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Binding of *Streptococcus pyogenes* to Soluble and Insoluble Fibronectin

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The interaction of soluble and insoluble fibronectin with *Streptococcus pyogenes* was investigated. Soluble fibronectin bound to *S. pyogenes* in a dose-dependent and irreversible manner. Lipoteichoic acid competitively inhibited the binding of fibronectin to *S. pyogenes* but had little effect on the binding of fibronectin to staphylococci or pneumococci. The phase of growth of the streptococci had a slight effect on binding of fibronectin, with optimal binding occurring in the late log phase. *S. pyogenes* cells bound to fibronectin immobilized on microtiter plates in a dose-dependent and saturable manner. Both soluble fibronectin and lipoteichoic acid inhibited the binding of streptococci to immobilized fibronectin, suggesting that streptococci interact with soluble and insoluble fibronectin in a similar manner. Antibodies to fibronectin blocked the attachment of streptococci to immobilized fibronectin, whereas normal serum had no effect. Adherence of streptococci to buccal epithelial cells was inhibited by antibodies to fibronectin, but not by normal sera or by antibodies to buccal epithelial cells. The data suggest that lipoteichoic acid on the surface of *S. pyogenes* binds to fibronectin exposed on the host cell and that such binding mediates the attachment of streptococci to host cells.

Fibronectins are a family of glycoproteins found in a soluble form in many body fluids (2, 13, 17, 20) and in an insoluble form as a component of cell surfaces, basement membranes, and the extracellular matrices (1, 2, 11, 17, 20, 30). Soluble plasma fibronectin interacts with various bacteria (3, 12, 14, 15, 18), and it has been suggested that cell surface fibronectin may serve as a receptor in the adherence of bacteria to host epithelial cells (24, 29). Fibronectin is a likely candidate as a receptor in the adherence of group A streptococci to host cells, since fibronectin binds to streptococci (14, 15, 18, 25, 26, 28) and inhibits adherence of the organism to host cells (23). In previous studies (1, 23), we have shown that streptococci bind only to epithelial cells coated with fibronectin and that fibronectin inhibits binding in a dose-related manner.

Structural and functional differences have been noted between the various forms of fibronectin. Placental fibronectin exhibits a higher degree of glycosylation than plasma fibronectin (11), and cell surface fibronectin is more potent in hemagglutination and in restoration of normal cell morphology to transformed cells than plasma fibronectin (17). Functional differences have also been noted between the soluble and insoluble forms of plasma fibronectin (10, 16, 22). Because of the possibility that the different forms of fibronectin may interact with streptococci in different fashions, the present investigation was undertaken to compare the binding of viable streptococci to soluble and insoluble fibronectin and to determine the role such interactions may play in the adherence of streptococci to host cells.

MATERIALS AND METHODS

Preparation of bacteria. *Streptococcus pyogenes* type M5, *Streptococcus pneumoniae* EF3114, and *Staphylococcus aureus* Cowan I strain were grown in Todd-Hewitt broth for 18 h at 37°C, washed, and suspended in saline to the indicated concentration. Unless specified otherwise these preparations were used in all subsequent binding experiments.

Preparation of LTA. Lipoteichoic acid (LTA) was extracted from *S. pyogenes* type M5 with aqueous phenol as previously described (6). The bacteria were grown in 60-liter batches of Todd-Hewitt broth for 16 h at 37°C and harvested by filtration with the Pellicon Cassette system (Millipore Corp., Bedford, Mass.). The bacteria were washed and suspended in distilled water (10 g [wet weight]/100 ml of water) and extracted with an equal volume of 95% phenol (Fisher Scientific Co., Fairlawn, N.J.) for 1 h at 4°C. The aqueous phase was removed and extracted with chloroform-methanol (2:1). LTA was purified by molecular sieve chromatography on a column of Sepharose 6B. The fractions containing erythrocyte-sensitizing activity (4) were pooled and lyophilized. Chemical analysis of the purified material performed as previously described (6) revealed a glycerol/phosphate/glucose ratio of 1:1.05:0.037. Quantitative amino acid analysis indicated a protein contamination of less than 0.5%.

