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Fibronectin Binding to a *Streptococcus pyogenes* Strain

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In previous studies, *Staphylococcus aureus* has been shown to bind fibronectin (P. Kuusela, Nature (London) **276**:718–720, 1978), an interaction that may be important in bacterial attachment and opsonization. Recently some strains of streptococci of serological groups A, C, and G were also found to bind fibronectin. The binding to one selected strain of *Streptococcus pyogenes* has been characterized here. The binding of [^{125}I]fibronectin to streptococcal cells resembles that to staphylococcal cells and was found to be time dependent, functionally irreversible, and specific in the sense that unlabeled proteins other than fibronectin did not block binding. Bacteria incubated with proteases largely lost their ability to bind fibronectin, and material released from the streptococci by a brief trypsin digestion contained active fibronectin receptors. This material inhibited the binding of [^{125}I]fibronectin to the streptococci. The inhibitory activity was adsorbed on a column of fibronectin-Sepharose but not on a column of unsubstituted Sepharose 4B or egg albumin Sepharose. The receptor appeared to be a protein nature since the inhibitory activity of the trypsinate was destroyed by papain and was not adsorbed on a column containing monoclonal antibodies directed against lipoteichoic acid bound to protein A-Sepharose. Binding sites in fibronectin for streptococci and staphylococci, respectively, were localized by analyzing the ability of isolated fragments to inhibit [^{125}I]fibronectin binding to bacteria and by adsorbing ^{125}I-labeled tryptic fragments with staphylococcal and streptococcal cells. Both species of bacteria appeared to preferentially bind a fragment (M_r = ~25,000) originating from the N-terminal region of the protein. In addition, streptococci also bound a slightly smaller fragment (M_r = ~23,000). Fibronectin receptors solubilized from either streptococci or staphylococci inhibited the binding of fibronectin to both species of bacteria.

Fibronectin is a class of closely related glycoproteins found in connective tissue and in blood plasma (for recent reviews, see references 11 and 14). The ability of fibronectin to serve as a substrate for the adherence of eucaryotic cells is the most prominent biological activity known for the protein, although fibronectin affects many other cellular reactions, e.g., it may act as an unspecific opsonin mediating the phagocytosis by cultured macrophages of gelatin-coated latex beads (3, 21, 23). Fibronectin binds to a number of other biological macromolecules, such as collagen, actin, fibrin/fibrinogen, heparin, and related glycosaminoglycans. By studying the binding properties of fibronectin peptides generated by limited proteolysis of the protein, different binding sites have been identified and mapped along the fibronectin molecule (see the reviews cited above and the references therein).

In 1978, Kuusela reported that fibronectin binds to heat-killed and Formalin-fixed *Staphylococcus aureus* cells (7), and later Mosher and Proctor located the staphylococcal binding site to a peptide derived from the N-terminal region of the protein (12). Recent studies in our and other laboratories have confirmed and extended these findings (2, 20, 22). Some staphylococcal strains contain a fibronectin receptor protein that can be solubilized by treatment with bacteriolytic enzymes (lysostaphin) (15). Also, many streptococcal strains have been shown to bind fibronectin (1, 16, 19). In the present communication, the binding of fibronectin to a *Streptococcus pyogenes* strain is characterized and compared with that between fibronectin and staphylococci. The biological implications of the fibronectin-bacteria interactions are discussed.

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MATERIALS AND METHODS

Chemicals. Fibronectin was purified from human plasma as described by Vuento and Vaheri (24). The protein was ^{125}I-labeled by using the chloramine T method (4). The specific activity of the subsequently isolated protein was estimated to be 5×10^6 cpm/μg. Egg albumin, fetuin (type IV), alpha$_1$-acid glycoprotein, bovine serum albumin, human immunoglobulin G (IgG), trypsin (type III), papain, soybean trypsin inhibitor (type IS), and lysostaphin were purchased from Sigma Chemical Co., St. Louis, Mo. Fibrinogen was obtained from AB KABI, Stockholm, Sweden, and Percoll, CNBr-activated Sepharose, and protein A-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Egg albumin and fibronectin were coupled to CNBr-activated Sepharose by following the recommendations of the manufacturer.

The N-terminal peptide of fibronectin (M_r = 25,000 [25k]), purified after elastase digestion of the protein (8), was kindly provided by J. McDonald, Washington University, St. Louis, Mo. A monoclonal antibody (7 B 11) reacting with polyglycerol-phosphate and classified as an IgG 3 (D. E. Jackson, W. Wong, M. T. Largen, and G. D. Shockman, manuscript in preparation) was generously provided by Jackson, Wong, and Shockman, Temple University, Philadelphia, Pa.

Bacterial strains and culture conditions. *Streptococcus pyogenes* 1321, from a patient with pharyngeal infection, was kindly supplied by A. Ljungh, Central Microbiological Laboratory, Stockholm, Sweden. The strain was classified as a group A streptococcus by the Phadebact coaggutination test (Pharmacia Diagnostics, Uppsala, Sweden). This strain was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C.

Staphylococcus aureus Cowan 1 was cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville,

Md.) under constant rotation for 18 h at 37°C. Both strains of bacteria were harvested by centrifugation (800 × *g*, 20 min, 20°C), resuspended, and washed twice in phosphate-buffered saline (PBS) (0.13 M sodium chloride and 0.02% sodium azide in 10 mM phosphate buffer, pH 7.4). Unless otherwise indicated, bacteria were killed by being heated to 88°C for 20 min. Cells were then suspended in PBS to a cell density of 10^{10}/ml and stored at −20°C. The concentration of bacteria was determined by counting cells in a Petroff-Hausser chamber.

