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# Binding Sites for Streptococci and Staphylococci in Fibronectin

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**Purified cathepsin G fragments of fibronectin were used to locate the binding sites for streptococci and staphylococci in the fibronectin molecule. The iodinated, $NH_2$-terminal, 30-kilodalton (kd) fragment bound to group A and G streptococci and to *Staphylococcus aureus*. The $^{125}I$-labeled, COOH-terminal, 120- to 140-kd fragment bound weakly to group A streptococcus strain and to *S. aureus* when tested in a buffer of low ionic strength. The 30- and 120- to 140-kd fragments inhibited the binding of iodinated fragments to bacteria. The two fragments were, on a molar basis, equally effective, and they were more potent inhibitors than intact fibronectin. The gelatin-binding 40-kd fragment neither bound to any of the bacterial strains nor inhibited the binding of $^{125}I$-labeled 30-kd or $^{125}I$-labeled 120- to 140-kd fragments to bacteria. The results indicate that fibronectin has at least two separate binding sites for streptococci and staphylococci, one in the $NH_2$-terminal region and another in the COOH-terminal region of the molecule, both capable of specific interaction with a complementary structure exposed on streptococcal and staphylococcal cell surfaces.**

Fibronectin is a high-molecular-weight glycoprotein (5, 17, 20, 25) found in association with basement membranes, in interstitial connective tissue matrix (23), and in soluble form in plasma (15) and other body fluids (3, 11, 12, 28, 29). The soluble form of fibronectin is a dimer of two disulfide-bonded, 210- to 250-kilodalton (kd) polypeptides (9, 14, 16, 32). Fibronectin interacts with several kinds of macromolecules, cells, and bacteria. The latter include beta-hemolytic group A, C, and G streptococci and *Staphylococcus aureus* (10, 19, 24). Limited proteolysis has been an important approach in identifying structural and functional features of fibronectin, and many of the interactions have been located in specific domains in the molecule. The binding site for staphylococci and streptococci has been located to the $NH_2$-terminal end of fibronectin (18, 24), but there is also evidence for two other possible binding sites for staphylococci in the 120-kd, COOH-terminal region of fibronectin (7).

For the present communication, we studied the interaction of purified cathepsin G-cleaved fragments of fibronectin with different strains of streptococci and with *S. aureus*. Our results indicate that there are at least two separate binding sites in the fibronectin, both capable of interaction with a complementary structure on streptococcal and staphylococcal cell surfaces.

## MATERIALS AND METHODS

**Bacterial strains.** A group A streptococcus (A-11), a bovine group G streptococcus (DG-26), a human group G streptococcus (G-16), and an *S. aureus* strain (Cowan I) were included as fibronectin binding organisms identified in a previous study (19). The group B streptococcus (B-1) was used as a nonbinding strain (19). Bacteria were maintained on sealed blood agar plates and transferred every 4 to 6 weeks or stored at −80°C suspended in fetal calf serum. For experiments, bacteria were grown for 16 h in Todd-Hewitt broth, washed with 0.01 M phosphate-buffered saline (PBS) containing 0.02% sodium azide, stabilized by heating to 80°C for 5 min, rewashed, and finally suspended in the PBS-azide buffer (19). The concentrations of bacteria were determined by a microhematocrit analysis, in which it was assumed that the 0.5% suspension contained $10^9$ bacteria per ml.

**Cathepsin G and fibronectin.** Cathepsin G (EC 3.4.21.20), a gift from Jeremy Saklatvala, Strangeways Research Laboratory, Cambridge, United Kingdom, had been purified from human neutrophil leukocytes as described previously (27) and had an activity of 350 U/mg in the azocasein-hydrolysis assay described by Starkey (22). Fibronectin was purified from human plasma by the gelatin-Sepharose method (4), modified as described previously (30).

**Isolation of fragments of fibronectin.** Purified fibronectin (1,500 to 3,000 μg/ml) was digested with cathepsin G in 50 mM Tris-hydrochloride (pH 7.5) at 37°C for 10 s, using an enzyme-to-substrate ratio of 1:200 (wt/wt). The digestion was terminated by adding phenylmethylsulfonyl fluoride to give a final concentration of 1 mM. Defined fragments (the $NH_2$-terminal 30-kd, the gelatin-binding 40-kd, and the gelatin-nonbinding COOH-terminal 120- to 140-kd fragments) were isolated by sequential affinity chromatography, using gelatin-Sepharose, spermine-Sepharose, and heparin-Sepharose as described previously (26, 27). The purified fragments were analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (13; see Fig. 1).

