*Clin. exp. Immunol.* (1985) **60**, 347–354.

# Studies on fibronectin in inflammatory *vs* non-inflammatory polymorphonuclear leucocytes of patients with rheumatoid arthritis. II. Synthesis and release of fibronectin *in vitro*

C. MENARD, A. D. BEAULIEU, MARIE AUDETTE, J. CORBEIL, & L. LATULIPPE *Unité de recherche en Immunologie-Rhumatologie, Department of Medicine, University Laval, Quebec, Canada*

*(Accepted for publication 30 November 1984)*

## SUMMARY

We conducted studies dealing with the synthesis and release of fibronectin *in vitro* by polymorphonuclear leucocytes (PMNL). The specific purpose of our study was to look for any changes in these events as they happen in inflammatory *vs* non-inflammatory PMNL. We used PMNL isolated from the synovial fluid of patients with rheumatoid arthritis as a source of inflammatory cells and PMNL isolated from peripheral blood as a source of non-inflammatory cells. Marked differences were observed. Using $^{35}$S-methionine metabolic labelling and SDS-polyacrylamide gel analysis, we were first able to clearly observe an increased synthesis of fibronectin by inflammatory PMNL when compared to non-inflammatory PMNL. Furthermore, the release of fibronectin *in vitro* by these cells was increased by factors of up to 20 when compared to non-inflammatory peripheral blood PMNL. Experimental evidence was also obtained which strongly suggests that fibronectin exists in a stored form inside the inflammatory PMNL we used in this study. Finally, we observed that PMNL are capable of synthesizing a 95 kD gelatin binding protein which appears to be distinct from fibronectin.

**Keywords** fibronectin polymorphonuclear leucocytes synthesis release inflammation

## INTRODUCTION

Using an indirect immunofluorescence procedure, we have recently made the observation that antibodies to human fibronectin react strongly with polymorphonuclear leucocytes (PMNL) isolated from inflammatory synovial fluids of patients with rheumatoid arthritis (RA) (Beaulieu *et al.*, 1985). This was in contrast to non-inflammatory peripheral blood PMNL where reactivity of the antibody with the cells was found to be much weaker. These findings suggested the presence of higher fibronectin levels in inflammatory PMNL. Although some surface fibronectin was found to be present on the cell membrane, strongest reactivity was found to be localized inside the cell. The studies which we now present were undertaken in part to investigate the possibility that synthesis of fibronectin by PMNL isolated from the synovial fluid of patients with RA might be increased. We performed metabolic labelling experiments and compared fibronectin synthesis by non-inflammatory peripheral blood PMNL to synthesis by inflammatory synovial fluid PMNL.

In the second part of this study, we present experimental evidence which strongly suggests that inflammatory PMNL of synovial fluid store large amounts of fibronectin which can be released massively under *in vitro* conditions.

Correspondence: Dr André D. Beaulieu, Unité de recherche en Immunologie-Rhumatologie, Department of Medicine, University Laval, 2705 Boulevard Laurier, Quebec G1V 4G2, Canada.

## MATERIALS AND METHODS

*Patient population.* Patients selected for these studies had either definite or classical RA diagnosed according to American Rheumatism Association criteria (Ropes *et al.*, 1958). All patients were on short acting non-steroidal anti-inflammatory therapy and none were on long acting therapy. Normal controls were recruited among healthy hospital personnel and were sex and age matched with RA patients.

*Preparation and* in vitro *labelling of cells.* Cells were prepared as previously described (Beaulieu *et al.*, 1985) and labelled with $^{35}$S-methionine (800–1,200 Ci/mmol, New England Nuclear, Lachine, Canada). Before labelling, the cells were washed in cold methionine free RPMI 1640. Five million cells were labelled in this medium with 50 $\mu$Ci of $^{35}$S-methionine. At the end of the labelling periods (2 or 16 h) the cells were centrifuged and the supernatants were kept for further usage at $-70$°C in the presence of 1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were rinsed and extracts were prepared for electrophoretic analysis.

