutions of each domain were mixed with M type 5 streptococci, added to buccal cells, and assayed as described above. The data were plotted as percentage of inhibition, and the concentration required for 50% inhibition was estimated by determining the *y* intercept for 50% inhibition. The level of adhesion without inhibitors was ~24 streptococci per buccal cell.

All of the assays of adhesion to host cells were done in triplicate in a single assay. The assays were repeated twice with essentially the same results, and only the results from a single experiment are shown.

Production of antisera. New Zealand White rabbits were immunized subcu-

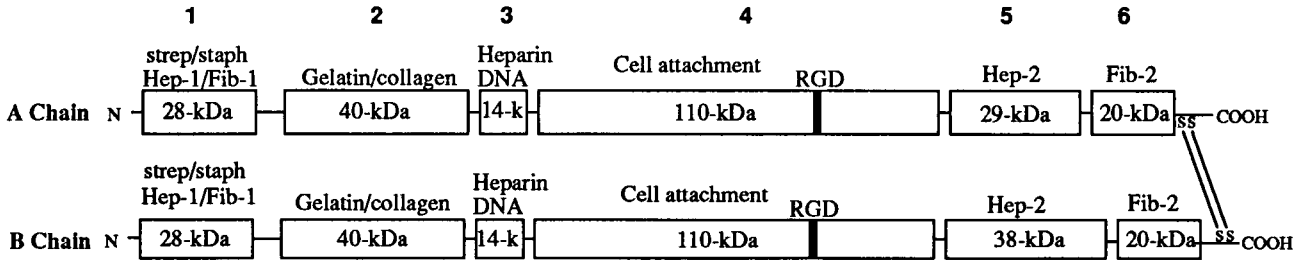

FIG. 1. Schematic diagram of the domains of fibronectin and their functions. Domain 1 is a 28-kDa N-terminal fragment that reacts with streptococci (strep), staphylococci (staph), heparin (Hep-1), and fibrinogen (Fib-1). Domain 2+3 is a 54-kDa fragment that contains the collagen binding domains. Domain 4+5 consists of a doublet of 139- and 148-kDa fragments that contain the cell attachment domain and a second heparin binding domain. Domain 6 is a 20-kDa C-terminal fragment that contains a second fibrinogen binding domain. RGD, tripeptide responsible for cell attachment; ss, disulfide bond between the two polypeptide chains.

taneously with 50 μg of Fp 1-89 emulsified in the adjuvant Imject alum (Pierce, Rockford, Ill.) at a ratio of 4:1. Booster injections were given at 2 and 4 weeks. Antisera were collected every 2 weeks. The production of antisera to rFBP54 has been described elsewhere (7).

Collection of AGN and ARF sera. The AGN and ARF sera were collected from patients in Saudi Arabia under the direction of Walter Norton. There were approximately equal numbers of males and females in each group (the AGN and ARF groups consisted of 47 and 49% females, respectively). Sera were also collected from household contacts of similar ages. Those contacts with indications of current or prior infections due to group A streptococci were excluded from the control group.

Purification of antibodies. Rabbit antiserum to Fp 1-89 was first subjected to QAE-Sephadex chromatography to remove fibronectin and fibrin fragments that could bind to rFBP54 and prevent deposition of antibodies onto the FBP54 affinity matrix. The antiserum was dialyzed against 0.10 M Tris-HCl (pH 6.8) and added to the QAE-Sephadex column equilibrated with the same buffer. The flowthrough containing the antibodies was subjected to affinity chromatography utilizing rFBP54 covalently linked to Reacti-Gel (6X) (Pierce) as specified by the manufacturer. The bound antibodies were eluted with 0.1 M glycine–0.15 M NaCl (pH 3.5) and dialyzed against 0.05 M sodium carbonate (pH 9.5).

Enzyme-linked immunosorbent assays (ELISAs). M type 5 *S. pyogenes* cells were biotinylated as described previously (24). Microtiter wells were coated with affinity-purified antibodies to Fp 1-89 (50 μg/ml) dissolved in 0.05 M sodium carbonate (pH 9.5). Controls consisted of wells coated with bovine serum albumin (BSA) or immunoglobulin G purified from normal rabbit serum (Sigma Chemical Co., St. Louis, Mo.). After coating, the wells were blocked with BSA, reacted with biotinylated streptococci, and then subjected to multiple washes and reaction with Neutralite avidin peroxidase (Molecular Probes Inc., Eugene, Oreg.). The substrate, tetramethylbenzidine, was added, color was allowed to develop, and stop solution was added. The A_{450} was then recorded. Controls were also performed to determine if soluble FBP54 was released from the bacteria during incubation in the microtiter wells. The streptococci were incubated in buffer for 30 min and centrifuged to pellet the bacteria, and the supernatant was used to coat microtiter wells. The wells were then reacted with antibodies to Fp 1-89. There was no reaction, indicating that there was no significant release of FBP54 by the streptococci under the conditions of the assay.