Binding of [^{125}I]fibronectin to bacteria. The method used for quantitating the binding of [^{125}I]fibronectin to bacteria was essentially as described (15). Bacteria (3×10^9 cells) were incubated with 10^5 cpm of ^{125}I-labeled fibronectin in 1.2 ml of PBS containing 0.1% bovine serum albumin. The tubes containing the mixtures were incubated end over end at 20°C for 3 h unless otherwise stated. Subsequently, 200 μl of the incubation mixture was carefully added to 0.5 ml of PBS layered on top of 3 ml of Percoll in 0.15 M NaCl (density, 1.020 g/ml). The samples were centrifuged at 1,350 × *g* for 15 min in a swinging bucket rotor. During centrifugation, bacteria sedimented through the gradient medium and formed a visible pellet at the bottom of the tube, whereas the incubation medium remained as a layer on top of the Percoll. The supernatants were aspirated off, and the radioactivity associated with the pellet was measured in a gamma counter (LKB Wallac, Turku, Finland). Radioactivity recovered after centrifugation of mixtures lacking bacteria (usually 200 to 400 cpm) was considered as background and subtracted from values obtained from incubations containing bacteria. Control experiments showed that the presence of bovine serum albumin did not affect the binding of [^{125}I]fibronectin to bacteria. The plastic tubes used in these experiments (Sarstedt, Princeton, N.J.) were preincubated overnight with 1 ml of 0.1% bovine serum albumin in PBS to minimize unspecific binding of proteins and bacteria to the tubes.

Digestion of fibronectin by trypsin. The purified protein (2.6 mg/ml) was incubated with trypsin (1 μg/ml) in PBS containing no azide at 37°C. After 2 h, soybean trypsin inhibitor (2 μg/ml) was added. Fibronectin fragments were ^{125}I-labeled by using the chloramine T method (4).

Incubation of bacteria with proteolytic enzymes. Two hundred milligrams (wet weight) of cells were suspended in 1 ml of PBS containing no azide and digested with trypsin (25 μg/ml) or papain (10 U/ml) at 37°C. At the indicated times, samples were removed from the incubation mixture, and soybean trypsin inhibitor (50 μg/ml) was added to the trypsin-digested samples. All samples were heated for 10 min at 90°C. Cells were then pelleted by centrifugation, washed once, and suspended in PBS. Cell wall components of *Streptococcus pyogenes* 1321, solubilized by a 1-h trypsin digestion (referred to as "trypsinate"), were used in inhibition experiments. Cells of *Staphylococcus aureus* Cowan 1 were lysed with lysostaphin as described (15).

Electrophoresis in polyacrylamide gels. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Studier (17). The running gel consisted of a linear 5 to 15% acrylamide gradient, and the stacking gel was 3% acrylamide. Protein samples were dissolved in 0.065 M Tris-hydrochloride buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, and 0.001% bromophenol blue, with or without 5% 2-mercaptoethanol, and heated at 100°C for 5 min. The following proteins were used as molecular weight standards (Pharmacia), with molecular weights in parentheses: phosphorylase *b* (94k), bovine serum albumin (67k), egg albumin (43k), carbonic anhydrase (30k), soybean trypsin inhibitor (20.1k), and alpha-lactalbumin (14.4k). The gels were stained with Coomassie brilliant blue G after electrophoresis. Radioactive components were visualized by autoradiography of dried gels with X-omat AR film (Kodak, Rochester, N.Y.).

Estimation of protein concentration. Fibronectin concentration was estimated by determining the absorbance at 280 nm by using an $E_{mg/ml}$ (1-cm) value of 1.28 (10).

RESULTS

Binding of [^{125}I]fibronectin to *Streptococcus pyogenes* 1321. Incubation of *Streptococcus pyogenes* cells with ^{125}I-labeled fibronectin resulted in a time-dependent binding of the radioactive protein to the bacteria. The reaction was rapid; binding of [^{125}I]fibronectin was essentially completed after 10 to 20 min of incubation (Fig. 1). Binding to live and killed streptococci followed the same kinetics, and similar amounts of fibronectin bound to the two forms of bacteria. In future experiments bacteria were always heat killed to avoid possible degradation of cell wall components by bacterial enzymes. The binding capacity of streptococci stored at −20°C remained unchanged for several months.

Incubation of streptococcal cells with increasing amounts of [^{125}I]fibronectin was reflected in a biphasic increase of radioactivity associated with the bacteria (Fig. 2). At low concentrations of added fibronectin, the amounts of labeled protein bound to the bacteria increased rapidly, whereas additions above 6 μg per sample (corresponding to 30 μg/ml) resulted only in a marginal additional increase of bound fibronectin. These results are compatible with a specific binding of fibronectin to a limited number of receptor molecules present on the surface of the bacteria and also to a binding of fibronectin to bacteria that is unspecific and that increases with the amount of radioactivity added.

Under the conditions used, 5×10^8 bacteria bound 0.38 μg of fibronectin when incubated with 30 μg of the protein per ml. If we assume that fibronectin is bound only to specific receptors and that these receptors are saturated with ligand, and further assume a molecular weight of 440k for the

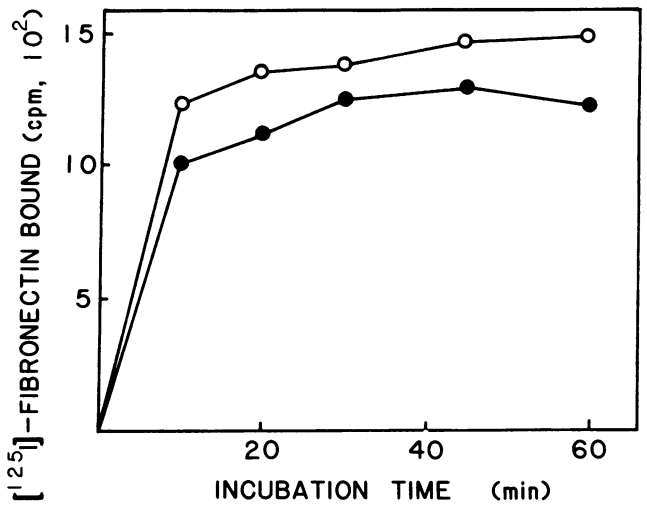

FIG. 1. Time course of binding of ^{125}I-labeled fibronectin to *Streptococcus pyogenes* 1321. Live (○) and heat-killed (●) cells of *Streptococcus pyogenes* 1321 were incubated with [^{125}I]fibronectin for the indicated periods of time as described in the text. Data refer to the amount of protein bound by 5×10^8 bacteria.