*SDS-PAGE and handling of samples.* Electrophoresis was performed on a 7% SDS-polyacrylamide gel in the discontinuous buffer system of Laemmli (1970). All samples including cells to be analysed on the gels were boiled 5 min in a buffer containing Tris-HCl 0·0625 M pH 6·8, 1·25% SDS, 1·25% 2-mercaptoethanol, 12·5% glycerol. In order to isolate the $^{35}$S-labelled gelatin binding proteins, supernatants were incubated with 100 $\mu$l of gelatin–Sepharose 4B (Pharmacia Fine Chemicals, Dorval, Canada). After washing the gelatin–Sepharose 4B with 20 vols of Tris-HCl 50 mM pH 7·4 containing 1 M NaCl, it was re-equilibrated with three successive washes of Tris-HCl 50 mM pH 7·4. The SDS-PAGE gels containing $^{35}$S-labelled proteins were stained with Coomassie brilliant blue to detect mol. wt markers. Six different mol. wt markers were used (SDS-6H, Sigma Chemical Co., St Louis, Missouri, USA). The gels were next subjected to fluorography and exposed to Kodak XAR-2 films. Blotting of samples was performed on nitrocellulose paper as previously described (Towbin, Staehelin & Gordon, 1979). Fibronectin was revealed by autoradiography after incubating the nitrocellulose transfer paper with a $^{125}$I-labelled IgG fraction of a goat antibody to human fibronectin (Cappel, West Chester, Pennsylvania, USA). A replica of the transfer paper was stained with amido black in order to check for effective transfer of proteins.

*Culture conditions.* Cells were cultured in RPMI 1640 supplemented with glutamine (0·3 mg/ml), penicillin (100 u/ml), streptomycin (100 $\mu$g/ml) and 20% fibronectin depleted fetal calf serum (FCS). Depletion in fibronectin was achieved by two successive adsorptions to gelatin–Sepharose 4B. Experiments were performed in triplicates with $5 \times 10^6$ cells in 0·5 ml of culture medium. Cultures were established in 24 well flat bottom culture plates (Flow Labs, Mississauga, Canada) and incubated in an atmosphere of 5% $CO_2$ at 37°C. To inhibit proteolysis, 1 mM PMSF was immediately added to the collected supernatants and these were stored at $-70$°C until ready for testing. In selected experiments, parallel cultures were established in the presence of 10 $\mu$M cycloheximide (Sigma). Cell viability was verified by trypan blue dye exclusion. Viable cells constituted over 90% of the population in culture after 16 h.

*Quantification of fibronectin in supernatants.* Quantification was performed by an enzyme linked immunosorbent assay (ELISA). Seventy-five microlitres of a F(ab')$_2$ fragment of a rabbit antibody to human fibronectin (Cappel) diluted 1:2,000 with phosphate-buffered saline (PBS) were used to coat wells of polystyrene microtitre plates. Coating was allowed to proceed for 3 h at room temperature. Each well then received 150 $\mu$l of a solution of 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After three washes with PBS were performed, 50 $\mu$l of cell culture supernatant were added for 1 h at 37°C. Plates were then washed and 50 $\mu$l of a peroxidase conjugated IgG fraction of a rabbit antibody to human fibronectin (Cappel) were added for 1 h at 37°C. After washing the plates with PBS + TWEEN 0·1%, 50 $\mu$l of a peroxidase substrate solution (*o*-phenylediamine in 0·1 M sodium citrate buffer, pH 5) were added to the wells. The reaction was stopped by the addition of 50 $\mu$l of 4N $H_2SO_4$ and optical densities were read at 492 nm in a Titertek Multiskan ELISA reader. For each quantification, a standard curve was established with human plasma fibronectin, purified by gelatin Sepharose 4B chromatography (Engvall & Ruoslahti, 1977). Each supernatant was measured at three different dilutions.

