Clin. exp. Immunol. (1983) **52**, 305–310.

Fibronectin binds to C1q: possible mechanisms for their co-precipitation in cryoglobulins from patients with systemic lupus erythematosus

I. KONO, T. SAKURAI, T. KABASHIMA, K. YAMANE & H. KASHIWAGI
Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan

(Accepted for publication 10 December 1982)

SUMMARY

Fibronectin and C1q frequently co-precipitated in cryoglobulins from patients with SLE. As a portion of the C1q molecule is similar to collagen to which fibronectin has a high affinity, we studied whether fibronectin specifically bound to C1q. Fibronectin was found to bind to both native and heat-inactivated C1q. The binding was enhanced by Ca^{++} and low ionic strength. We have also demonstrated that fibronectin is capable of binding to C1q fixed to immune complexes. The interaction between fibronectin and C1q may play a role in cryoglobulin formation and in clearance of immune complexes by reticuloendothelial system.

INTRODUCTION

Cryoglobulins are frequently found in serum from patients with SLE. Their main components have been reported to be immunoglobulins and complement components (Hanauer & Christian, 1967; Winfield, Koffler & Kunkel, 1975; Wilson *et al.*, 1977). Recently fibronectin, in addition to these components, has been shown to be present in cryoglobulins (Wood *et al.*, 1980; Anderson *et al.*, 1981; Beaulieu, Valet & Strevey, 1981). Fibronectin precipitates in the cold when reacting with certain substances such as fibrin–fibrinogen complexes (Stathakis *et al.*, 1978). It is conceivable that fibronectin reacts with a specific component in cryoglobulins. C1q, a subunit of the first component of complement, is known to have a collagen like portion (Porter & Reid, 1978). Since fibronectin has a high affinity for collagen, we investigated whether fibronectin was found with C1q in cryoglobulins and studied optimum conditions for their binding *in vitro*.

MATERIALS AND METHODS

Patients. Sera were collected from 10 patients who met the preliminary American Rheumatism Association criteria for the classification of SLE (Cohen *et al.*, 1971).

Collection of sera and isolation of cryoglobulin. Venous blood was allowed to clot at 37°C for 2 h. The clot was removed by centrifugation at 1,500 *g* for 10 min at 37°C, and the serum was placed at

Abbreviations: ACD = acid–citrate–dextrose; BSA = bovine serum albumin; EDTA = ethylenediaminetetraacetic acid; PBS = phosphate-buffered saline; SLE = systemic lupus erythematosus; TBS-Tween = 50 mM Tris buffer pH 7·5 containing 100 mM NaCl and 0·05% of Tween 20.

Correspondence: Ichiro Kono, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Sakuramura, Niihari-gun, Ibaraki-ken 305, Japan.

0009-9104/83/0500-0305$02.00

4°C for 72 h. The cryoprecipitates obtained were washed three times at 4°C with 50 mM PBS pH 7·4. They were then solublized by constantly stirring in PBS at 37°C for 2 h.

Analysis of cryoglobulins. Cryoglobulins were analysed for the presence of fibronectin, immunoglobulins and complement components by the Ouchterlony double immunodiffusion technique with monospecific antisera to each of the proteins. Antiserum to fibronectin was raised in rabbits by immunizing with a purified fibronectin. Antisera to immunoglobulins were purchased from Behringwerke AG, Marburg, West Germany. Antisera to complement components were kindly provided by Dr Noboru Tamura, Institute of Basic Medical Sciences, University of Tsukuba, Japan.

Purification of fibronectin. Plasma fibronectin was purified by the method described by Engvall & Ruoslahti (1977) using gelatin–Sepharose affinity chromatography. ACD–plasma was applied to the gelatin–Sepharose column, and fibronectin was eluted with 4 M urea in 50 mM Tris buffer pH 7·5. Phenylmethylsulphonylfluoride (0·1 mM) was added to all buffers.

Purification of C1q. C1q was purified by the method of Tenner, Lesavre & Cooper (1981). In brief, fresh serum was applied to a Bio-Rex 70 column equilibrated with 50 mM sodium phosphate buffer pH 7·3 containing 82 mM NaCl and 2 mM EDTA. After washing, the column was eluted using a NaCl gradient. Fractions containing C1q were further purified by gel filtration on a Biogel A5m column.