Preparation of antisera. Antisera against M protein from type M5 *S. pyogenes* were prepared by an intracutaneous injection behind the necks of New Zealand White rabbits of 100 μg of purified pepsin-extracted M5 protein (pepM5, [7]) emulsified in Freund complete adjuvant. Serum was collected every 2 weeks and stored at 4°C. The production of monoclonal antibodies to pepM5 protein has been described previously (7).

Antibodies to human oral epithelial cells were prepared in New Zealand White rabbits by intravenous injection of 5×10^5 epithelial cells suspended in phsophate-buffered saline (PBS; 0.02 M PO_4, 0.15 M NaCl, pH 7.4) followed by a second injection of the same dose 2 weeks later. Mouse

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antisera to human epithelial cells were prepared by intraperitoneal injection of 10^6 epithelial cells in PBS followed by a second injection 2 weeks later. Sera were collected every 2 weeks and stored at 4°C. The titers of the anti-epithelial cell sera were determined by an enzyme-linked immunosorbent assay (ELISA) with microtiter plates containing immobilized epithelial cells.

Antisera to human plasma fibronectin were prepared by subcutaneous injection of 100 μg of purified human plasma fibronectin emulsified in complete Freund adjuvant into New Zealand White rabbits. A second injection of fibronectin emulsified in Freund incomplete adjuvant was given 2 weeks after the primary injection. Sera were collected every 2 weeks and stored at −70°C. Mouse antisera to human plasma fibronectin were prepared by immunizing mice with 50 μg of purified fibronectin emulsified in Freund complete adjuvant. Sera were collected weekly and stored at 4°C. The titers of the anti-fibronectin sera were determined by an ELISA with human plasma fibronectin immobilized on microtiter plates.

Preparation of fibronectin. Fibronectin was purified by affinity chromatography of human plasma on columns of gelatin-Sepharose as described by Engvall and Ruoslahti (8). In some experiments the fibronectin was iodinated by the lactose-peroxidase and glucose-oxidase technique (New England Nuclear Corp., Boston, Mass.) by the manufacturer's protocol. The [^{125}I]fibronectin was repurified over a gelatin-Sepharose column and dialyzed against saline. The specific activity of the various preparations of labeled fibronectin ranged from 133,000 to 300,000 cpm/μg.

Assays for the effect of phase of growth on the binding of soluble fibronectin to streptococci. *S. pyogenes* cells were grown in Todd-Hewitt broth at 37°C. Samples of the bacteria were removed at the times indicated in the figures, and the absorbance at 530 nm (A_{530}) was measured. Each sample was washed three times in saline and suspended in saline to an A_{530} of 0.4. Iodinated fibronectin (1 μg; specific activity, 133,000 cpm/μg) was added to each sample, and the volume was adjusted to 1 ml. The mixtures were rotated for 30 min at the ambient temperature, and the bacteria were washed three times in saline. The bacterial pellet was resuspended in 100 μl of water and counted in a gamma scintillation counter. Polypropylene tubes that were pretreatd with bovine albumin were used in all binding experiments to minimize nonspecific binding of fibronectin to the walls of the tubes. In these and subsequent binding experiments, nonspecific binding was defined as the amount of labeled fibronectin bound in the presence of a 100-fold excess of unlabeled fibronectin. Nonspecific binding was never greater than 15% of the total and was subtracted from the total binding to obtain values for specific binding.

Assays for the effect of LTA on the binding of soluble fibronectin to *S. pyogenes*, *S. pneumoniae*, and *S. aureus*. Various concentrations of LTA (0 to 200 μg) were added to 10 μg of [^{125}I]fibronectin (specific activity, 280,000 cpm/μg), and the mixtures were added to the bacterial suspensions to obtain a final volume of 1.0 ml and an A_{530} of 0.4. The bacteria were rotated for 1 h at ambient temperature, and the unbound fibronectin was removed by sequential washings in saline. The bacteria were resuspended in 100 μl of water, and associated radioactivity was assayed in a gamma scintillation counter. All experiments were performed in duplicate.