## RESULTS

PMNL were incubated with $^{35}$S-methionine in culture for 2 or 16 h, and the profiles of the proteins synthesized were analysed on SDS-PAGE under reducing conditions. Before loading onto the gels however, the supernatants from the cultures were reacted with gelatin–Sepharose 4B and centrifuged in order to isolate out the gelatin binding proteins. The different protein profiles obtained are shown in Fig. 1. Tracks A and B contain the gelatin binding proteins from culture supernatants of PMNL isolated from synovial fluid after 2 and 16 h of labelling, respectively. Track C shows a similarly treated culture supernatant of PMNL isolated from the peripheral blood of the

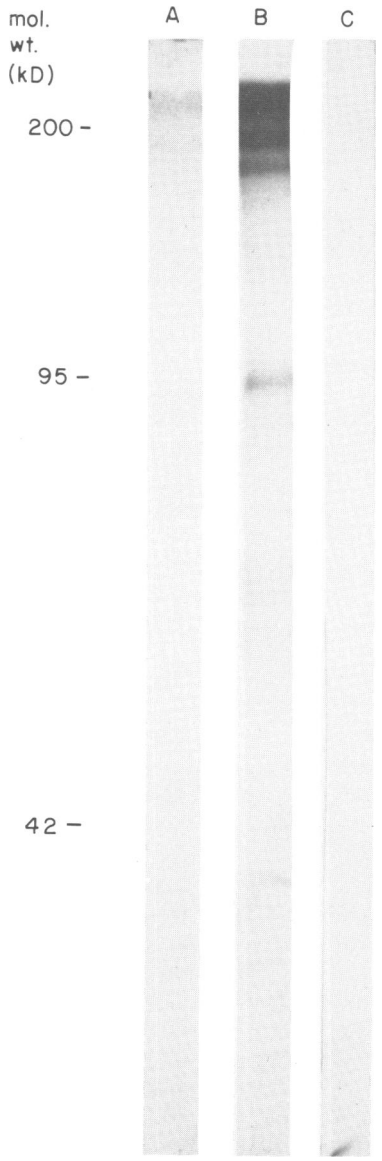


**Fig. 1.** Autoradiogram of a 7% SDS-PAGE. Track A: gelatin Sepharose 4B precipitate of a supernatant from a 2 h culture of PMNL isolated from synovial fluid and labelled with $^{35}$S-methionine. Track B: same as A except for culture time (16 h). Track C: same as B except for PMNL isolated from peripheral blood.

same patient (16 h labelling). As can be seen in A, bands strongly suggesting the presence of fibronectin, and therefore active synthesis by PMNL of synovial fluid, are revealed in the first 2 h of culture. After 16 h of culture and labelling, the bands are stronger and a better definition is obtained. One first notes that at the position where fibronectin usually migrates (approximately 200 kD), three close bands are seen varying between 220 and 175 kD. Fibronectin usually gives two close bands

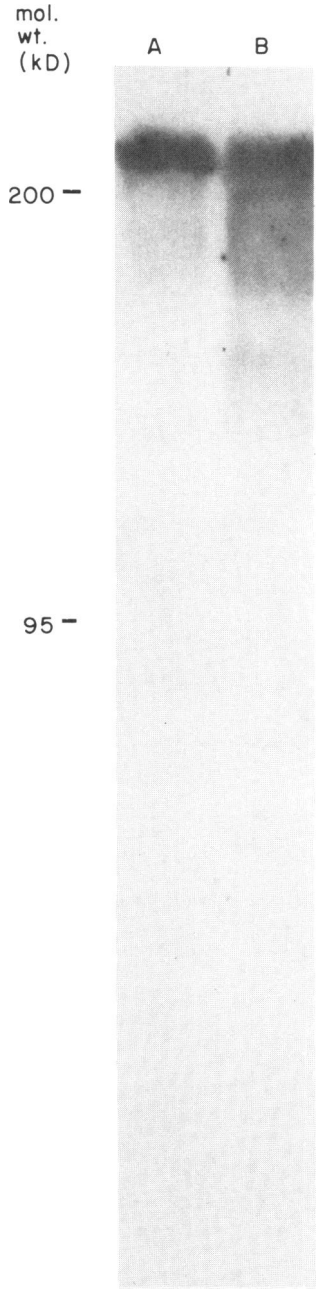


**Fig. 2.** Autoradiogram of an immunoblotting procedure using $^{125}$I-labelled IgG antibody to fibronectin. Track A: plasma fibronectin. Track B: gelatin binding proteins isolated from a 16 h culture of PMNL isolated from synovial fluid.