Binding assay. The binding of fibronectin to C1q was assessed using an enzyme linked immunosorbent assay. The wells of microtitre plates (Immulon II, Dynatech Lab. Inc., Alexander, Virginia, USA) were coated with purified C1q by incubation at 4°C for 20 h with 200 μl of C1q solution (5 μg/ml in 20 mM carbonate buffer pH 9·6). The wells were washed three times with 250 μl of TBS-Tween and then 250 μl of 0·5% of BSA in TBS-Tween were added to each wells. After incubation at room temperature for 2 h, the wells were washed three times with TBS-Tween. The C1q coated wells were immediately used for the binding assay. Purified fibronectin was serially diluted in TBS-Tween containing 20 mM $CaCl_2$. Two hundred microlitres of the diluted fibronectin were added to the C1q coated wells in triplicate. After incubation at 37°C for 90 min, followed by three washes with TBS-Tween, 200 μl of peroxidase labelled antibody to fibronectin were added to each wells. The antibody was labelled with horseradish peroxidase (Sigma Chemical Co., St Louis, Missouri, USA) as described by Wilson & Nakane (1978). Following incubation at 37°C for 90 min, the wells were washed three times with TBS-Tween, filled with 200 μl of *o*-phenylendiamine as the enzyme substrate and then placed in the dark at 37°C for 30 min. The enzyme reaction was stopped by the addition of 50 μl of 4N H_2SO_4 and the absorbance at 492 nm was measured.

Preparation of immune complexes. The wells of microtitre plates were coated with BSA (Sigma) by incubation at 4°C for 20 h with 200 μl of BSA solution (200 μg/ml in 20 mM carbonate buffer pH 9·6). The wells were washed three times with 250 μl of TBS-Tween and then 250 μl of 0·5% of ovalbumin (Miles Laboratories, Elkhart, Indiana, USA) in TBS-Tween were added to each wells. After incubation at room temperature for 2 h, the wells were washed three times with TBS-Tween, and then 200 μl of IgG antibody to BSA (200 μg/ml in TBS-Tween) were added to each wells. The IgG antibody was prepared by passage through a column of DEAE–cellulose from rabbit antisera raised by immunization with BSA. After incubation at 37°C for 2 h, the wells were washed three times with TBS-Tween and immediately used.

RESULTS

Analysis of cryoglobulins

Cryoglobulins from patients with SLE were reacted with antisera to a variety of components. IgG and IgM were detected in all fractions (Table 1). Fibronectin was present in eight of 10 fractions; all but two of these fibronectin positive fractions were found to contain C1q.

Binding of fibronectin to C1q

Purified fibronectin was assessed at concentrations ranging from 10 to 1,000 ng/ml for binding to C1q. Fibronectin was found to bind to C1q coated wells in a dose-dependent fashion (Fig. 1). The

Table 1. Components detected in cryoglobulins

Patient	FN	C1q	IgG	IgA	IgM	C4	C3	FB
1	+	+	+	−	+	+	+	−
2	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	+	−
5	+	+	+	−	+	+	+	−
6	+	+	+	−	+	+	+	−
7	+	−	+	+	+	−	−	−
8	+	−	+	−	+	−	−	−
9	−	−	+	−	+	−	−	−
10	−	−	+	−	+	−	−	−

FN = fibronectin; FB = factor B.

binding increased when the wells were coated with C1q pre-heated at 56°C for 30 min. Fibronectin did not bind to wells coated with BSA.

To obtain the optimum conditions for the binding, we investigated first the effect of divalent cations on the binding. Fibronectin (1 mg/ml) was diluted to 1,000 ng/ml with TBS-Tween containing various concentrations of Ca^{++} or Mg^{++}, and then assessed for binding to C1q. As shown in Fig. 2, the binding was enhanced by Ca^{++} but not by Mg^{++}. The optimum Ca^{++} concentration for the binding ranged from 20 to 40 mM. To test whether Ca^{++} was essential for the binding, we determined the effect of EDTA on the binding. After fibronectin bound to the C1q coated wells in the presence of 20 mM Ca^{++}, the wells were filled with 200 μl of TBS-Tween with or without 20 mM EDTA, incubated at room temperature for 5 min and then the buffer was removed. The wells were treated twice more with TBS-Tween with or without 20 mM EDTA in a similar manner as above. After the wells were washed with TBS-Tween, the bound fibronectin was measured. As shown in Fig. 3, the treatment of EDTA resulted in a marked decrease of the binding, but a considerable amount of fibronectin still bound to the C1q coated wells without Ca^{++}.