**Iodination of fragments.** Iodination was performed by the chloramine-T method (6). The specific radioactivities for the 30-, 40-, and 120- to 140-kd fragments were $1.6 \times 10^7$, $7.5 \times 10^6$, and $5.1 \times 10^6$ cpm/μg, respectively. In the case of the 120- to 140-kd fragment, the solid-phase lactoperoxidase-catalyzed iodination was also used, yielding a specific radioactivity of $2.6 \times 10^7$ cpm/μg of protein (8). The labeled proteins were separated from the free isotope by gel filtration on Sepharose G-25 columns equilibrated with PBS containing 1% (wt/vol) bovine serum albumin (PBS-BSA buffer).

**Binding assay.** $^{125}I$-labeled cathepsin G fragments (3 to 4 ng; $2.8 \times 10^4$ to $4.5 \times 10^4$ cpm, chloramine-T iodinated) were mixed and incubated with $2 \times 10^8$ to $2.5 \times 10^8$ bacterial cells in 0.5 ml of 50 mM Tris-hydrochloride (pH 7.5) containing 1% BSA (Tris-BSA buffer) or PBS-BSA buffer with or without 100 μl of normal human serum for 2 h at room temperature. Serum was used as a source of fibronectin to inhibit the binding and to determine the binding independent

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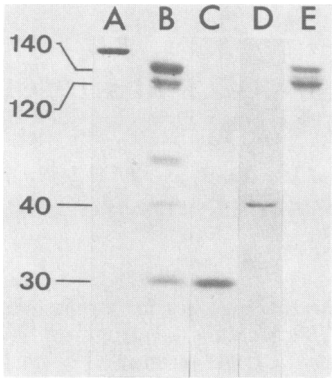


FIG. 1. Purified fibronectin and its cathepsin G fragments analyzed in 9% SDS-polyacrylamide gel electrophoresis. Lanes: A, intact fibronectin; B, cathepsin G digest of fibronectin; C, 30-kd $NH_2$-terminal fragment; D, 40-kd gelatin-binding fragment; E, 120- to 140-kd COOH-terminal fragment. Molecular weight markers (in kd) are shown on the left.

of fibronectin. After incubation, the bacteria were pelleted by centrifugation and washed once with 2.0 ml of PBS containing 0.1% BSA, and the radioactivity associated with the bacteria was counted. The bound radioactivity was expressed as the percentage of the added radioactivity after subtraction of the reactivity associated with bacteria tested in the presence of normal human serum.

**Inhibition assay.** Bacterial cells ($2 \times 10^8$ to $5 \times 10^8$) were incubated with a $^{125}$I-labeled, 30-kd fragment (2 ng; $3.8 \times 10^4$ cpm, chloramine-T iodinated) or with a $^{125}$I-labeled, 120- to 140-kd fragment (18 ng; $4.8 \times 10^5$ cpm, lactoperoxidase iodinated) together with fibronectin or its fragments in 250 or 300 μl of PBS-BSA buffer for 2 h at room temperature, and the radioactivity bound to bacteria was measured as described above. The binding of the label was calculated according to the formula [(counts per minute bound in the presence of inhibitor)/(counts per minute bound in the absence of inhibitor)] × 100. Molar concentrations of the 30-, 40- and 120- to 140-kd fragments and of intact fibronectin were calculated by using molecular weights 30,000, 40,000, 130,000 and 440,000, respectively.

## RESULTS

**Binding assays.** Binding of cathepsin G fragments of fibronectin to heat-stabilized bacteria was studied by using purified labeled fragments (Fig. 1). Initial experiments were performed with bacteria suspended in PBS-BSA buffer. The $^{125}$I-labeled, 30-kd, $NH_2$-terminal fragment was reactive with an uptake of 39% to the group A streptococcus and to *S. aureus* (Fig. 2A). A low but definite uptake was noted with the human group G streptococci. The binding of the 30-kd fragment to the bovine group G streptococcus was only slightly higher than that recorded with the group B *Streptococcus* strain. None of the bacterial strains bound the $^{125}$I-labeled, 120- to 140-kd, COOH-terminal fragment better than the nonreactive group B streptococcus when the chloramine-T method was used for the iodination of the fragment (Fig. 2A). Lactoperoxidase-catalyzed iodination, however, produced the label that bound slightly better to the group A streptococcus (2%) and *S. aureus* (4%) than to the group B streptococcus (0.2%)(data not shown).

