Fibronectin and immune complexes in rheumatic diseases

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SUMMARY The relation between fibronectin and immune complexes in rheumatic diseases was examined in a series of linked studies. Fibronectin was present in immune complexes formed *in vitro* in the absence of C1q. Gel filtration chromatography showed complexed fibronectin was present in the serum of a patient with rheumatoid vasculitis, but not in normal serum; the complexed fibronectin coeluted with IgA and C3. Two dimensional immunoelectrophoresis showed a single fibronectin component was present in normal serum, but a number of components were present in serum from a rheumatoid patient. Polyacrylamide gel electrophoresis followed by immunoblotting for fibronectin showed that polyethylene glycol precipitates of synovial fluid contained immunoreactive components of a variety of sizes, indicating the presence of fragments of the molecule. An analysis of fibronectin in polyethylene glycol precipitates of paired serum and synovial fluid samples from 17 patients with rheumatoid arthritis and 16 with osteoarthritis showed more fibronectin was present in rheumatoid samples, especially in synovial fluid. More fibronectin was also present in synovial fluid than in serum polyethylene glycol precipitates; there was no direct relationship with C1q levels. All these results suggest that fibronectin is an integral component of immune complexes. This has potential pathogenic significance because it shows that a product of connective tissue cells may influence the functions of the immune system.

Immune complexes (IC) are involved in the pathogenesis of rheumatoid arthritis and other connective tissue diseases. Their persistence in pathological fluids is influenced by the efficiency of their phagocytosis by cells. Recent evidence has suggested that a connective tissue protein, fibronectin, may be associated with immunoglobulins and complement components in IC.¹⁻⁴ Fibronectin is a high molecular weight glycoprotein (440 000-500 000) of plasma, tissue fluids, and tissues. It binds to macromolecules such as collagen, heparin, and fibrin,⁵⁻⁸ adheres to cells,⁹ and is a major opsonin of particulate material in plasma.¹⁰⁻¹² This opsonic role could be important in facilitating removal of IC from tissue fluids.

Several studies have provided evidence that fibronectin is a potential constituent of immune complexes; it binds to C1q,¹³ to IgM rheumatoid

factor,⁴ and to complexes formed experimentally by heating IgG²; and it is a component of serum polyethylene glycol (PEG) precipitates.¹ These results may have other explanations unrelated to a role for fibronectin in IC formation. The presence of fibronectin in PEG precipitates could be explained by its cryoprecipitable properties^{14 15}; by precipitation of large proteins by PEG and by adherence of fibronectin to denatured protein.

Because of these questions we have further studied in detail the relation between fibronectin and IC formation in rheumatic diseases. Our objectives were to define the relation between fibronectin and circulating IC, to evaluate the nature of complexed fibronectin, and to investigate the relation between fibronectin and C1q in IC.

Materials and methods

FORMATION OF IN VITRO IMMUNE COMPLEXES

IC were formed by mixing purified human IgM

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(Sigma, Poole, Dorset, UK) at a final concentration of 20 µg/ml in phosphate buffered saline (PBS), pH 7.4, with goat antihuman IgM (Dakopatts, High Wycombe, UK) 1:40 in PBS (total volume 1 ml), in the presence or absence of fibronectin (0.33 mg/ml), for four hours at room temperature. These amounts of antigen and antibody were those required to reach the equivalence point as indicated by the manufacturer. Complexed proteins were precipitated by 2% (w/v) polyethylene glycol 6000 (PEG) (Sigma) at 4°C for three hours. We have shown previously that purified fibronectin is not precipitated by PEG (4% w/v).¹ The resultant precipitate was washed once in 2% PEG and redissolved in PBS.

IC were also formed in the presence of normal human serum, which acted as a source of complement. Fibronectin was extracted from human serum by affinity chromatography (see below) to yield fibronectin depleted serum (containing 5% of the original fibronectin concentration measured by enzyme linked immunosorbent assay (ELISA)). This was diluted to give a final IgM concentration of 20 µg/ml and mixed with goat antihuman IgM antiserum and fibronectin as before.