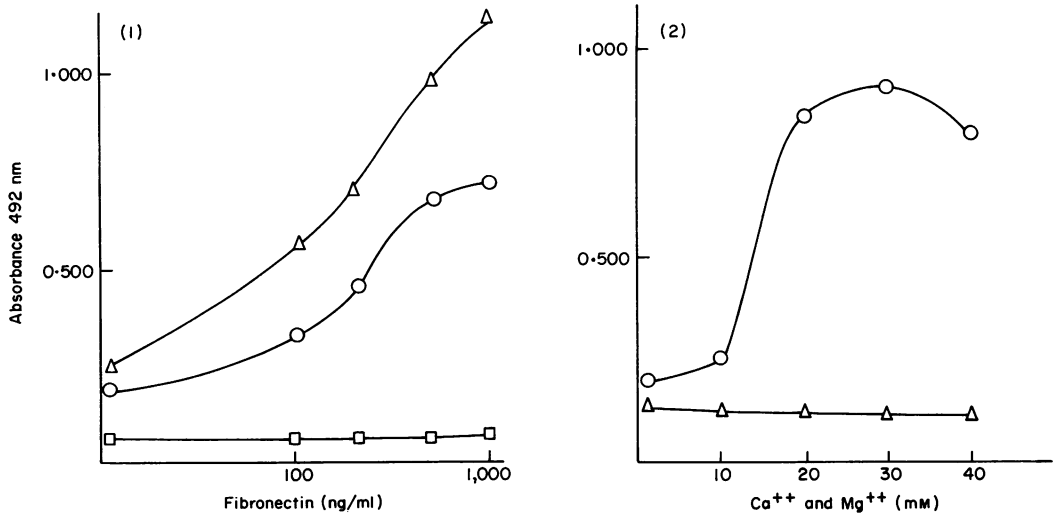

Fig. 1. Binding of fibronectin to wells coated with C1q (○), heat-inactivated C1q (△) and BSA (□).
Fig. 2. Effect of Ca^{++} (○) and Mg^{++} (△) on the binding of fibronectin to C1q.

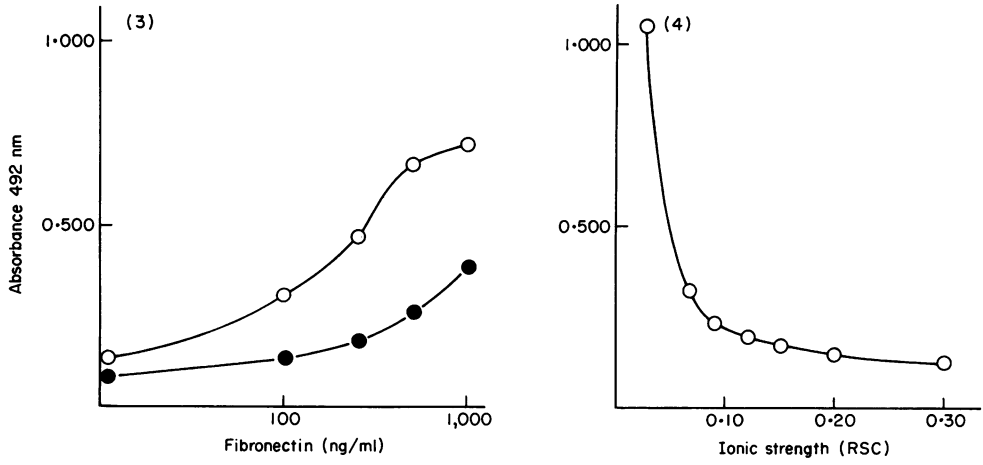

Fig. 3. Reduction of the binding of fibronectin to C1q by EDTA. o——o = EDTA (−); ●——● = EDTA (+).
Fig. 4. Effect of ionic strength on the binding of fibronectin to C1q. The ionic strength was expressed as relative concentration of NaCl (RSC).

The effect of ionic strength on the binding was also determined. Fibronectin (1 mg/ml) was diluted to 1,000 ng/ml with 50 mM Tris buffer pH 7·5 containing various amounts of NaCl and then assessed for binding to C1q. The binding of fibronectin to the C1q coated wells was markedly dependent on ionic strength, as indicated in Fig. 4. As the ionic strength was increased, there was a steep reduction in the amount of fibronectin bound to C1q coated wells.