under reducing conditions. Furthermore, it becomes clear that a 95 kD gelatin binding protein is also synthesized by the PMNL. No gelatin binding protein could be detected in the supernatant of PMNL isolated from peripheral blood and labelled for 16 h (track C). Ten micromoles of cycloheximide abolished all $^{35}$S-methionine incorporation. These findings clearly illustrate the important difference which exists between inflammatory and non-inflammatory PMNL in terms of synthesis.

An immunoblotting procedure was next performed. We used a $^{125}$I-labelled antibody to human fibronectin in order to reveal the fibronectin bands. An autoradiogram of the nitrocellulose transfer sheet is presented in Fig. 2. Track A contains a transferred sample of normal human plasma fibronectin and track B, a sample of the transferred gelatin binding proteins isolated from cultures of synovial fluid PMNL. As can be seen, all of the material migrating between 220 and 175 kD reacted with the antibody to fibronectin. The 95 kD band showed no reactivity, thus strongly suggesting that this 95 kD gelatin binding protein is distinct from fibronectin.

We next proceeded to establish cultures of PMNL in order to assess fibronectin release in culture supernatants. The PMNL from five patients with RA were isolated from their peripheral blood and synovial fluid. The amount of fibronectin which was released in the supernatant was measured by ELISA. The PMNL isolated from the peripheral blood of five normal individuals were also studied. The results are presented in Fig. 3. There was no significant difference between normal individuals and patients when PMNL from peripheral blood were compared. In each patient however, there was a significant difference ($P < 0{\cdot}025$) in the amount of fibronectin released by PMNL of synovial fluid and peripheral blood. A difference of up to 20-fold was obtained in certain patients. Furthermore, differences between patients were observed as to the quantity of fibronectin released by a fixed number of PMNL ($5 \times 10^6$). Patients 6 and 10 released considerably less fibronectin than patients 7, 8 and 9.

The kinetic of fibronectin release in culture supernatants was next studied. During these studies, parallel cultures were established in the presence and absence of the protein synthesis inhibitor cycloheximide in order to address the question of fibronectin stocking *vs* rapid *de novo* synthesis. It was assumed that cycloheximide would not significantly affect the amount of fibronectin being released in the supernatants if fibronectin already existed in a stored form. Results are presented in Fig. 4. Cultures of PMNL isolated from two synovial fluids and one peripheral blood of a normal individual were established. Fibronectin measurements were performed at various time intervals. Fibronectin release was found to be a rapid phenomenon (maximum in less than 30 min) and in all

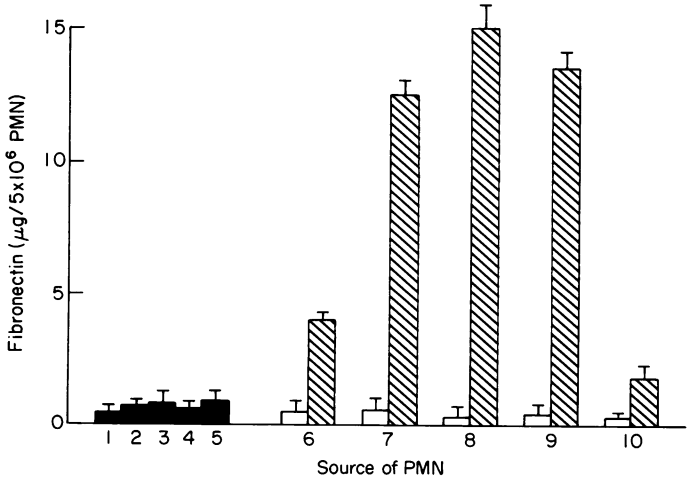


**Fig. 3.** Quantity of fibronectin released by $5 \times 10^6$ PMNL isolated from five normal individuals (1–5) and five patients with active RA (6–10). ■ = peripheral blood, normals; □ = peripheral blood, RA patients; ▨ = synovial fluid, RA patients.