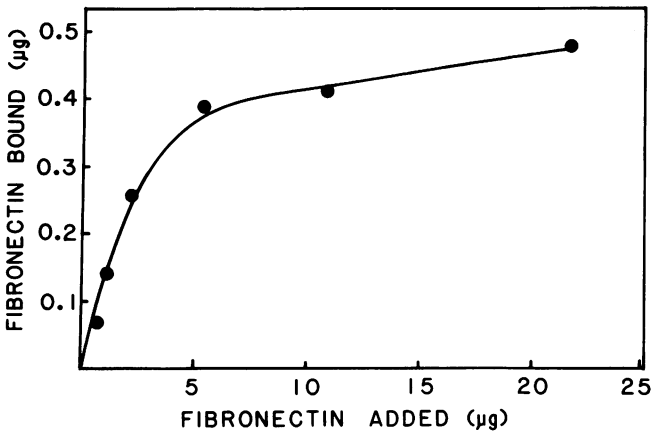

FIG. 2. Binding of [^{125}I]fibronectin to *Streptococcus pyogenes* 1321 as a function of added protein. Increasing amounts of ^{125}I-labeled fibronectin were added to bacteria, and the radioactivity bound to the cells was quantitated. Background values were determined for each concentration of added [^{125}I]fibronectin and were subtracted. Data refer to the samples containing 5×10^8 bacteria.

fibronectin, we can calculate an average of 10^3 fibronectin binding sites per cell.

Binding of fibronectin to both live and heat-killed bacteria was not readily reversible, i.e., ^{125}I-labeled fibronectin bound to the bacteria during an initial incubation was not displaced from the receptors by continuing the incubation in the presence of excess amounts (400 μg/ml) of unlabeled fibronectin (data not shown). This finding indicates that the fibronectin receptor complex is kept together by strong forces. Since the binding of fibronectin to streptococcal cells is functionally irreversible, the necessary requirements for Scatchard plot analysis are not fulfilled. However, an apparent dissociation constant of 5×10^{-9} M can be estimated from the concentration required for half-maximal binding.

When an excess of unlabeled fibronectin was added to the bacteria at the same time as the labeled protein, the binding of [^{125}I]fibronectin to streptococci was inhibited (Table 1), demonstrating that unlabeled fibronectin can successfully compete with the iodinated protein for the receptor. A number of other proteins, including alpha$_1$-acid glycoprotein, egg albumin, fibrinogen, IgG, and fetuin were tried as potential inhibitors of the fibronectin binding to streptococcal cells (Table 1). These proteins were not as effective as unlabeled fibronectin itself, indicating the presence of receptors on the streptococcal cell surface that preferentially bound fibronectin. However, in some cases, other plasma proteins inhibited the [^{125}I]fibronectin binding more than 30%. The significance of these results is unclear (see below).

Localization of bacterial binding site in fibronectin. To localize the binding sites in fibronectin for streptococcal and staphylococcal cells, we studied the bacterial binding activity of peptides obtained by trypsin digestion of fibronectin. To make sure that all bacterial binding sites are conserved in the generated peptides, intact and fragmented fibronectins were compared as inhibitors of the binding to streptococci and staphylococci, respectively (Fig. 3A and 3B). The fragments inhibited the binding to both species of bacteria as well or better than the intact protein. In control experiments, a mixture of trypsin and soybean trypsin inhibitor was not found to affect the binding of [^{125}I]fibronectin to bacteria. Therefore, all bacterial binding sites appear to be retained in the trypsin-generated fragments.

TABLE 1. Effect of some proteins as potential inhibitors of [^{125}I]fibronectin binding to *Streptococcus pyogenes* 1321[a]

Protein	% Inhibition of binding by proteins added to the 1.2-ml incubation mixture	
	100 μg	500 μg
Control, no additives	0	0
Fibronectin	89	100
Fibrinogen[b]	19	34
IgG	13	32
Egg albumin	9	27
Alpha$_1$-acid glycoprotein	20	22
Fetuin	10	9

[a] Various proteins were tested as potential inhibitors of the binding of [^{125}I]fibronectin. The concentrations of the added proteins exceeded 5,000 (100 μg) and 25,000 (500 μg) times the concentration of [^{125}I]fibronectin. The amount of radioactivity recovered in the tubes in the absence of tested proteins after subtraction of the background value was set as 0% inhibition, and all data are expressed as percentages of the control.

[b] Immunochemical analysis showed that the fibrinogen preparation contained a small fibronectin contamination (less than 2% of the total proteins) which could be at least partly responsible for the inhibitory activity of the preparation.

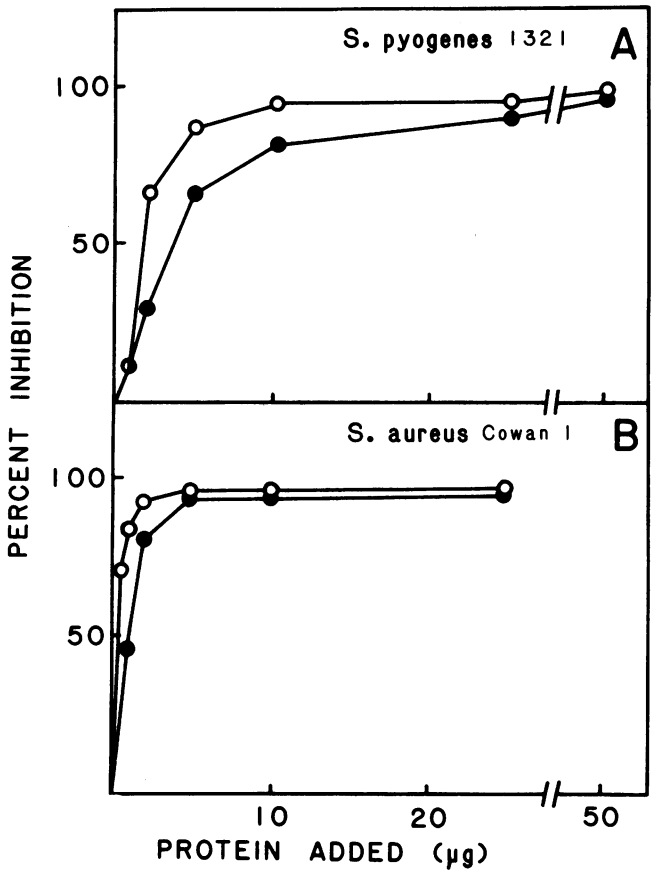

FIG. 3. Inhibition of binding of [^{125}I]fibronectin to *Streptococcus pyogenes* 1321 and *Staphylococcus aureus* Cowan I with intact and trypsin-digested fibronectin. Bacteria (panel A, *Streptococcus pyogenes* 1321; panel B, *Staphylococcus aureus* Cowan I) were incubated with [^{125}I]fibronectin in the presence of an excess of unlabeled intact fibronectin (●) or the same batch of fibronectin peptides generated by trypsin (○). Data are expressed as percentages of the control, i.e., incubation performed in the absence of unlabeled fibronectin or its peptides. The amount of added protein refers to the 1.2-ml incubation mixture.