Binding experiments carried out at low ionic strength in Tris-BSA buffer showed a slightly different picture. The $^{125}$I-labeled, 30-kd, $NH_2$-terminal fragment demonstrated high binding reactivity, with an uptake of 35 to 55% to group A and G streptococci as well as to *S. aureus* (Fig. 2B). The $^{125}$I-labeled, 120- to 140-kd fragment bound moderately to *S. aureus* (9%) and very weakly to the group A streptococcus and to the human group G streptococcus (5%). In this buffer, the 120- to 140-kd fragment labeled by the lactoperoxidase-catalyzed iodination displayed a similar binding pattern (data not shown). The $^{125}$I-labeled, 40-kd, gelatin-binding fragment showed only background binding in both buffer systems as recorded with the group B *Streptococcus* strain (Fig. 2A and B).

**Inhibition assays.** The binding activity detected in direct uptake experiments was studied further in an inhibition assay in which intact fibronectin and its 30-, 40-, and 120- to 140-kd fragments were used to inhibit the binding of either the $^{125}$I-labeled 30-kd or the $^{125}$I-labeled 120- to 140-kd fragment to streptococci and staphylococci.

The 30-kd and the 120- to 140-kd fragments inhibited in a dose-dependent way the uptake of the labeled 30-kd fragment to group A and G streptococci and to *S. aureus* (Fig. 3). The two fragments were, on a molar basis, equally effective. They were more potent inhibitors than the intact fibronectin molecule. Half-maximal (50%) inhibiton of the uptake of the $^{125}$I-labeled 30-kd fragment to group A and G streptococci was obtained with unlabeled fragments at 10 to 20 nM and with purified fibronectin at 70 to 80 nM. In the case of *S. aureus*, the corresponding concentrations were 4 to 5 nM for the 30-kd and 120- to 140-kd fragments, and approximately 10 nM for intact fibronectin. Analogous experiments demonstrated that both the 30-kd and the 120- to 140-kd fragment inhibited the uptake of $^{125}$I-labeled 120- to 140-kd fragment to the group A streptococcus and *S. aureus* (Fig. 4). Half-maximal inhibition was achieved with unlabeled fragments at 12 to 17 nM. The 40-kd gelatin-binding fragment inhibited the uptake of neither the labeled 30-kd fragment nor the 120- to 140-kd fragment (Fig. 3 and 4).

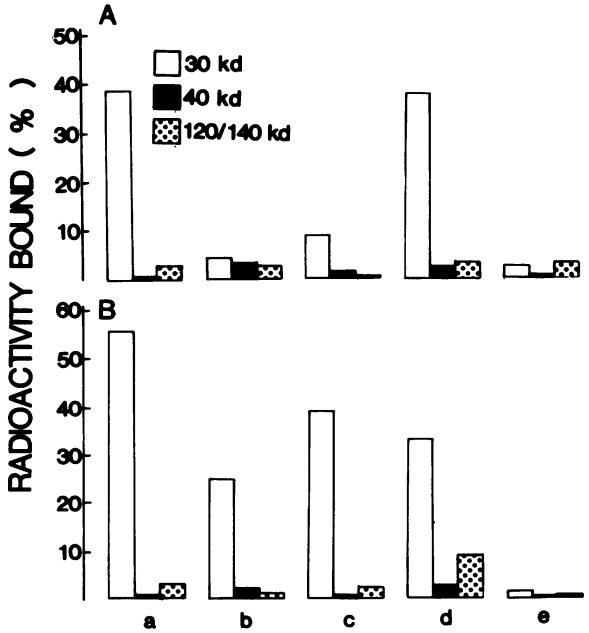


FIG. 2. Binding of $^{125}$I-labeled 30-, 40-, and 120- to 140-kd cathepsin G fragments of fibronectin to group A (a), bovine group G (b), and human group G (c) streptococci, to *S. aureus* (d), and to group B streptococcus (e) in PBS-BSA (A) and in Tris-BSA (B) buffer. For experimental details, see the text.