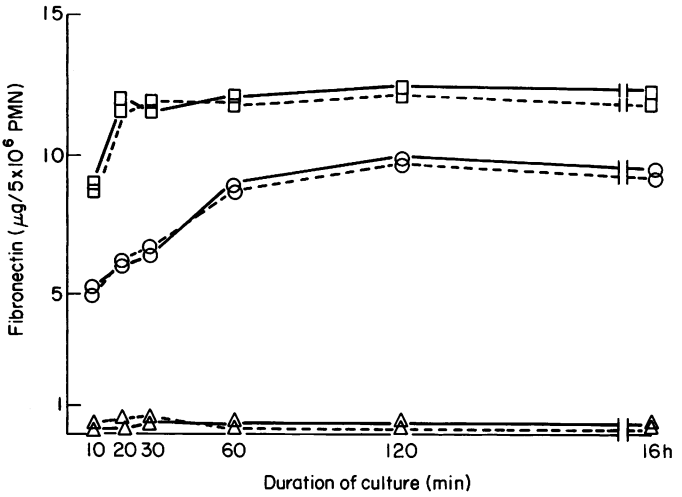


**Fig. 4.** Kinetic of fibronectin release in culture supernatant with $5 \times 10^6$ PMNL from synovial fluid of two patients with RA (□ and ○) and the peripheral blood of one patient with RA (△). Cultures were performed in the absence (——) or presence (– – –) of the protein synthesis inhibitor cycloheximide.

cases, cycloheximide did not modify the levels released in supernatants. Therefore, these findings strongly suggest that the large majority of the fibronectin released in supernatants existed in a stored form in PMNL.

Finally, in order to investigate whether fibronectin release by PMNL is an active energy requiring process or not, we performed experiments analysing the effect of temperature on fibronectin release. Results showed a dramatic decrease in fibronectin release by PMNL of synovial fluid at 4°C compared to 37°C. In the three patients studied, less than 10% of the fibronectin released at 37°C was released at 4°C (results not shown).

## DISCUSSION

In this paper, we compared PMNL isolated from an inflammatory and a non-inflammatory environment in terms of synthesis and release of fibronectin *in vitro*. The major findings which emerge are: (1) fibronectin synthesis and stocking is considerably increased in inflammatory PMNL isolated from the synovial fluid of patients with RA; (2) PMNL synthesize a 95 kD gelatin binding protein which appears to be distinct from fibronectin and (3) inflammatory PMNL of synovial fluid release large quantities of fibronectin *in vitro* by an energy requiring process.

Active synthesis of fibronectin by PMNL was shown to occur through $^{35}$S-methionine labelling experiments. It was identified by SDS-PAGE analysis and an immunoblotting procedure (Figs 1 & 2). While keeping experimental conditions identical, active incorporation of $^{35}$S-methionine into the fibronectin released in supernatants was only observed when PMNL isolated from inflammatory synovial fluid were studied. The most obvious explanation for this is a greater rate of synthesis in these cells as compared to non-inflammatory peripheral blood PMNL. Evidence of synthesis was seen as early as after 2 h of culture and labelling. The gelatin binding proteins isolated from the supernatants of PMNL gave an unexpected electrophoretic profile (Fig. 1). One first observes the appearance of three close bands migrating between 220 and 175 kD. It is presently not clear as to why fibronectin migrated in such a manner on SDS-PAGE. Although a protease inhibitor was added to the supernatant immediately after culture, it cannot be excluded that some limited proteolysis still occured during the culture period. Further studies will be needed to clarify this point. An additional finding was the presence of a band migrating at the 95 kD position (Fig. 1). By an immunoblotting procedure, it was shown that all of the heavier bands reacted with an antibody

to fibronectin whereas this lighter band did not (Fig. 2). This suggested that a 95 kD gelatin binding protein was also being synthesized by PMNL. It is not the first observation on the synthesis of such a protein. Fibroblasts are known to synthesize a 70 kD protein (Vartio & Vaheri, 1981) and macrophages, a 95 kD protein (Vartio, Hovi & Vaheri, 1982) both with gelatin binding properties. Both seem to be distinct from fibronectin.