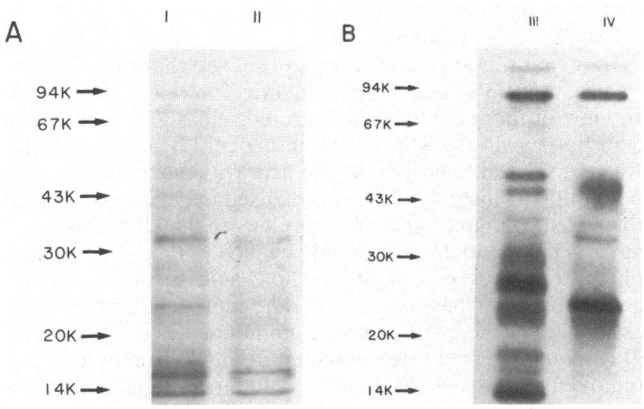

FIG. 4. Gel electrophoresis of fibronectin fragments obtained by trypsin digestion. (A) Coomassie brilliant blue staining or (B) autoradiography. Lanes I and III, samples reduced with 2-mercaptoethanol; lanes II and IV, unreduced. The arrows and numbers indicate the migration distances of standard proteins and their molecular weights. Fibronectin was digested with trypsin and labeled with [^{125}I]iodine as described in the text.

To identify the fibronectin fragments binding to bacteria, the mixture of peptides was labeled by ^{125}I-iodination. Most but not all of the fibronectin fragments were labeled by this procedure (Fig. 4). The radiolabeled peptide mixture was then incubated with streptococci or staphylococci. After separation of the cells from the incubation mixture, the fibronectin peptides bound to the bacteria and those peptides remaining in the incubation medium were analyzed by polyacrylamide gel electrophoresis followed by autoradiography (Fig. 5). Both streptococcal and staphylococcal cells bound one ^{125}I-labeled peptide with an apparent M_r of ~27k. Whereas this peptide appears to be the only fragment associated with staphylococci, streptococci bound an additional peptide (M_r, ~23k). These findings indicated that streptococcal cells bound the same peptide that previously had been shown to bind to staphylococci (12), as well as another somewhat smaller fragment. Since not all fibronectin fragments became ^{125}I-labeled (Fig. 4), it could not be excluded that the bacteria bound peptides other than those detectable in Fig. 5.

The relationship between the fibronectin binding sites for staphylococcal and streptococcal cells was further investigated. Fragmented, unlabeled fibronectin was incubated with streptococci and staphylococci, respectively. After the bacteria, along with associated fibronectin fragments, had been removed by centrifugation, the remaining fragments were tested for their ability to inhibit [^{125}I]fibronectin binding to both bacteria. The results show that fragments remaining after adsorption with streptococci were poor inhibitors of [^{125}I]fibronectin binding to streptococci (Fig. 6A). These remaining peptides had also largely lost their ability to inhibit the binding of [^{125}I]fibronectin to staphylococci (Fig. 6B). Likewise, fragments preadsorbed with staphylococci were poor inhibitors of [^{125}I]fibronectin binding to both staphylococci and streptococci. However, the relative inhibitory potency of the peptides remaining after adsorption with streptococci or staphylococci differed. Thus, binding of [^{125}I]fibronectin to staphylococci was more effectively inhibited with fragments remaining after adsorption with streptococci than with staphylococci (Fig. 6B). Furthermore, peptides preadsorbed with staphylococcal cells were slightly better inhibitors of [^{125}I]fibronectin binding to streptococci than were streptococci-adsorbed fragments. The data indicate that staphylococcal and streptococcal cells may not have exactly identical binding sites in the fibronectin molecule.

The binding site in fibronectin for staphylococci has previously been localized to a fragment with an apparent molecular weight of 25 to 27k obtained from the amino-terminal portion of the protein. Purified fragment of this type was found to be a potent inhibitor of the binding of [^{125}I]fibronectin to both streptococci and staphylococci (Fig. 7). However, whereas [^{125}I]fibronectin binding to staphylococci was completely inhibited by addition of the peptide at a low concentration, the fragment could not totally inhibit the binding to streptococci even when relatively high amounts of peptide were added. Taken together, these results suggest that the N-terminal peptide contains the major binding site(s) for both staphylococcal and streptococcal cells, although streptococci appear to have an additional binding site that may be located outside the N-terminal region represented by the 25k peptide.

Solubilization of fibronectin receptors from streptococcal cells. Recent results indicate that the fibronectin receptor on *Staphylococcus aureus* is a protein (15). The possibility that a similar component on the surface of *Streptococcus pyogenes* 1321 is responsible for the binding of fibronectin to these bacteria was therefore investigated. Streptococcal cells were incubated with trypsin or papain for increasing periods of time. Subsequently, binding of [^{125}I]fibronectin to