Binding of rFBP54 to domains of fibronectin was determined by coating microtiter wells for 1 h with 50 μg of each fragment per ml in 0.05 M carbonate (pH 9.5). This concentration is well above that required for saturation of the microtiter wells with these fibronectin domains. After coating, the wells were blocked with 1% BSA and reacted with purified rFBP54, 50 μg/ml in Tris-saline containing 1 mg of BSA per ml. The wells were washed and reacted with a 1:1,000 dilution of anti-FBP54 serum for 1 h at 37°C. The wells were then washed and incubated with a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin. Tetramethylbenzidine was added, color was allowed to develop, and stop solution was added. The A_{450} was then recorded. In control experiments, neither the anti-FBP54 serum nor the peroxidase conjugate reacted with any of the domains of fibronectin.

The reaction of ARF and AGN sera with rFBP54 was determined by coating microtiter wells with 50 μg of purified rFBP54 per ml in 0.05 M sodium carbonate (pH 9.5). The wells were then blocked with BSA and reacted with a 1:1,000 dilution of patient serum for 30 min at 37°C. The wells were washed and treated with peroxidase-conjugated goat anti-rabbit immunoglobulin as described above for FBP54.

RESULTS

We have previously demonstrated that FBP54 binds to fibronectin (7) and that fibronectin is involved in adhesion of group A streptococci to human buccal epithelial cells (1, 2, 8, 20, 31, 32). The domain of fibronectin that reacts with group A streptococci has been localized to an N-terminal 28-kDa fragment (10). To determine if this N-terminal domain also reacts with FBP54, various domains of fibronectin that collectively span the entire molecule were immobilized on microtiter wells and reacted with FBP54 (Fig. 2). The results indicate that FBP54 reacts primarily with the N-terminal domain 1 of fibronectin. The purified domains of fibronectin were also tested for their effects on streptococcal adhesion to buccal cells to determine if the domain that reacts with FBP54 and with group A streptococci also blocks adhesion to buccal cells. A concentration of only 0.62 μM domain 1 at was required to achieve 50% inhibition, whereas domains 2+3, 4+5, and 6 did not achieve 50% inhibition at concentrations of ≥3 μM. As expected, domain 1 was the most effective inhibitor of streptococcal adhesion to buccal cells.

These results suggest that FBP54 may be involved in streptococcal adhesion to host cells. Therefore, we tested the effects of FBP54 on adhesion of streptococci to human buccal epithelial cells and HEp-2 tissue culture cells (Fig. 3). FBP54 blocked streptococcal adhesion to buccal epithelial cells in a concentration-dependent fashion. However, FBP54 had little effect on adhesion of group A streptococci to HEp-2 cells.

Previous work indicated that FBP54 was expressed on the surface of streptococci, but its orientation on the surface is unknown (7). Therefore, to determine if the N terminus of FBP54 is exposed on the surface, streptococci were reacted with immobilized antibodies against the N-terminal residues 1 to 89 of FBP54. The rationale for this experiment is that if the N terminus of FBP54 is oriented on streptococci in a manner that allows it to interact with receptors on host cells, then the streptococci should also be bound by antibodies against the N

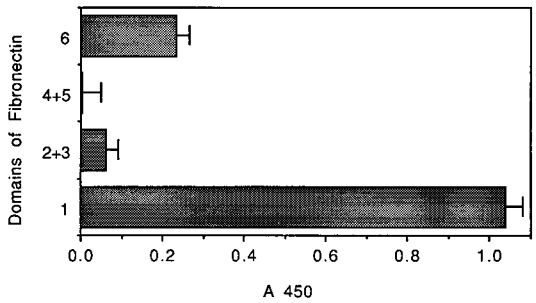

FIG. 2. Identification of the domain of fibronectin that interacts with FBP54. The purified domains of fibronectin were immobilized on microtiter wells and reacted with rFBP54. The levels of binding was assessed by ELISA, using antisera to rFBP54. Each bar represents the mean ± standard deviation of quadruplicate wells from a single experiment.