The results presented in Figs 3 & 4 provided additional evidence which suggest that PMNL of RA synovial fluid go through a dramatic change as far as their fibronectin handling is concerned. Release in culture medium is increased by a factor of up to 20 when inflammatory and non-inflammatory PMNL are compared. It was also observed that the majority of the fibronectin which was rapidly released in culture medium was not newly synthesized. This statement is based on the fact that cycloheximide did not influence the amount of fibronectin being released in culture supernatants. It would thus seem plausible that stocking is a way by which PMNL handle the increased production of fibronectin. It must be pointed out however that in Fig. 4 it was quite evident that the increased synthesis which was observed in PMNL of synovial fluid by $^{35}$S-methionine studies (Fig. 1) was not reflected on the levels of fibronectin which were measured in supernatants by the ELISA procedure over the time period studied. This is most likely explained by the difference in the levels of sensitivity which is obtained when using these two approaches.

One can only speculate at this point on the significance of our findings. Fibronectin, it is well known, has a large array of biological functions. The overall importance of this glycoprotein in inflammatory and immune responses remains difficult to assess. In relation to this however, it can be pointed out that fibronectin can function as an opsonin (Saba & Jaffe, 1980; Czop, Kadish & Austen, 1981, 1982; Pommier *et al.*, 1983), it has chemotactic properties for monocytes and fibroblasts (not neutrophils and lymphocytes) (Norris *et al.*, 1982; Postlethwaite *et al.*, 1981) and it may increase *in vitro* monocyte–macrophage-mediated tumoricidal activity (Perri *et al.*, 1982). Furthermore, it binds to the C1q component of complement (Pearlstein, Sorvillo & Gigli, 1982; Bing *et al.*, 1982) and to bacteria (Kuusela, 1978; Mosher & Proctor, 1980). Finally, it may also help in the adhesion and spreading of cells during inflammatory and immune responses. With all of these properties in mind, it becomes difficult to sort out what may be the major role of fibronectin. It is possible however that the main function of a given fibronectin may be related to its source. Already, it is known that biological (Hynes *et al.*, 1978; Yamada & Kennedy, 1979), biochemical (Alexander *et al.*, 1978; Yamada *et al.*, 1977; Hayashi & Yamada, 1981) an antigenic (Atherton & Hynes, 1981; Noonan, Noonan & Yamada, 1981) differences exist between cellular and plasma fibronectin. Further differences could exist based on cell source. In spite of this, our studies certainly stress the necessity to further explore the biological properties of fibronectin in relation to PMNL and to try to establish how PMNL fibronectin could differ from fibronectin produced by other cells. Of further obvious necessity is to explore separately the role, on cell function, of the other gelatin binding proteins which are now being identified.

The authors are grateful to Claudine Parent and Pierrette Côté for excellent technical and secretarial assistance.

## REFERENCES

ALEXANDER, S.S., COLONNA, G., YAMADA, K.M., PASTAN, I. & EDELHOCH, H. (1978) Molecular properties of a major cell surface protein from chick embryo fibroblasts. *J. biol. Chem.* **253,** 5820.

ATHERTON, B.T. & HYNES, R.O. (1981) A difference between plasma and cellular fibronectins located with monoclonal antibodies. *Cell,* **25,** 133.

BEAULIEU, A.D., AUDETTE, M., MENARD, C., PARENT, C. & DUVAL, M. (1985) Studies on fibronectin in inflammatory *vs* non-inflammatory polymorphonuclear leucocytes of patients with rheumatoid arthritis. I. Immunofluorescent and flow cytometric analysis. *Clin. exp. Immunol.* **60,** 339.

BING, D.H., ALMEDA, S., ISLIKER, H., LAHAV, J. & HYNES, R.O. (1982) Fibronectin binds to the C1q component of complement. *Proc. Natl. Acad. Sci. USA.* **79,** 4198.