To determine whether LTA competitively inhibited the binding of fibronectin to *S. pyogenes*, various concentrations of [^{125}I]fibronectin (0.5 to 16 μg; specific activity, 310,000 cpm/μg) were added to suspensions of *S. pyogenes* containing 0, 0.2, or 2.0 mg of LTA to obtain a final volume of 1.0 ml and an A_{530} 0.4. The mixtures were rotated for 30 min at ambient temperature, and unbound fibronectin was removed by sequential washings in saline. The amount bound was determined as described above. Precipitation of labeled fibronectin was not detected in control experiments without streptococci.

ELISA for the adherence of *S. pyogenes* to immobilized fibronectin. Purified human plasma fibronectin was immobilized onto microtiter plates (U16, high binding, microwell module; Nunc, Roskilde, Denmark) by adding 100 μl of fibronectin (100 μg/ml in 0.15 M NaCl, pH 9.5) to each well and incubating for 1 to 3 h at 37°C. Afterward, the plates were washed five times with PBS. Unoccupied plastic surfaces were blocked by adding hemoglobin (20 mg/ml in PBS) to each well and incubating for 1 h at 37°C. Hemoglobin was used to block nonspecific binding, since preliminary experiments indicated that 94% of the binding of streptococci to plastic was blocked by hemoglobin. After five washes with PBS, 100 μl of a streptococcal suspension (A_{530}, 0.6) was added to each well, and the microtiter plate was gently agitated by rotary shaking for 30 min at room temperature. The nonadherent bacteria were removed by five washes with PBS. The adherent bacteria were fixed to the plate by heating at 65°C for 10 min. A 1:400 dilution of rabbit anti-pepM5 serum (in 5% bovine serum albumin) was added to each well and incubated for 1 h at 37°C. The plates were washed five times with PBS, and a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG; Cooper Biomedical Inc., Malvern, Pa.) was added and incubated for 1 h at 37°C. The plates were washed five times with PBS, and 100 μl of 5-aminosalicylic acid solution was added to each well. The color was allowed to develop for 15 to 30 min, and the A_{450} was measured with a Dynatech microtiter plate reader. Control wells without streptococci were used as blank wells. An additional control consisted of wells treated with normal rabbit serum instead of rabbit anti-pepM5 sera. The A_{450}s in these wells were essentially the same as the blank wells.

The binding of streptococci to immobilized fibronectin was investigated by adding increasing concentrations of streptococci to wells containing immobilized fibronectin. The streptococci were suspended in PBS to obtain an A_{530} of 0.8. Serial twofold dilutions in PBS were prepared, and 100 μl of each dilution was added to wells containing immobilized fibronectin. The adherence assay was performed as described above.

The effect of LTA and soluble fibronectin on the adherence of streptococci to immobilized fibronectin was determined by mixing various concentrations of each with the streptococcal suspension before the addition of the bacteria to the wells. The specificity of the attachment of streptococci to the immobilized fibronectin was investigated by pretreating the immobilized fibronectin with a 1:10 dilution of normal rabbit serum or a 1:10 dilution of rabbit antifibronectin serum for 1 h at 37°C. The wells were then washed five times with PBS, and the adherence test was performed as described above, except the adherent bacteria were enumerated by using mouse monoclonal anti-pepM5 antibody and peroxidase-labeled rabbit anti-mouse IgG. In control wells without streptococci no absorbance was detected, indicating that the monoclonal antibodies to pepM5 did not react with any wells containing immobilized fibronectin.

ELISA for the adherence of *S. pyogenes* to immobilized oral epithelial cells. The immobilization of oral epithelial cells and