PURIFICATION OF FIBRONECTIN

Fibronectin was purified by affinity chromatography on gelatin-Sepharose 4B using citrated normal human plasma.¹⁶

TOTAL PROTEIN DETERMINATION

The concentration of protein in PEG (2% w/v) precipitates was determined using the bicinchoninic acid procedure.¹⁷

ELISA FOR FIBRONECTIN

The assay was based on that of Selmer *et al.*¹⁸ Plastic microtitre plate wells were coated (100 µl/well) with goat antihuman fibronectin (Sigma, Poole, Dorset, UK) (25 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6) and incubated at room temperature for 17 hours. Each well was then washed twice with 100 µl PBS (0.01 M) containing Tween 20 (0.05% v/v) (PBS-Tween). Excess binding sites on the plate were blocked with PBS-Tween containing 1% bovine serum albumin (BSA) (100 µl) for one hour at room temperature. Standards and samples were added in PBS-Tween-BSA (100 µl) and the plate incubated for one hour at room temperature. The standards used were in the range 1.6 to 205 µg/l of protein using fibronectin isolated from normal human plasma. Plates were washed three times with PBS-Tween and rabbit antihuman fibronectin (Dakopatts, Denmark) added (1:1000) in PBS-

Tween-BSA (100 µl) before further incubation for one hour at room temperature. After washing, peroxidase conjugated goat antirabbit IgG (Sigma) was added (1:1000) in PBS-Tween-BSA (100 µl). After incubation for one hour at room temperature and after washing (×4), freshly prepared substrate solution (100 µl) was added to each well and the plate incubated at room temperature for 10 minutes (substrate solution: Na₂HPO₄·2H₂O (0.1 M), citric acid (0.034 M) containing H₂O₂ (0.01%, w/v) (BDH, Poole, UK), and *o*-phenyldiamine (4.3 mM) (Sigma)). The reaction was terminated by adding 100 µl of sulphuric acid (2.5 M) and the absorbance at 492 nm read using a Uniscan plate reader (Flow Laboratories). Unknown values were calculated by reference to a semilogarithmic standard curve for purified fibronectin. The intra-assay coefficient of variation was 8%.

ROCKET IMMUNOELECTROPHORESIS ASSAY FOR FIBRONECTIN

This assay used a monospecific antiserum to fibronectin prepared by the immunodiagnostic research laboratory of the University of Birmingham. The assay has previously been described in detail.¹⁹

COMPARISON OF ANALYTICAL METHODS FOR FIBRONECTIN DETERMINATION

The amount of fibronectin in 45 whole sera and 42 PEG precipitates of sera was measured by both ELISA and rocket immunoelectrophoresis. The samples were from patients with either rheumatoid arthritis or systemic lupus erythematosus.

GEL FILTRATION

Elution profiles of serum fibronectin, complement component C3 (C3), and immunoglobulin A (IgA) were compared after gel filtration on Sephacryl S400 (Pharmacia, Uppsala, Sweden) of normal serum and serum from a rheumatoid arthritic patient with systemic vasculitis. Serum (1 ml) was applied to the column (50×1.5 cm) and proteins eluted with PBS, pH 7.4. The concentrations of fibronectin, C3, and IgA in the fractions were measured by single radial immunodiffusion using specific antisera and results expressed as relative concentrations for each protein.

TWO DIMENSIONAL IMMUNOELECTROPHORESIS

Two-dimensional immunoelectrophoresis was performed as described by Clarke and Freeman²⁰ on serum and PEG precipitates of serum, using monospecific antiserum to fibronectin in the second dimension.

**SODIUM DODECYL
SULPHATE-POLYACRYLAMIDE GEL
ELECTROPHORESIS (SDS-PAGE)**

PEG (2% w/v) precipitates of serum and synovial fluid samples were prepared as described above (for the formation of immune complexes *in vitro*). Precipitated proteins were separated by the SDS-PAGE system of Laemmli²¹ in vertical slabs, using a 4–12% polyacrylamide gradient containing SDS (0.1%) and a 5% polyacrylamide stacking gel. Typically electrophoresis was performed at a constant current of 12.5 mA/gel for 17 hours with cooling to 10°C. Proteins were transferred to nitrocellulose paper (Bio-Rad, California, USA) using electroblotting at 90 V for one hour at 5°C. Immunochemical localisation of fibronectin was performed using rabbit antihuman fibronectin antiserum (Dakopatts) and goat antirabbit peroxidase conjugated antiserum (Sigma), followed by colour formation with diaminobenzidine and hydrogen peroxide as substrates for the peroxidase.