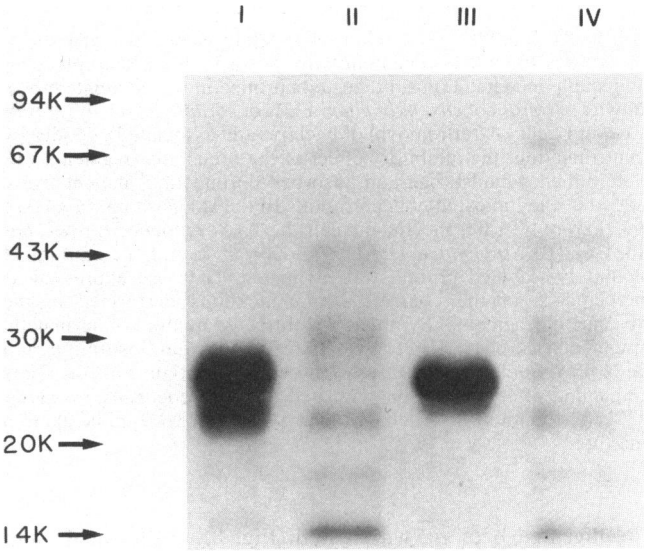

FIG. 5. Electrophoretic analysis of ^{125}I-labeled fragments binding to *Streptococcus pyogenes* 1321 and *Staphylococcus aureus* Cowan I. The ^{125}I-labeled fibronectin fragments were incubated with cells of *Staphylococcus aureus* Cowan I and *Streptococcus pyogenes* 1321 for 3 h. The mixture was then centrifuged, and the supernatant and bacterial pellet were boiled in the presence of 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Both the supernatant and material released from bacterial cells were analyzed by gel electrophoresis. Gels were dried and subjected to autoradiography. Lanes I and III, peptides adsorbed on the surface of *Streptococcus pyogenes* 1321 and *Staphylococcus aureus* Cowan I, respectively; lanes II and IV, peptides remaining in the supernatant of the incubation mixture with *Streptococcus pyogenes* 1321 and *Staphylococcus aureus* Cowan I, respectively. Arrows and numbers in the left margin indicate the migration distances and molecular weights of the standard proteins.

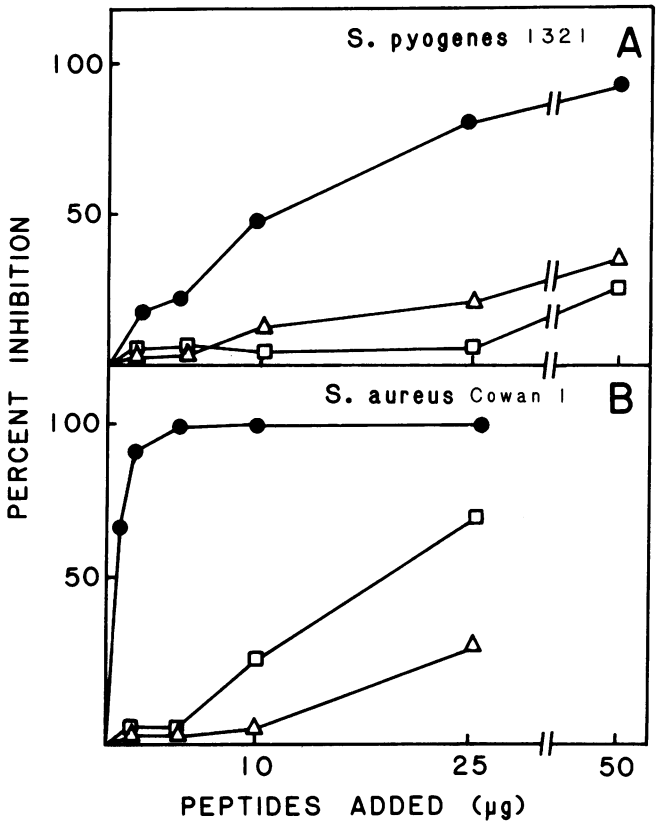

FIG. 6. Inhibition of binding of [^{125}I]fibronectin to bacteria by fibronectin fragments remaining after adsorption with streptococci and staphylococci. Trypsin-digested fibronectin was incubated for 3 h with *Streptococcus pyogenes* 1321 or *Staphylococcus aureus* Cowan I cells. After removal of bacteria and associated peptides by centrifugation, the inhibitory effect of the remaining fragments was determined. Unadsorbed and adsorbed fibronectin fragments were added at the indicated concentrations to the standard mixtures (see the text) of [^{125}I]fibronectin and cells of (A) *Streptococcus pyogenes* 1321 or (B) *Staphylococcus aureus* Cowan I, and the amount of bound ^{125}I-labeled protein was estimated. Data are expressed as percentages of the control, i.e., incubations performed in the absence of fragments. Symbols: ●, untreated mixture of fibronectin fragments; △, mixture of fragments remaining after absorption with cells of *Staphylococcus aureus* Cowan I; □, mixture of fragments remaining after absorption with cells of *Streptococcus pyogenes* 1321. The amount of added peptides refers to the 1.2-ml incubation mixture.

the digested bacteria was tested (Fig. 8). Both papain and trypsin were shown to destroy the fibronectin binding structures on the bacteria, suggesting that these structures are protein(s). The material released from the bacteria by incubation with trypsin was found to inhibit the binding of [^{125}I]fibronectin to streptococci, whereas material released by papain had no activity. The inhibitory activity of the trypsinate was not destroyed by heat inactivation (90°C for 10 min) but was abolished when the trypsin-released material was incubated with papain (data not shown). When the trypsinate was passed through a column of fibronectin-Sepharose, the inhibitory activity was adsorbed (Table 2). In control experiments, the trypsinate was found to retain its inhibitory activity after passage through columns of egg albumin-Sepharose 4B or unsubstituted Sepharose 4B (Table 2). In view of these findings, it seems reasonable to assume that the inhibitory activity in the trypsinate was due to released receptor fragments.

In recent reports (1, 16) it was proposed that the fibronectin receptor on streptococci is identical to lipoteichoic acid. In view of the recently demonstrated release of lipoteichoic acid by trypsin digestion of streptococci (9), the possibility was considered that lipoteichoic acid was the active component in the trypsinate. Trypsin-released material was passed through a column of protein A-Sepharose on which had been adsorbed a monoclonal antibody against poly-glycerol-phosphate. The presence of lipoteichoic acid in the trypsinate before and after passage through the immunoadsorbent was estimated by the enzyme-linked immunosorbent assay (Fig. 9). Lipoteichoic acid was detected in the trypsinate but was removed by passage through the column containing immobilized antibodies. However, the inhibitory activity of the trypsinate was not changed by passing this material through the immunoadsorbent (Table 2). These results demonstrate that lipoteichoic acid is not the major fibronectin receptor on *Streptococcus pyogenes* 1321.

Fibronectin receptors released from streptococcal cells by trypsin digestion and solubilized from staphylococci by incubation with lysostaphin were tried as inhibitors of fibronectin binding to both bacteria. The results of this experiment (Fig. 10) showed that receptor molecules solubilized from streptococci were as efficient as receptors obtained from staphylococci in inhibiting [^{125}I]fibronectin binding to both staphylococci and streptococci. Hence, staphylococcal and streptococcal cells appear to bind to the same site (or closely spaced structures) in the N-terminal region of the fibronection molecule.