We further investigated the specificity of binding of fibronectin to C1q. Increasing amounts of IgG antibody to C1q was incubated in C1q coated wells at 37°C for 90 min before binding assay for

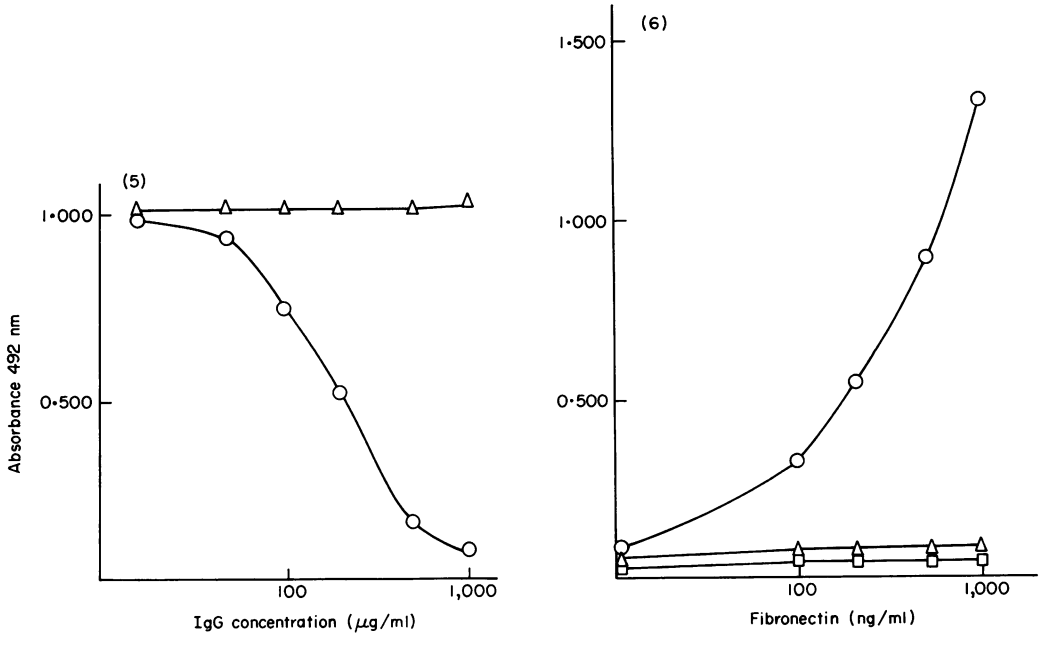

Fig. 5. Effect of IgG antibody to C1q (o) and non-specific rabbit IgG (△) on the binding of fibronectin to C1q.
Fig. 6. Binding of fibronectin to C1q fixed to BSA–anti-BSA antibody complexes (o), to BSA–anti-BSA antibody complexes without C1q (△) and to BSA (□).

1,000 ng/ml of fibronectin. The binding of fibronectin to C1q coated wells was blocked by IgG antibody to C1q in a dose-dependent manner (Fig. 5). Non-specific rabbit IgG did not block the binding.

Finally, we investigated whether fibronectin bound to C1q on immune complexes. Two hundred microlitres of C1q (50 μg/ml in TBS-Tween containing 20 mM $CaCl_2$) were added to BSA–anti-BSA antibody complexes prepared in the wells. After incubation at 37°C for 2 h, followed by three washes with TBS-Tween, 200 μl of dilutions of fibronectin in TBS-Tween containing 20 mM $CaCl_2$ were added to each wells and assayed for binding to C1q on the immune complexes. As shown in Fig. 6, fibronectin was found to bind to C1q on the BSA–anti-BSA antibody complexes in a dose-dependent fashion. Fibronectin did not bind to the immune complexes without C1q or to BSA alone.

DISCUSSION

The results of our studies clearly show that fibronectin frequently co-precipitates with C1q in cryoglobulins of patients with SLE and that fibronectin is capable of binding to C1q. This finding is consistent with the results of recent reports (Menzel *et al.*, 1981; Pearlstein *et al.*, 1982). Furthermore, we have demonstrated that this binding is enhanced by Ca^{++} and low ionic strength. The binding of fibronectin to heat-inactivated C1q was found to be greater than that to native C1q. It may be due to denaturation of the collagen like portion of C1q by heat-inactivation, because fibronectin has been shown to have a higher affinity for denatured collagen than for the native form (Engvall, Ruoslahti & Miller, 1978).

Fibronectin and C1q have been shown to be components of cryoglobulins (Wilson *et al.*, 1977; Wood *et al.*, 1980; Anderson *et al.*, 1981). However, the mechanism of their co-precipitation in cryoglobulins has not been fully understood. Our results may shed some light on this phenomenon. Recently fibronectin has been suggested to influence cryoglobulin formation (Beaulieu *et al.*, 1981). This influence of fibronectin must occur when fibronectin interacts with some components of cryoglobulin, since purified fibronectin is soluble at 0°C (Yamada *et al.*, 1977). Hardin has demonstrated that experimentally produced cold soluble immune complexes behave like cryoglobulins in the presence of certain normal serum proteins isolated with low concentrations of polyethylene glycol among which fibronectin was detected (Hardin, 1981). Together with this evidence, our findings that fibronectin is capable of binding to C1q fixed to immune complexes support the idea that the binding of fibronectin to C1q may play a role in cryoglobulin formation. The absence of Clq in fibronectin positive cryoglobulins may be explained by the low sensitivity of the assay method.