CLINICAL SPECIMENS

Paired samples of synovial fluid and serum were obtained from 17 patients with rheumatoid arthritis and 16 with osteoarthritis. Serum was also obtained from six normal controls. The specimens were centrifuged at 1000 g for 10 min and the supernatants removed and used immediately for studies of IC. PEG precipitates were produced as described above. Fibronectin was measured by the ELISA technique and C1q by single radial immunodiffusion using an assay kit supplied by Mercia Diagnostics.

Results**ASSOCIATION OF FIBRONECTIN WITH
IMMUNE COMPLEXES FORMED *IN VITRO***

The formation of IgM and anti-IgM IC was shown by measuring PEG precipitable protein (Table 1).

Table 1 *Precipitation of fibronectin by PEG during in vitro immune complex formation in the absence of serum (n=6)*

Proteins added to fibronectin	Total protein in PEG precipitate (mg/ml)	Fibronectin in PEG precipitates	
		µg/ml	% Control value
IgM alone	3.7 (1.7)	0.23 (0.06)	100 (26)
Anti-IgM and IgM	61.6 (11.7)*	1.48 (0.85)*	639 (368)

Values are mean (SD).

*Significantly higher by Mann-Whitney U test ($p<0.01$).

Table 2 *Precipitation of fibronectin by PEG during in vitro complex formation in the presence of fibronectin depleted human serum (n=8)*

Proteins added to serum	Fibronectin in PEG precipitate	
	µg/ml	% Control value
Fibronectin	0.07 (0.03)	100 (40)
Fibronectin and anti-IgM	0.43 (0.14)*	662 (220)

Values are mean (SD).

*Significantly higher by Mann-Whitney U test ($p<0.01$).

Analysis of the PEG precipitates by ELISA showed the amount of precipitated fibronectin was significantly increased when IC were produced ($p<0.01$ by Mann-Whitney U test). Exogenous fibronectin was a constituent of PEG precipitable complexes formed by the addition of anti-IgM to fibronectin depleted serum (Table 2). The amount of fibronectin precipitated was significantly greater when IC were formed than in the control where antibody was omitted (Table 2). The relative amount of fibronectin precipitated was similar when IC were synthesised using purified IgM or human serum.

GEL FILTRATION

Chromatography of normal serum showed IgA, C3, and fibronectin were only present in a single peak (Fig. 1). Serum from a patient with rheumatoid vasculitis showed a proportion of each of these proteins was present in high molecular weight forms (Fig. 1). This suggests that IC were present in the serum and contained IgA, C3, and fibronectin.

**TWO DIMENSIONAL
IMMUNOELECTROPHORESIS**

Normal serum gave a single peak with antifibronectin in the second dimension (Fig. 2). Serum from a rheumatoid patient showed a number of components were present, including a slow moving immunoreactive fibronectin moiety.

SDS-PAGE AND IMMUNOBLOTTING

SDS-PAGE of PEG precipitates of serum and synovial fluid followed by immunoblotting with antifibronectin antiserum was performed for patients with osteoarthritis (OA) and with rheumatoid arthritis (RA). Examples are shown in Figs 3 and 4. Fibronectin was present mainly as a polypeptide of molecular weight 220 000 in serum from a patient with OA (Fig. 3). There was evidence, however, of lower molecular weight forms of fibronectin in synovial fluid* from the same patient. In PEG

Fig. 1 Elution profiles of serum fibronectin, IgA, and C3 from Sephacryl S400 column (50×1.5 cm) eluted with PBS, pH 7.4. RA vasculitis serum (—), normal serum (·····). C = complexed; M = monomeric. RID = radial immunodiffusion.

precipitates of RA sera (Fig. 4) native fibronectin (molecular weight 220 000) and some smaller molecular weight polypeptides were observed. In contrast, a PEG precipitate of RA synovial fluid contained little native fibronectin, but many forms of lower molecular weight with immunoreactivity to antifibronectin antiserum were evident.