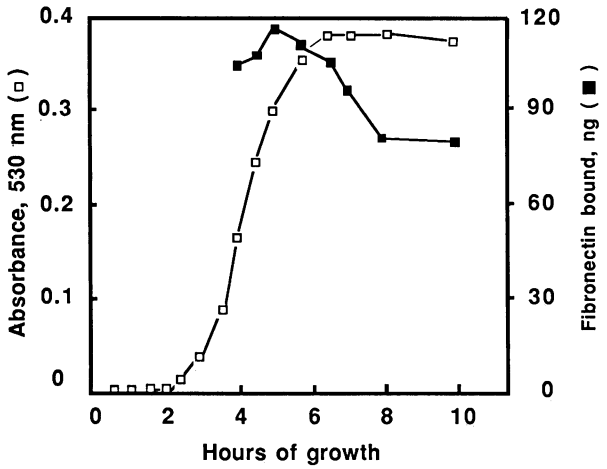

FIG. 1. Effect of the phase of growth of *S. pyogenes* on the binding of fibronectin. Samples of *S. pyogenes* growing in Todd-Hewitt broth were taken at timed intervals, and the A_{530} was measured (□). The samples of bacteria were washed in saline and adjusted to an A_{530} of 0.40. The bacteria were mixed with [^{125}I]fibronectin for 30 min at ambient temperature and washed three times in saline. The radioactivity associated with the bacteria (■) was measured by suspending the bacteria in 100 μl of water and counting in a gamma scintillation counter.

the assay for bacterial adherence to the immobilized cells will be described in detail elsewhere (Ofek et al., submitted for publication). Briefly, microtiter plates (96 wells, flat bottomed; Linbro/Titertek; Flow Laboratories, McLean, Va.) were pretreated consecutively with 1.0 M lysine and 1.25% glutaraldehyde. The plates were then washed with water. Buccal epithelial cells were collected and washed in PBS, and the concentration was adjusted to 1.5×10^5 cells per ml. A sample (100 μl) of the epithelial cell suspension was added to each well of the plate. The plates were centrifuged for 10 min at 2,000 × *g*, and the supernatant was carefully removed. The plates were dried overnight at 37°C and stored at room temperature for up to 3 weeks. Control plates were prepared as described above, except that the epithelial cells were omitted. Unoccupied plastic surfaces were blocked by treating each well with hemoglobin (20 mg/ml in PBS) as described above.

The effect of normal sera, anti-epithelial cell sera, and antifibronectin sera on the adherence of streptococci to the immobilized cells was determined by pretreating the monolayer of cells with a 1:10 dilution of each of the respective sera from either mice or rabbits for 30 min at 37°C. The plates were then washed five times with PBS, and 100 μl of a suspension of *S. pyogenes* (A_{530}, 0.6) was added to all wells and rotated horizontally for 1 h at ambient temperature. The plates were then washed five times with PBS and heated to 65°C for 10 min. In wells pretreated with mouse antisera or rabbit antisera the adherent bacteria were detected with a 1:400 dilution of rabbit anti-pepM5 serum or a 1:400 dilution of mouse monoclonal anti-pepM5 ascites fluid, respectively. The plates were incubated for 1 h at 37°C and then washed five times with PBS. A 1:1,000 dilution of peroxidase-labeled goat anti-rabbit IgG or rabbit anti-mouse IgG was added to appropriate wells and incubated for 1 h at 37°C followed by five washes in PBS. Substrate (5-aminosalicylic acid) was added to each well, the color was allowed to develop for 15 to 30 min, and the A_{450} was measured with a Dynatech microtiter plate reader. Controls consisted of wells devoid of epithelial cells to determine nonspecific binding, and wells without streptococci were used as blanks.

RESULTS

Interaction of soluble fibronectin with *S. pyogenes*. Fibronectin bound to *S. pyogenes* in a dose-dependent but essentially irreversible manner, consistent with the findings of others (26). The phase of growth of the bacteria appeared to have a slight effect on the binding of fibronectin. Optimal binding of fibronectin occurred in the late log phase of growth, with a decrease in binding as the organisms entered the stationary phase (Fig. 1).

The effect of LTA on the binding of fibronectin to *S. pyogenes* was compared with the effect of LTA on the binding of fibronectin to *S. pneumoniae* and *S. aureus*. LTA blocked the binding of fibronectin to group A streptococci but had little effect on the binding of fibronectin to pneumococci or staphylococci (Fig. 2). To determine whether the inhibition by LTA was competitive, various concentrations of labeled fibronectin were added to a suspension of streptococci in the presence or absence of LTA, and the binding was analyzed with a double-reciprocal plot (Fig. 3). The lines converge at the abscissa, indicating that LTA competitively inhibited the binding of fibronectin to *S. pyogenes*.