Fibronectin is known to enhance reticuloendothelial system clearance of certain proteins and bacteria to which fibronectin has affinity (Saba & Jaffe, 1980). However, it might be difficult to assign biological significance to the binding of fibronectin to C1q *in vivo*, because very little free C1q is present in normal serum. Ziccardi & Cooper (1979) have shown that $\overline{C1r}$ and $\overline{C1s}$ are rapidly dissociated from C1q by $\overline{C1}$ inactivator when C1 is activated, exposing C1q alone bound on macromolecules such as immune complexes. Under such circumstances, fibronectin could bind to C1q attached to immune complexes and thereby could enhance clearance of immune complexes *in vivo*.

We wish to thank Dr Noboru Tamura and Dr Teizo Fujita for providing us with antisera to complement components and for their valuable suggestions.

REFERENCES

ANDERSON, B., RUCKER, M., ENTWISTLE, R., SCHMID, F.R. & WOOD, G.W. (1981) Plasma fibronectin is a component of cryoglobulins from patients with connective tissue disease and other diseases. *Ann. rheum, Dis.* **40,** 50.

BEAULIEU, A.D., VALET, J.P. & STREVEY, J. (1981) The influence of fibronectin on cryoprecipitate formation in rheumatoid arthritis and systemic lupus erythematosus. *Arthrit. Rheum.* **24,** 1383.

COHEN, A.S., REYNOLDS, W.E., FRANKLIN, E.L.,

KULKA, J.P., ROPES, M.W., SHULMAN, L.E. & WALLACE, S.L. (1971) Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. rheum. Dis.* **21,** 643.

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

ENGVALL, E., RUOSLAHTI, E. & MILLER, E.J. (1978) Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J. exp. Med.* **147,** 1584.

HANAUER, L.B. & CHRISTIAN, C.L. (1967) Studies of cryoproteins in systemic lupus erythematosus. *J. clin. Invest.* **46,** 400.

HARDIN, J.A. (1981) Cryoprecipitogogue from normal serum: Mechanism for cryoprecipitation of immune complexes. *Proc. Natl. Acad. Sci. USA.* **78,** 4562.

MENZEL, E.J., SMOLEN, J.S., LIOTTA, L. & REID, K.B.M. (1981) Interaction of fibronectin with C1q and its collagen-like fragment (CLF). *FEBS Letters*, **129,** 188.

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.

PORTER, R.R. & REID, K.B.M. (1978) The biochemistry of complement. *Nature*, **275,** 699.

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

STATHAKIS, N.E., MOSESSON, M.W., CHEN, A.B. & GALANAKIS, D.K. (1978) Cryoprecipitation of fibrin–fibrinogen complexes induced by the cold-insoluble globulin of plasma. *Blood*, **51,** 1211.

TENNER, A.J., LESAVRE, P.H. & COOPER, N.R. (1981) Purification and radiolabeling of human C1q. *J. Immunol.* **127,** 648.

WILSON, M.R., ARROYAVE, C.M., MILES, L. & TAN, E.M. (1977) Immune reactants in cryoproteins. *Ann. rheum. Dis.* **36,** 540.

WILSON, M.B. & NAKANE, P.K. (1978) In *Immunofluorescence and related staining techniques* (ed. by W. Knapp, K. Holubar, & G. Wick) pp. 215. Elsevier/North-Holland Biomedical Press, Amsterdam.

WINFIELD, J.R., KOFFLER, D. & KUNKEL, H.G. (1975) Specific concentration of polynucleotide immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. *J. clin. Invest.* **56,** 563.

WOOD, G., RUCKER, M., DAVIS, J.W., ENTWISTLE, R. & ANDERSON, B. (1980) Interactions of plasma fibronectin with selected cryoglobulins. *Clin. exp. Immunol.* **40,** 358.

YAMADA, K.M., SCHLESINGER, D.H., KENNEDY, D.W. & PASTAN, I. (1977) Characterization of a major cell surface glycoprotein. *Biochemistry*, **16,** 5552.

ZICCARDI, R.J. & COOPER, N.R. (1979) Active disassembly of the first complement component, $\bar{C}1$, by $\bar{C}1$ inactivator. *J. Immunol.* **123,** 788.