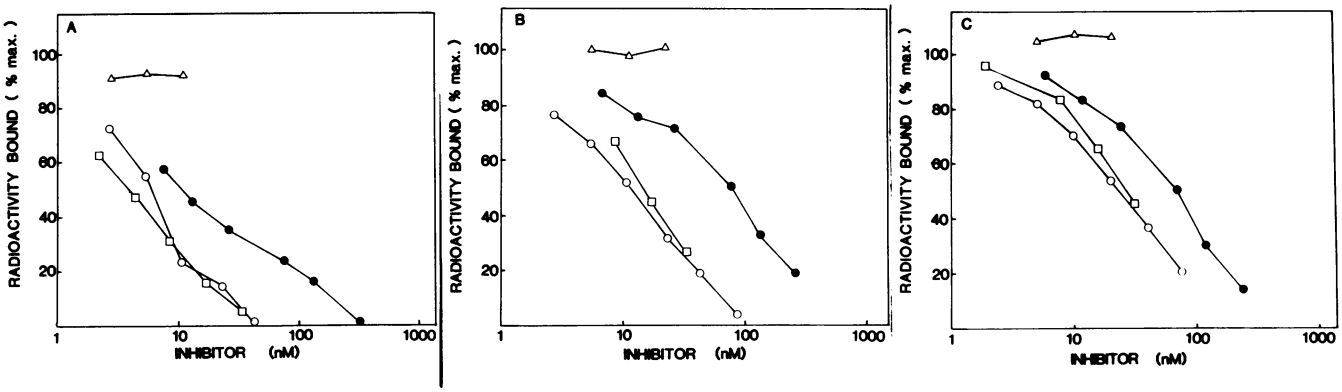


FIG. 3. Inhibition of the binding of the $^{125}$I-labeled 30-kd, $NH_2$-terminal fragment to *S. aureus* (A), bovine group G streptococci (B), and group A streptococci (C) by 30-kd $NH_2$-terminal fragment (○), by 40-kd gelatin-binding fragment (△), by 120- to 140-kd COOH-terminal fragment (□), and by intact fibronectin (●). For experimental details, see the text.

## DISCUSSION

In the present investigation, defined fragments of fibronectin were produced by controlled proteolysis and used to locate the binding sites for streptococci and staphylococci in the fibronectin molecule. Data obtained by direct binding assays and in inhibition experiments confirmed and extended earlier studies by other investigators, who reported that fibronectin binds to beta-hemolytic streptococci and to *S. aureus* via the $NH_2$-terminal end of the molecule (18, 24).

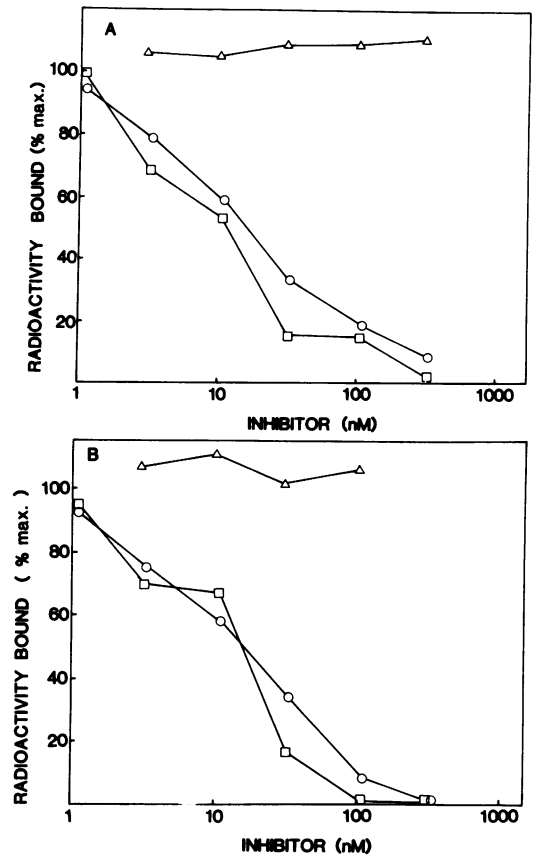


FIG. 4. Inhibition of the binding of the $^{125}$I-labeled, 120- to 140-kd COOH-terminal fragment to group A streptococcus (A) and *S. aureus* (B) by cathepsin G fragments of fibronectin. Symbols are the same as in Fig. 3.