Interaction of insoluble fibronectin with *S. pyogenes*. The ability of *S. pyogenes* to bind to insoluble fibronectin is illustrated in Fig. 4. Streptococci bound to immobilized fibronectin in a dose-related and saturable fashion. The binding was inhibited by soluble fibronectin or LTA (Fig. 5), suggesting that soluble and insoluble fibronectin interact with *S. pyogenes* in a similar manner. The specificity of the interaction was demonstrated by the ability of rabbit antifibronectin serum to inhibit the interaction of streptococci with immobilized fibronectin. Pretreatment of the immobilized fibronectin with antifibronectin serum decreased the attachment of streptococci by >80%, whereas normal rabbit serum had no inhibitory effect.

Adherence of *S. pyogenes* to epithelial cells. To ascertain whether fibronectin on the surface of buccal epithelial cells plays a role in the adherence of *S. pyogenes*, the epithelial

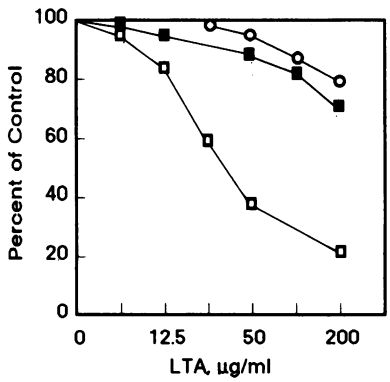

FIG. 2. Effect of LTA on the binding of fibronectin to *S. pyogenes*, *S. pneumoniae*, and *S. aureus*. *S. pyogenes* (□), *S. pneumoniae* (■), and *S. aureus* (○) cells were mixed with various concentrations of LTA and mixed with 10 μg of [^{125}I]fibronectin. The bacteria were rotated for 1 h at ambient temperature, and unbound fibronectin was removed by sequential washings in saline. The bacteria were suspended in 100 μl of water, and associated radioactivity was counted in a gamma scintillation counter. Experiments were performed in duplicate.

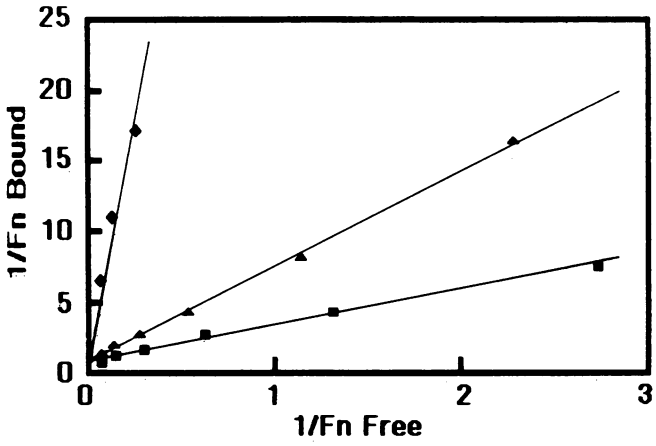

FIG. 3. Competitive inhibition of fibronectin binding to *S. pyogenes* by LTA. Various amounts of [^{125}I]fibronectin (0.5 to 16 μg) were added to streptococci in the presence of 0.2 (▲) or 2.0 (◆) mg of LTA or in the absence of LTA (■). The mixtures were rotated for 30 min and washed three times in saline. The bacteria were suspended in 100 μl of water and counted in a gamma scintillation counter. Experiments were performed in duplicate.