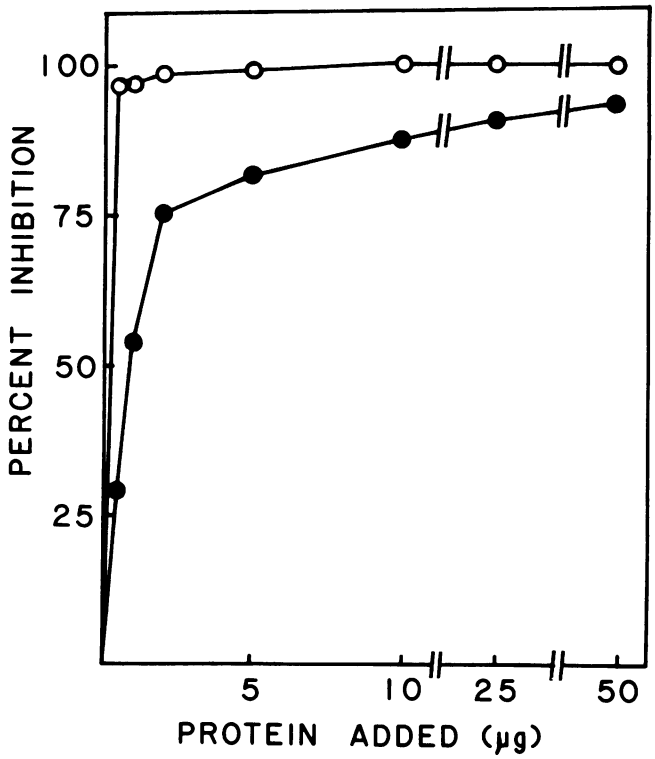

FIG. 7. Inhibition of ^{125}I-labeled fibronectin binding to *Staphylococcus aureus* and *Streptococcus pyogenes* by the N-terminal 25k fibronectin fragment. Increasing amounts of the purified N-terminal fibronectin fragment were added to cells of *Streptococcus pyogenes* 1321 (●) or *Staphylococcus aureus* Cowan I (○). The procedure was the same as that described in the legend to Fig. 6.

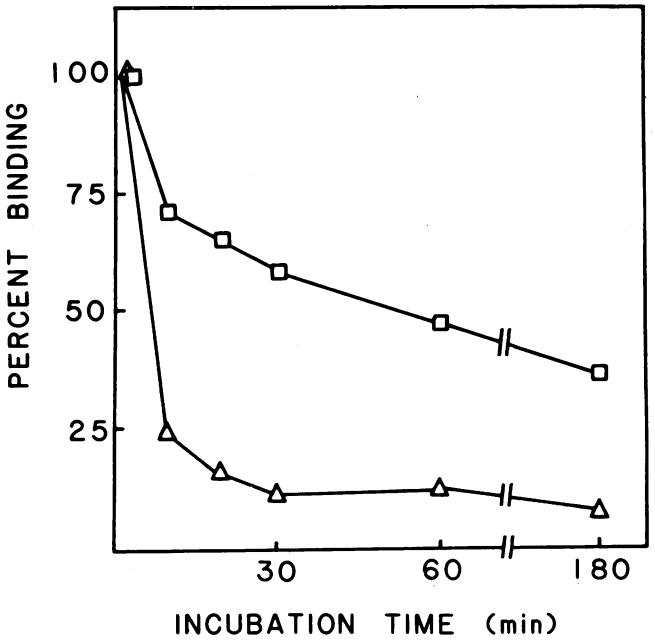

FIG. 8. Binding of [^{125}I]fibronectin to *Streptococcus pyogenes* 1321 preincubated with trypsin or papain. Bacteria were incubated with papain (10 U/ml) (△) or trypsin (25 μg/ml) (□) for the indicated times (for details see the text). Digested bacteria were assayed for [^{125}I]fibronectin binding activity, and binding was expressed as a percentage of the activity of an untreated control.

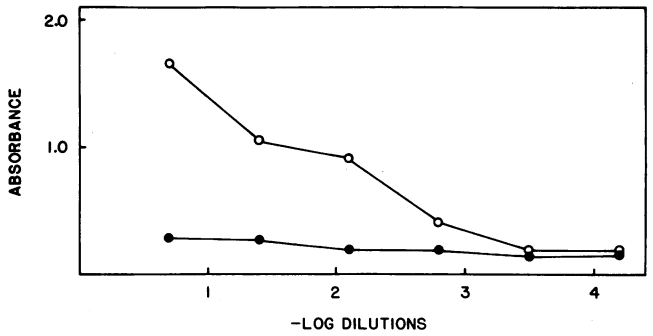

FIG. 9. Lipoteichoic acid in material released from streptococci by trypsin digestion before and after immunoadsorption. *Streptococcus pyogenes* 1321 cells were digested with trypsin as described in the text, and a portion of the released material was adsorbed on a column of anti-poly-glycerol-phosphate antibodies bound to protein A-Sepharose (see Table 2, footnotes *a* and *b*). Samples (200 μl) of dilutions of the trypsinate before (○) and after (●) immunoadsorption were transfered to a multiwell plate (Linbro E.I.A. microtitration plate) and incubated at 4°C overnight. The wells were washed three times with PBS and incubated with 200 μl of 1% bovine serum albumin in PBS for 2 h at 37°C to block remaining protein binding sites on the plastic surface. The wells were washed and incubated at 37°C for 2 h with ascites fluid from a mouse injected with the hybridoma 7 B 11 diluted 100-fold in PBS containing 1% ovalbumin. After extensive washing, 200 μl of the second antibody, phosphatase-conjugated goat anti-mouse IgG + IgM (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), was added at a 100-fold dilution, and the incubation was continued for 1.5 h at 37°C. The wells were washed, and the substrate *p*-nitrophenyl phosphate (Sigma) was added. The absorbance at 405 mm was determined in a Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.).