The existence of other binding site(s) for *S. aureus*, but not for other bacterial species, has been suggested by Hayashi and Yamada (7). The weak but detectable binding of the COOH-terminal, 120- to 140-kd fragment to bacteria observed in the present experiments was further substantiated in inhibition experiments. The capacity of the COOH-terminal fragment to inhibit the uptake of the 30-kd amino-terminal fragment to group A and G streptococci and to *S. aureus*, and vice versa, clearly demonstrates the presence of an additional binding site in the COOH portion of the molecule. There are, therefore, at least two separate binding sites in the fibronectin molecule capable of interaction with complementary component exposed on the surface of group A and G streptococci and on *S. aureus*. These sites are located in the 30-kd $NH_2$-terminal and in the 120- to 140-kd COOH-terminal regions of the molecule. The observation that the two reactive fragments were equipotent inhibitors indicates that these sites did not differ appreciably in receptor affinity. In these experiments, only heat-stabilized bacteria were used. Though in our previous study, living and heat-stabilized bacteria behaved similarly (19), stabilized and nontreated bacterial cells may express different types of receptors for fibronectin. The capacity of the reactive fragments to inhibit both fragments suggests that the amino-terminal and carboxy-terminal fragments interact with the same component exposed on the bacterial cell surface. It is therefore reasonable to think that the two binding sites identified in the present studies might be structurally related, possessing a common three-dimensional structure complementary to the bacterial cell receptor. Whether the streptococci and staphylococci interact with identical binding sites or with the separate ones is not yet known, but the many similarities observed between these bacteria in the present study are in favor of identical or closely related sites.

The labeled 120- to 140-kd fragment showed a weak binding compared with the high reactivity noted in inhibition experiments performed with the unlabeled fragment. The impaired binding could be due to the iodination procedure causing alteration of the tertiary protein structure and leading to the reduced binding. This possibility is further supported by the finding that staphylococci attach very efficiently on glass cover slips coated with the 120- to 140-kd fragment (unpublished data). Intact fibronectin was, on a molar basis, a less potent inhibitor than were the 30- and 120- to 140-kd fragments. This may be due to self-association of fibronectin (31) reducing the number of available binding sites.

Fibronectin may help streptococci to bind dental tissues (2, 21), and fibronectin present on the surface of human oropharyngeal cells may modulate the colonization of cellular surfaces by pathogenic bacteria (1, 33). Binding sites identical or analogous to those described in the present report may be involved in the host-parasite interaction between bacteria and fibronectin-carrying cells and tissue components.