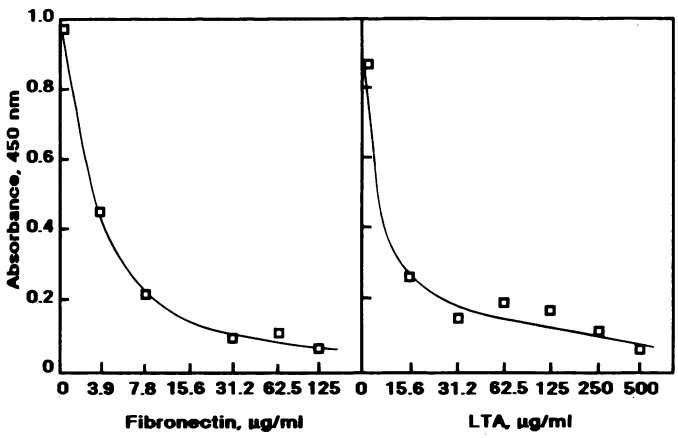

FIG. 5. Effect of soluble fibronectin and LTA on the interaction of *S. pyogenes* with insoluble fibronectin. *S. pyogenes* cells (A_{530} = 0.6) were mixed with various concentrations of purified human plasma fibronectin (0 to 125 μg) or LTA (0 to 500 μg) and added to microtiter plate wells containing immobilized fibronectin. The adherent bacteria were enumerated by an ELISA assay with rabbit anti-pepM5 sera as described in Materials and Methods.

cells were pretreated with antisera raised against human plasma fibronectin. Mouse and rabbit antifibronectin sera inhibited the adherence of *S. pyogenes* by 58 and 76%, respectively, whereas normal sera from mice and rabbits had little or no effect on adherence (Fig. 6). An additional control consisted of pretreating the epithelial cells with antisera against human oral epithelial cell raised in both mice and rabbits. These antisera did not contain any detectable antibodies to fibronectin as measured by an ELISA assay but did contain antibodies to human epithelial cells (A_{450} of 0.6 ELISA). The anti-epithelial cell sera failed to inhibit the adherence of *S. pyogenes* to epithelial cells, suggesting that the inhibition by antifibronectin is specific.

DISCUSSION

Since the initial description of the binding of fibronectin to *S. aureus* by Kuusela (12), various other organisms have been found capable of interacting with fibronectin (3, 18, 29). Previous reports from this and other laboratories have demonstrated that soluble fibronectin also binds to *S. pyogenes* (14, 18, 25, 28). From these and other observations (1, 30), it has been postulated that fibronectin may serve as a host cell receptor for these bacteria (24, 29). However, the binding of soluble fibronectin to bacteria does not necessarily indicate that immobilized fibronectin will also bind. Plasminogen binds to immobilized fibronectin but not to soluble fibronectin (22), and hyaluronate binds only to immobilized fibronectin (16).

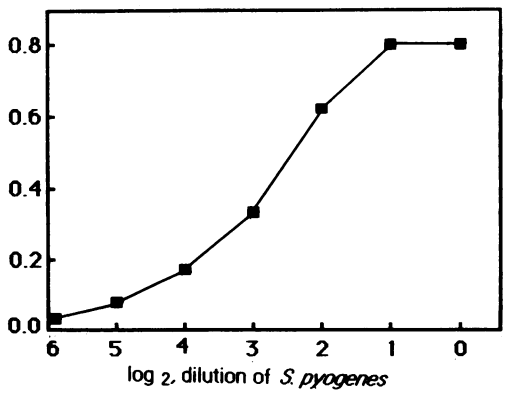

FIG. 4. Interaction of *S. pyogenes* with insoluble fibronectin. Serial twofold dilutions of *S. pyogenes* cells (A_{530}, 0.8) were added to microtiter wells containing immobilized fibronectin. The adherent bacteria were enumerated by an ELISA as described in the text.

In the present investigation, we found that streptococci bound to immobilized fibronectin and that the binding was inhibited by soluble fibronectin and LTA in a dose-related manner. The data suggest that streptococci bind to soluble and insoluble fibronectin by similar mechanisms. Our data are in contrast with those of Kuusela et al. (15), who reported that group A streptococci adhere poorly to immobilized fibronectin. The reason for the discrepancy may be the differences in the organisms used; heat-treated organisms were used by Kuusela et al., whereas live organisms were used in our studies. Alternatively, the type of solid surface used for immobilization may account for the differences. Grinnel and Feld (10) found that fibronectin immobilized on different surfaces can exhibit differences in reactivity with ligands. Kuusela et al. used glass cover slips, whereas plastic microtiter plates were used in our studies. The finding that streptococci can attach to immobilized fibronectin agrees with the results of Simpson et al. (23), who found that immobilization of exogenous fibronectin onto epithelial cells resulted in an increase in adherence of *S. pyogenes* to these cells and that streptococci induced the aggregation of fibronectin-coated beads. Thus, it appears that fibronectin immobilized on three different surfaces can interact with streptococci. Moreover, in each case, the interaction was inhibited by LTA, the streptococcal surface molecule previously shown to interact with fatty acid binding sites of fibronectin (5).