TABLE 2. Inhibition of binding of [^{125}I]fibronectin to *Streptococcus pyogenes* 1321 by trypsin-released streptococcal cell wall components and adsorption of inhibitory activity[a]

Expt	% Inhibition
No trypsinate added	0
Expt I	
Crude trypsinate	94.5
Trypsinate passed through:	
Sepharose 4B	86.0
Egg albumin-Sepharose 4B	90.0
Fibronectin-Sepharose 4B	7.0
Expt II[b]	
Crude trypsinate	64.5
Trypsinate passed through:	
Protein A-Sepharose complexed with anti-poly-glycerol-phosphate antibodies (7 B 11)	65.0

[a] Trypsinate was produced by digestion of cells of *Streptococcus pyogenes* 1321 as described in the text. Materials was adsorbed on columns of substituted or unsubstituted gels. Three hundred microliters (experiment I) or 200 μl (experiment II) of crude trypsinate or trypsinate passed through the columns was tried as an inhibitor of [^{125}I]fibronectin binding to *Streptococcus pyogenes* 1321 in the standard assay. Data are expressed as percentages of inhibition of binding, relative to the control.

[b] Ascites fluid (0.4 ml) from a mouse injected with the cloned hybridoma 7 B 11 was mixed with 0.5 ml of 1% ovalbumin in PBS and 0.5 ml of protein A-Sepharose. The mixture was incubated (end over end) for 3 h at 20°C. After the gel was washed with PBS, 1.5 ml of the trypsinate was added, and this mixture was incubated for 2.5 h. The gel containing adsorbed proteins was removed by filtration, and the inhibitory activity of samples (200 μl) of the trypsinate before and after adsorption was analyzed.

DISCUSSION

In the present report, the binding of [^{125}I]fibronectin to a strain of *Streptococcus pyogenes* is characterized. The selected strain originated from a collection of strains of streptococci that were isolated from human infections and that previously had been screened for [^{125}I]fibronectin binding (19).

Labeled fibronectin bound to the bacteria could not be displaced by subsequent incubation with a large excess of unlabeled fibronectin, demonstrating that the fibronectin-bacteria interaction is very strong. The binding of [^{125}I]fibronectin was specific in the sense that it was completely inhibited by the presence of unlabeled fibronectin. Other proteins tested were not as efficient inhibitors of [^{125}I]fibronectin binding to the bacteria, although a significant reduction of the bacteria-bound [^{125}I]fibronectin was observed in the presence of some proteins. These proteins may not necessarily compete with fibronectin for its receptor. It is possible that the observed inhibitory effect of the proteins in Table 1 has sterical grounds. Streptococci have been reported to bind galactose units (T. Wadström, unpublished observations), as well as fibrinogen (5) and IgG (6), and so did the selected strain (data not shown). When these components become bound on the surface of the streptococci, they may partly cover the fibronectin receptors and, for sterical reasons, interfere with the fibronectin receptor interaction. Along this line, egg albumin was found to partially inhibit the binding of fibronectin to streptococci, although solubilized fibronectin receptors showed no affinity for egg albumin Sepharose (Table 2). On the other hand, the presence of bovine serum albumin did not interfere with the binding of fibronectin.

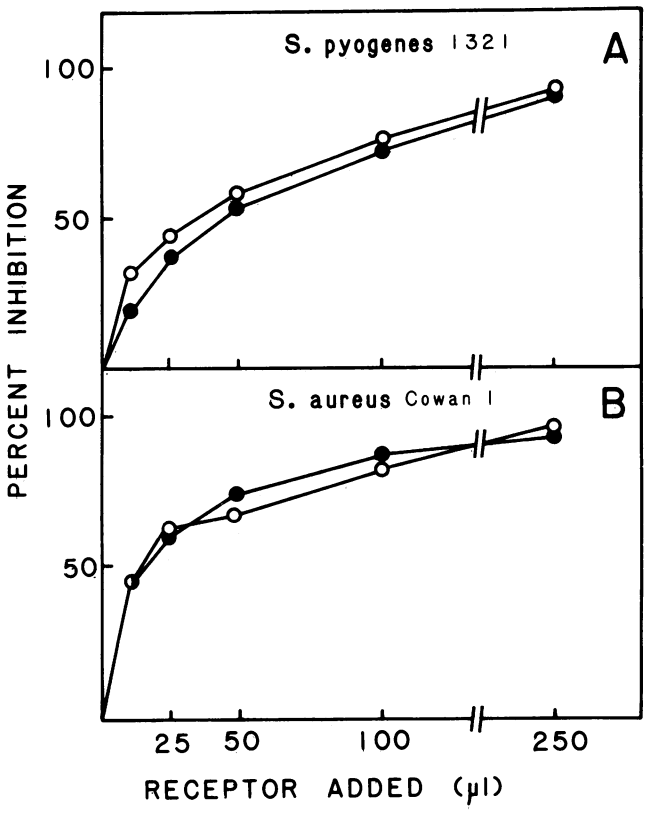

FIG. 10. Inhibition of fibronectin binding to bacteria by solubilized receptors. Fibronectin receptors solubilized from staphylococcal (○) and streptococcal (●) cells by digestion of the bacteria with lysostaphin and trypsin, respectively, were tested as inhibitors of [^{125}I]fibronectin binding to (A) *Streptococcus pyogenes* 1321 and (B) *Staphylococcus aureus* Cowan I. Data are expressed as percentages of inhibition where binding to bacteria incubated in the absence of potential inhibitors was set as 0%. The volume of added solubilized receptors refers to the 1.2-ml incubation mixture.

Digestion of streptococci with proteolytic enzymes reduced fibronectin binding to the cells, and the trypsinate inhibited binding of fibronectin to bacteria. In addition to peptides, the trypsinate contained lipoteichoic acid (9). The latter component could be removed without loss of inhibitory activity. These results demonstrate that lipoteichoic acid is not the major fibronectin receptor on *S. pyogenes* 1321. The inhibitory activity of the trypsinate was lost after papain digestion, suggesting that the major streptococcal receptor is protein.

Fibronectin receptors solubilized from streptococci or staphylococci inhibited the binding of fibronectin to both species of bacteria. Furthermore, an isolated fibronectin fragment inhibited the binding of intact [^{125}I]fibronectin to both streptococcal and staphylococcal cells. These findings indicate that both species of bacteria bind to the same structure (or to closely spaced sites) in the fibronectin molecule. The receptors on streptococci and staphylococci differed in susceptibility to proteolytic digestions; the staphylococcal receptor was released by trypsin digestion but in an inactive form (15), whereas the same treatment released the major streptococcal receptor in an active form. This difference suggests that the primary structures of the major receptor proteins from staphylococci and streptococci may differ.