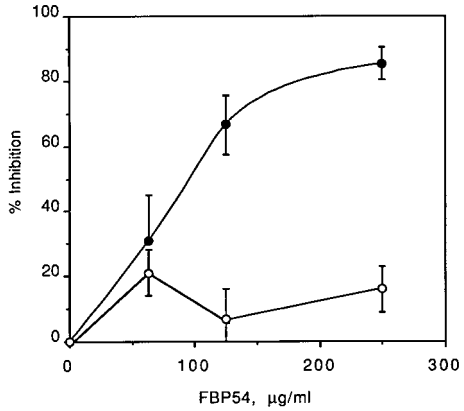

FIG. 3. Inhibition of group A streptococcal adhesion to host cells by FBP54. M type 5 *S. pyogenes* cells were mixed with rFBP54 to obtain the indicated concentrations and tested for effects on adhesion to human buccal epithelial cells (solid circles) and to HEp-2 tissue culture cells (open circles). The symbols represent means, and the bars represent standard deviations. The level of adhesion of streptococci in absence of inhibitors was ~19 streptococci per cell for buccal cells and 55 streptococci per cell for HEp-2 cells.

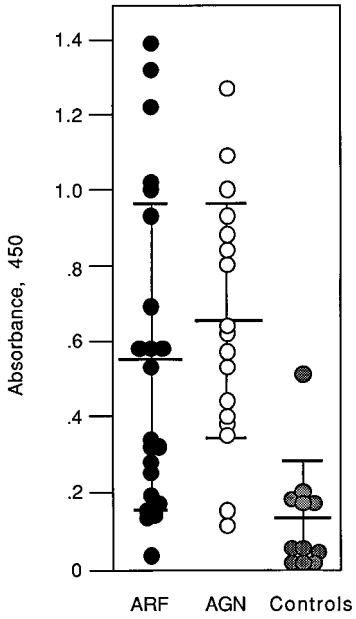

FIG. 5. Reactions of sera from ARF and AGN patients with FBP54. Sera from patients or controls were diluted 1:1,000 and reacted with rFBP54 immobilized on microtiter wells. Individual values are shown. Each bar indicates the mean ± standard deviation. Both the ARF and AGN sera are significantly different from control sera ($P < 0.05$, Student's t test). There is no significant difference between ARF and AGN sera.

terminus of FBP54 immobilized on a surface. Antibodies were raised against Fp 1-89 and affinity purified on an FBP54 affinity column. A nonfusion, intact form of rFBP54 was used as the affinity matrix, thereby eliminating any antibodies directed against the polyhistidine leader sequence and any antibodies that did not react with intact FBP54. The purified antibodies were immobilized on microtiter wells and reacted with streptococci (Fig. 4). The streptococci bound to the immobilized antibodies, indicating that the N-terminal region of FBP54 from residues 1 to 89 is not only exposed on the surface of *S. pyogenes* but also oriented in a manner such that it could interact with immobilized receptors.

It is possible that FBP54 is expressed by group A streptococci in culture but not in the human host. Therefore, we screened sera from ARF and AGN patients to determine if antibodies were made against FBP54 (Fig. 5). Whereas only background levels of antibodies were found in controls, sera from both ARF and AGN patients had significant levels of antibodies.

DISCUSSION

We have postulated that group A streptococci utilize multiple mechanisms to attach to host cells and that the mechanisms of attachment utilized by a particular strain will depend on the strain of streptococci and on the array of receptors expressed by a particular type of host cell (11, 21). For example, we recently found that although M24 protein is not involved in mediating attachment to buccal cells, it does mediate streptococcal attachment to human HEp-2 tissue culture cells and is required for colonization in a mouse model (5, 11). M protein also mediates attachment to keratinocytes but not to Langerhans' cells, whereas protein F mediates adhesion to Langerhans' cells but not to keratinocytes (25, 27).

In this report, we provide further evidence that attachment of group A streptococci to different host cells involves different adhesins. Human buccal epithelial cells and HEp-2 tissue culture cells, a human respiratory epithelial cell line derived from a patient with laryngeal carcinoma, were used to compare the adhesive interactions between streptococci and various types of host cells. HEp-2 cells have been widely used to study bacterial adhesion, invasion, and effects of adhesion on host cells (3a, 22a, 34a) and to investigate streptococcal adhesion in particular (11, 18, 39). A tissue culture cell line such as HEp-2 may provide a convenient source for identification of streptococcal receptors (40). Probes for such receptors could then be used to screen for the presence of these receptors on other human cell types that may have limited availability.