The binding of soluble fibronectin to streptococci was inhibited by LTA in a competitive manner, suggesting that fibronectin binds to LTA exposed on the surface of the organism. This concept is supported by the finding that penicillin induced the release of LTA-fibronectin complexes from streptococci (19).

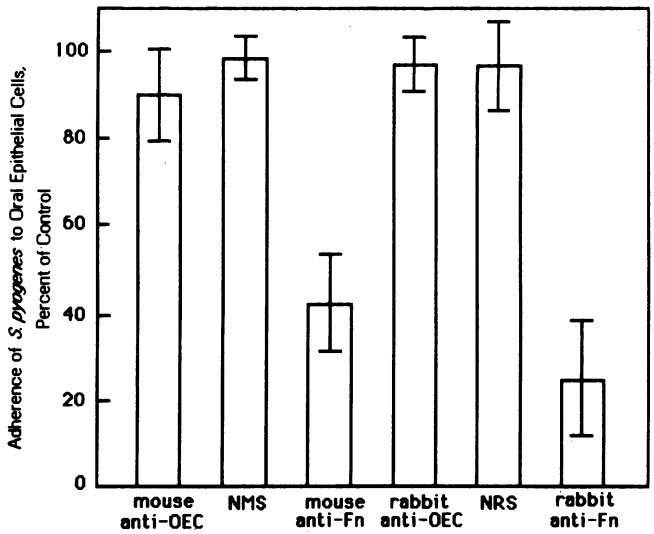

FIG. 6. Effect of anti-fibronectin on the adherence of *S. pyogenes* to epithelial cells. Oral epithelial cells (OEC) immobilized in wells of a microtiter plate were pretreated with 1:10 dilutions of mouse or rabbit antifibronection (anti-Fn) sera, anti-oral epithelial cell sera, normal sera (from mice [NMS] or rabbits [NRS]), or PBS, incubated for 1 h at 37°C, and washed in PBS. A suspension of streptococci (A_{530} = 0.6) was then added to all wells, and the adherence assay was performed as described in the text. The percentage of control was calculated according to the formula [A_{450} of test well)/(A_{450} of PBS control well)] × 100. All experiments were performed in quadruplicate. Bars indicate standard deviations.

To determine the role that endogenous surface fibronectin plays in the adherence of streptococci to epithelial cells, the buccal epithelial cells were pretreated with antifibronectin. Antifibronectin from mice and rabbits inhibited the adherence of streptococci to epithelial cells. The failure of antifibronectin to completely inhibit the interaction raises the possibility that a receptor(s) other than fibronectin may also be involved in the adherence of group A streptococci. It is possible that higher concentrations of antibodies to fibronectin might enhance the inhibition of adherence to epithelial cells. However, Fitzgerald and Repesh (9) achieved partial inhibition of adherence of treponemes to cultured cells with a 1:50 dilution of antifibronectin sera, and this partial inhibition could not be improved by higher concentrations of antifibronectin or by longer incubation times. These authors speculated that, in addition to fibronectin, other receptors may also be involved in the adherence of treponemes.

Nevertheless, the inhibition by antifibronectin suggests that fibronectin is a major receptor on buccal cells for *S. pyogenes*. This concept is reinforced by the observations that (i) *S. pyogenes* selectively adheres to cells rich in fibronectin (1, 28), (ii) the number of streptococci attached to epithelial cells correlates with the amount of fibronectin on the surface of these cells (23), and (iii) fibronectin in solution inhibits the adherence of streptococci to buccal cells (23).

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