By analyzing the ability of fibronectin peptides to bind to bacteria or to inhibit the fibronectin-bacteria binding, we located a major bacterial binding site to one peptide (M_r = ~25 to 27k) that is derived from the N-terminal region of the molecule. However, streptococci appear to bind an additional peptide of slightly smaller size M_r = ~23k). Since this 23k peptide apparently does not bind to staphylococci, it is somewhat surprising that receptors solubilized from the two species of bacteria were equally efficient in inhibiting fibronectin binding to either strain of bacteria. This finding could be explained if two types of fibronectin receptors are present on streptococcal cells: one receptor class that is solubilized by trypsin and binds the 25k peptide and a second class which binds to the smaller 23k peptide and is neither solubilized nor destroyed by trypsin treatment.

A large number of streptococcal and staphylococcal strains express fibronectin receptors, although fibronectin receptor-deficient strains have also been isolated (13, 19, 20). It is noteworthy that staphylococci and streptococci, which are not genetically closely related, both express distinct surface proteins that have the ability to specifically bind fibronectin. Staphylococci and streptococci are also the major causes of bacterial wound infections. In view of these observations, it was proposed that fibronectin binding is advantageous to the bacteria and represents a mechanism of bacterial adherence which enables tissue colonization and development of an infection. In a wounded tissue, fibronectin in the intracellular matrix or fibronectin incorporated into the fibrin clot during blood coagulation may become exposed to bacteria in the environment and serve as a substrate to which bacteria may adhere. It should be noted that some staphylococci and streptococci also often bind to fibrin/fibrinogen (5, 18), which may represent a parallel mechanism of bacterial adherence in the wound.

Since fibronectin has been demonstrated to act as an opsonin in some systems, it is tempting to speculate that fibronectin mediates the phagocytosis of bacteria. However, the possible role of fibronectin as an opsonin remains unclear and should be investigated.

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LITERATURE CITED

1. **Courtney, H. S., W. A. Simpson, and E. H. Beachey.** 1983. Binding of streptococcal lipoteichoic acid to fatty acid-binding sites on human plasma fibronectin. J. Bacteriol. **153**:763–770.
2. **Doran, J. E., and R. H. Raynor.** 1981. Fibronectin binding to protein A-containing staphylococci. Infect. Immun. **33**:683–689.
3. **Gudewicz, P. W., J. Molnar, M. Z. Lai, D. W. Beezhold, G. E. Siefring, Jr., R. B. Credo, and L. Lorand.** 1980. Fibronectin mediated uptake of gelatin coated latex particles by peritoneal macrophages. J. Cell Biol. **87**:427–433.
4. **Hunter, W. M.** 1978. Radioimmunoassay, p. 14.1–14.40. *In* D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford.
5. **Kronvall, G., L. Björck, E. B. Myhre, and L. W. Wannamaker.** 1979. Binding of aggregated β_2-microglobulin, IgG, IgA, and fibrinogen to group A, C, and G streptococci with special reference to streptococcal M protein, p. 74–78. *In* M. T. Parker (ed.), Pathogenic streptococci. Reedbooks, Ltd., Chertsey, Surrey, England.

6. **Kronvall, G., A. Simmons, E. B. Myhre, and S. Jonsson.** 1979. Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. Infect. Immun. **25**:1–10.
7. **Kuusela, P.** 1978. Fibronectin binds to *Staphylococcus aureus*. Nature (London) **276**:718–720.
8. **McDonald, J. A., and D. G. Kelley.** 1980. Degradation of fibronectin by human leukocyte elastase. Release of biologically active fragments. J. Biol. Chem. **255**:8848–8858.
9. **Miörner, 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.
10. **Mosesson, M. W., and R. A. Umfleet.** 1970. The cold-insoluble globulin of human plasma. J. Biol. Chem. **245**:5728–5736.
11. **Mosher, D. F.** 1980. Fibronectin. Prog. Hemostasis Thromb. **5**:111–151.
12. **Mosher, D. F., and R. A. Proctor.** 1980. Binding and factor $XIII_a$-mediated cross-linking of a 27 kilodalton fragment of fibronectin to *Staphylococcus aureus*. Science **209**:927–929.
13. **Myhre, E. B., and P. Kuusela.** 1983. Binding of human fibronectin to group A, C, and G streptococci. Infect. Immun. **40**:29–34.
14. **Ruoslahti, E., E. Engvall, and E. Hayman.** 1981. Fibronectin: current concepts of its structure and functions. Coll. Relat. Res. **1**:95–128.
15. **Rydén, C., K. Rubin, P. Speziale, M. Höök, M. Lindberg, and T. Wadström.** 1983. Fibronectin receptors from *Staphylococcus aureus*. J. Biol. Chem. **258**:3396–3401.
16. **Simpson, W. A., and E. H. Beachey.** 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. **39**:275–279.
17. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
18. **Świtalski, L. M.** 1976. Isolation and purification of staphylococcal clumping factor. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. **5**(Suppl.):413–426.
19. **Świtalski, L. M., Å. Ljungh, C. Rydén, K. Rubin, M. Höök, and T. Wadström.** 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.
20. **Świtalski, L. M., C. Rydén, K. Rubin, Å. Ljungh, M. Höök, and T. Wadström.** 1983. Binding of fibronectin to *Staphylococcus* strains. Infect. Immun. **42**:628–633.
21. **van de Water III, L., S. Schroeder, E. B. Crenshaw III, and R. O. Haynes.** 1981. Phagocytosis of gelatin-latex particles by a murine macrophage line is dependent on fibronectin and heparin. J. Cell Biol. **90**:32–39.
22. **Verbrugh, H. A., P. K. Peterson, D. E. Smith, B. T. Nguyen, J. R. Hoidal, B. J. Wilkinson, J. Verhoef, and L. T. Furcht.** 1981. Human fibronectin binding to staphylococcal surface protein and its relative inefficiency in promoting phagocytosis by human polymorphonuclear leukocytes, monocytes, and alveolar macrophages. Infect. Immun. **33**:811–819.
23. **Villiger, B., D. G. Kelley, W. Engleman, C. Kuhn III, and J. McDonald.** 1981. Human alveolar macrophage fibronectin: synthesis, secretion, and ultrastructural localization during gelatin-coated latex particle binding. J. Cell Biol. **90**:711–720.
24. **Vuento, M., and A. Vaheri.** 1979. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. Biochem. J. **183**:331–337.