We found that FBP54 differentially affects streptococcal adhesion to these host cells. FBP54 blocked streptococcal adhesion to human buccal epithelial cells but not to HEp-2 cells. That FBP54 does not block streptococcal attachment to HEp-2 cells is not surprising since M proteins have been shown to mediate adhesion of streptococci to this host cell type (5, 11, 39). Furthermore, we found that fibronectin had little effect on adhesion of streptococci to HEp-2 cells, suggesting that fibronectin is not involved in streptococcal adhesion to HEp-2 cells (unpublished data). In contrast, fibronectin does block streptococcal adhesion to human buccal cells (31, 32).

In order for FBP54 to mediate attachment of group A strep-

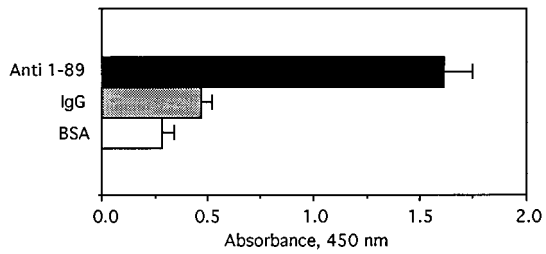

FIG. 4. Binding of group A streptococci to immobilized antibodies to FBP54. Affinity-purified antibodies to Fp 1-89 were immobilized on microtiter wells and reacted with biotinylated M type 5 *S. pyogenes*. Each bar represents the mean ± standard deviation of quadruplicate wells from a single experiment. IgG, immunoglobulin G.

tococci to host cells, it must be expressed in the host. The finding that sera from patients contain antibodies to FBP54 indicates that FBP54 is not only expressed in the human host but also immunogenic in the human host. Sera from a few patients had little or no reactivity with FBP54, possibly because not all strains express FBP54 or the primary structure of FBP54 varies among strains. In addition, immune responses to specific antigens vary among individuals.

For FBP54 to be an adhesin, it must be not only expressed in vivo but also oriented on the bacterial surface in a manner such that it can interact with its receptor immobilized on host cells. The binding of group A streptococci to immobilized antibodies to the N-terminal 89 residues of FBP54 indicates that this region of FBP54 is exposed on the streptococcal surface in an orientation that permits interactions with immobilized receptors. However, these data do not exclude the possibility that other domains of FBP54 are also exposed on the streptococcal surface.

Previous work from our laboratory indicating that fibronectin is a major receptor for group A streptococci on human buccal cells (1, 8, 31, 32) and the present findings that FBP54 blocks adhesion to buccal cells suggest that FBP54 may mediate streptococcal adhesion to buccal cells by interactions with fibronectin. This concept is supported by the observation that FBP54 interacts primarily with the same domain of fibronectin that binds to streptococci and blocks streptococcal adhesion to buccal cells.

Another surface component of group A streptococci that is involved in adhesion to host cells is lipoteichoic acid (LTA). LTA not only blocked streptococcal adhesion to buccal cells but also blocked adhesion to human pharyngeal cells and HEp-2 tissue culture cells (3, 11). Moreover, LTA blocked adhesion of streptococci to oral epithelial cells from mice and blocked colonization of the oral cavity of mice (12). We have proposed that both LTA and a second adhesin are required for optimal adhesion to host cells (11, 21). We believe that the type of second adhesin utilized by streptococci depends on the strain of streptococci and on the type of host cell. This concept is supported by the present studies. In the case of human buccal cells, FBP54 may serve as the second adhesin. Both LTA and FBP54 bind to fibronectin via interactions with the N terminus of fibronectin (10). Whether group A streptococci adhere to buccal cells by the sequential binding of LTA and FBP54 to the same site within the N-terminal domain of fibronectin or by the simultaneous interaction of LTA and FBP54 to different sites in the N terminus is not known. In any case, the presence of two adhesins on the streptococcal surface that interact with fibronectin may increase the avidity of binding.

Streptococcal adhesion to host cells also probably involves other adhesins such as M protein or protein F/Sfb. Not all strains of group A streptococci express the same array of adhesins in a particular environment. FBP54 is not expressed by all strains of group A streptococci (7), and approximately 48% of the strains tested lacked the gene for protein F (23). Furthermore, various adhesins are differentially regulated in response to environmental signals (36). Thus, all group A streptococci probably do not use the same mechanism to adhere to a particular type of host cell. The molecular mechanism(s) that streptococci utilize to adhere to host cells depends on the characteristics of the strain, the nature of the host cell, and the environment in which adhesion takes place.

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