## ACKNOWLEDGMENTS

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## LITERATURE CITED

1. **Abraham, S. N., E. H. Beachey, and W. A. Simpson.** 1983. Adherence of *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* to fibronectin-coated and uncoated epithelial cells. Infect. Immun. **41**:1261–1268.
2. **Babu, J. P., W. A. Simpson, H. S. Courtney, and E. H. Beachey.** 1983. Interaction of human plasma fibronectin with cariogenic and non-cariogenic oral streptococci. Infect. Immun. **41**:162–168.
3. **Chen, A. B., M. W. Mosesson, and G. I. Solish.** 1976. Identification of the cold-insoluble globulin of plasma in amniotic fluid. Am. J. Obstet. Gynecol. **125**:958–961.
4. **Engvall, E., and E. Ruoslahti.** 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer **20**:1–5.
5. **Furcht, L.** 1983. Structure and function of the adhesive glycoprotein fibronectin. Mod. Cell Biol. **1**:53–117.
6. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of $^{131}$I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
7. **Hayashi, M., and K. M. Yamada.** 1981. Differences in domain structures between plasma and cellular fibronectins. J. Biol. Chem. **256**:11292–11300.
8. **Karonen, S.-L., P. Mörsky, M. Siren, and U. Senderling.** 1975. An enzymatic solid-phase method for trace iodination of proteins and peptides with $^{125}$Iodine. Anal. Biochem. **67**:1–10.
9. **Kurkinen, M., T. Vartio, and A. Vaheri.** 1980. Polypeptides of human plasma fibronectin are similar but not identical. Biochim. Biophys. Acta **624**:490–498.
10. **Kuusela, P.** 1978. Fibronectin binds to *Staphylococcus aureus*. Nature (London) **276**:718–720.
11. **Kuusela, P., M. Seppälä, D. H. J. Brock, L. Ruusuvaara, and E. Ruoslahti.** 1978. Amniotic fluid fibronectin in normal pregnancy and in pregnancies with anencephalic fetus. Biomedicine **29**:296–299.
12. **Kuusela, P., A. Vaheri, J. Palo, and E. Ruoslahti.** 1978. Demonstration of fibronectin in human cerebrospinal fluid. J. Lab. Clin. Med. **92**:595–601.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Mosesson, M. W., A. B. Chen, and R. M. Huseby.** 1975. The cold-insoluble globulin of human plasma: studies of its essential structural features. Biochim. Biophys. Acta **386**:509–524.
15. **Mosesson, M. W., and R. A. Umfleet.** 1970. The cold-insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold-insoluble fraction components. J. Biol. Chem. **245**:5728–5736.
16. **Mosher, D. F.** 1975. Crosslinking of cold-insoluble globulin by fibrin stabilizing factor. J. Biol. Chem. **250**:6614–6621.
17. **Mosher, D. F., and L. T. Furcht.** 1981. Fibronectin: review of its structure and possible functions. J. Invest. Dermatol. **77**:175–180.
18. **Mosher, D. F., and R. A. Proctor.** 1980. Binding and factor XIII-mediated cross-linking of a 27-kilodalton fragment of fibronectin to *Staphylococcus aureus*. Science **209**:927–929.
19. **Myhre, E. B., and P. Kuusela.** 1983. Binding of human fibronectin to group A, C, and G streptococci. Infect. Immun. **40**:29–34.
20. **Ruoslahti, E., E. Engvall, and E. G. Hayman.** 1981. Fibronectin: current concepts of its structure and functions. Collagen Relat. Res. **1**:95–128.
21. **Simpson, W. A., and E. H. Beachey.** 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. **39**:275–279.
22. **Starkey, P. M.** 1977. Elastase and cathepsin G: the serine proteinases of human neutrophil leukocytes and spleen, p. 57–89. *In* A. J. Barrett (ed.), Proteinases in mammalian cells and tissues. Elsevier/North-Holland Publishing Co., Amsterdam.
23. **Stenman, S., and A. Vaheri.** 1978. Distribution of a major connective tissue protein, fibronectin. J. Exp. Med. **147**:1054–1064.
24. **Switalski, L. M., A. Ljungh, C. Ryden, 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.
25. **Vaheri, A., J. Keski-Oja, T. Vartio, K. Alitalo, K. Hedman, and M. Kurkinen.** 1980. Structure and functions of fibronectin. Dev. Biochem. **15**:161–178.
26. **Vartio, T.** 1982. Characterization of the binding domains in the fragments cleaved by cathepsin G from human plasma fibronectin. Eur. J. Biochem. **123**:223–233.
27. **Vartio, T., E.-M. Salonen, G. DePetro, S. Barlati, V. Miggiano, C. Stahli, G. Virgallita, B. Takacs, and A. Vaheri.** 1983. Monoclonal antibody against the N-terminal end of human plasma fibronectin. Biochem. J. **215**:147–151.
28. **Vartio, T., R. von Essen, H. Isomäki, and S. Stenman.** 1981. Fibronectin in synovial fluid and tissue in rheumatoid arthritis. Eur. J. Clin. Invest. **11**:207–212.
29. **Vuento, M., E. Salonen, A. Koskimies, and U.-H. Stenman.** 1980. High concentrations of fibronectin-like antigens in human seminal plasma. Hoppe-Seyler's Z. Physiol. Chem. **361**:1453–1456.
30. **Vuento, M., and A. Vaheri.** 1979. Purification of fibronectin from human plasma by affinity chromatography under nondenaturing conditions. Biochem. J. **183**:331–337.
31. **Vuento, M., T. Vartio, M. Saraste, C. H. von Bonsdorff, and A. Vaheri.** 1980. Spontaneous and polyamine-induced formation of filamentous polymers from soluble fibronectin. Eur. J. Biochem. **105**:33–42.
32. **Wagner, D. D., and R. O. Hynes.** 1979. Domain structure of fibronectin and its relation to function. J. Biol. Chem. **254**:6746–6754.
33. **Woods, D. E., D. C. Strauss, W. G. Johanson, Jr., and J. A. Bass.** 1980. Role of fibronectin in the prevention of adherence of *Pseudomonas aeruginosa* to buccal cells. J. Infect. Dis. **143**:784–790.