CZOP, J.K., KADISH, J.L. & AUSTEN, K.F. (1981) Augmentation of human monocyte opsonin-independent phagocytosis by fragments of human plasma fibronectin. *Proc. Natl. Acad. Sci. USA.* **78,** 3649.

CZOP, J.K., KADISH, J.L. & AUSTEN, K.F. (1982) Purification and characterization of a protein with fibronectin determinants and phagocytosis-enhancing activity. *J. Immunol.* **129,** 163.

ENGVALL, E. & RUOSLAHTI, E. (1977) Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer*, **20,** 1.

HAYASHI, M. & YAMADA, K.M. (1981) Differences in domain structures between plasma and cellular fibronectins. *J. biol. Chem.* **256,** 11292.

HYNES, R.O., ALI, I.U., DESTREE, A.T., MAUTNER, V., PERKINS, M.E., SENGER, D.R., WAGNER, D.D. & SMITH, K.K. (1978) A large glycoprotein lost from the surface of transformed cells. *Ann. N.Y. Acad. Sci.* **312,** 317.

KUUSELA, P. (1978) Fibronectin binds to *Staphylococcus aureus*. *Nature*, **276,** 718.

LAEMMLI, V.K. (1970) Cleavage of structural proteins during assembly of the head of the bacteriophage $T_4$. *Nature*, **227,** 680.

MOSHER, D.F. & PROCTOR, R.A. (1980) Binding and factor XIIIa-mediated crosslinking of a 27-kilodalton fragment of fibronectin to *Staphylococcus aureus*. *Science*, **209,** 927.

NOONAN, K.D., NOONAN, N.E. & YAMADA, K.M. (1981) Monoclonal antibodies directed against fragments of fibronectin. *J. Supramol. Struct. Cell. Biochem.* **Suppl. 5,** 302.

NORRIS, D.A., CLARK, R.A.F., SWIGART, L.M., HUFF, J.C., WESTON, W.L. & HOWELL, S.E. (1982) Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. *J. Immunol.* **129,** 1612.

PEARLSTEIN, E., SORVILLO, J. & GIGLI, I. (1982) The interaction of human plasma fibronectin with a subunit of the first component of complement, C1q. *J. Immunol.* **128,** 2036.

PERRI, R.T., KAY, N.E., MCCARTHY, J., VESSELLA, R.L., JACOB, H.S. & FURCHT, L. (1982) Fibronectin enhances *in vitro* monocyte–macrophage-mediated tumoricidal activity. *Blood*, **60,** 430.

POMMIER, C.G., INADA, S., FRIES, L.F., TAKAHASHI, T., FRANK, M.M. & BROWN, E.J. (1983) Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. exp. Med.* **157,** 1844.

POSTLETHWAITE, A.E., KESKI-OJA, J., BALIAN, G. & KANG, A.H. (1981) Induction of fibroblast chemotaxis by fibronectin. *J. exp. Med.* **153,** 494.

ROPES, M.W., BENNET, G.A, COBB, S., JACOX, R.F. & JESSAR, R.A. (1958) Revision of the diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* **9,** 175.

SABA, T.M. & JAFFE, E. (1980) Plasma fibronectin (opsonic glycoprotein): its synthesis by endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelial function. *Am. J. Med.* **68,** 577.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76,** 4350.

VARTIO, T., HOVI, T. & VAHERI, A. (1982) Human macrophages synthesize and secrete a major 95,000-dalton gelatin-binding protein distinct from fibronectin. *J. biol. Chem.* **10,** 8862.

VARTIO, T. & VAHERI, A. (1981) A gelatin-binding 70,000-dalton glycoprotein synthesized distinctly from fibronectin by normal and malignant adherent cells. *J. biol. Chem.* **256,** 13085.

YAMADA, K.M. & KENNEDY, D.W. (1979) Fibroblast and cellular plasma fibronectins are similar but not identical. *J. Cell Biol.* **80,** 492.

YAMADA, K.M., SCHLESENGER, D.H., KENNEDY, D.W. & PASTAN, I. (1977) Characterization of a major fibroblast cell surface glycoprotein. *Biochemistry*, **16,** 5522.