FIBRONECTIN AND C1q IN RA AND OA PEG PRECIPITATES

Significantly more fibronectin was present in PEG

precipitates from RA synovial fluid than from OA synovial fluid ($p < 0.02$ by Student's unpaired *t* test). The differences in serum were less pronounced (Table 3). The level of fibronectin in PEG precipitates of synovial fluid was greater than in serum for both groups of patients ($p < 0.01$ by Student's paired *t* test). Conversely C1q concentrations in serum PEG precipitates were greater than those of synovial fluid. Therefore the ratio of serum/synovial fluid PEG precipitable fibronectin was low (less than 1) and for C1q was high (greater than 1).

Fig. 2 Two dimensional immunoelectrophoresis using antifibronectin antiserum in the second dimension gel. (a) Normal human serum; (b) SLE serum. N = native fibronectin; C = complexed fibronectin; L = low molecular weight fibronectin.

COMPARISON OF ELISA AND ROCKET TECHNIQUES

The presence of fibronectin fragments in some PEG precipitates raises a potential analytical problem: different antibodies may possess different specificities for fibronectin fragments. A second analytical difficulty is interference in the ELISA assay by rheumatoid factor. A comparison of ELISA and rocket assays using antisera to fibronectin from different sources showed these problems were not important. The two assays gave similar correlations with serum and with PEG precipitates: the Pearson correlation coefficients were $r=0.69$ ($n=45$) for serum and $r=0.89$ ($n=42$) for PEG precipitates. Overall their correlation was $r=0.86$ ($n=87$).

Fig. 3 PAGE (4–12%) of 2% PEG precipitates of serum and synovial fluid, immunoblotted with antifibronectin antiserum. Paired serum (a) and synovial fluid (b) were prepared from a patient with osteoarthritis. MW=molecular weight in kilodaltons.

Fig. 4 PAGE (4–12%) of 2% PEG precipitates of serum and synovial fluid, immunoblotted with antifibronectin antiserum. (a) Serum from a patient with rheumatoid vasculitis; (b) and (c) serum and synovial fluid respectively taken from a patient with rheumatoid arthritis. MW=molecular weight in kilodaltons.

Table 3 Comparison of fibronectin and C1q in serum and synovial fluid PEG precipitates in RA and OA

	n	Fibronectin (µg/ml)	C1q (µg/ml)
RA serum	17	12 (16)	120 (59)
RA synovial fluid	17	234 (224)	68 (37)
RA serum/synovial fluid ratio	17	0.05	1.76
OA serum	16	7 (12)	73 (40)
OA synovial fluid	16	76 (111)	10 (12)
OA serum/synovial fluid ratio	16	0.09	7.30

Values are mean (SD).

Discussion

These results strongly suggest that fibronectin is a component of IC. The coelution of fibronectin with high molecular weight C3 and IgA in the rheumatoid vasculitic serum shows its presence in immune complexes is not merely an *in vitro* artefact produced by IC isolation by PEG. The amount of fibronectin in IC depends on their site of formation. Synovial fluid complexes contained more fibronectin than serum IC. This may be due to differences in the nature of the fibronectin molecule in synovial fluid. Although fibronectins are formed from a single gene, there are post-transcriptional differences in its polypeptide chain structure at different sites.^{22 23} Thus synovial fluid fibronectin is different from the serum form.²⁴⁻²⁷ Using SDS-PAGE with immunoblotting, however, we estimated the molecular weight of reduced serum and synovial fluid fibronectin each to be 220 000, which agrees with previous reports on plasma⁶ and cell surface fibronectins.²⁸

The immunoblotting studies show fibronectin in IC may be partially or completely degraded, most notably in synovial fluid complexes. Inflamed joints contain free proteolytic enzymes and macrophages and polymorphonuclear leucocytes capable of degrading fibronectin both enzymically and by a free radical mechanism. The propensity of fibronectin to damage by these mechanisms may be more marked in the synovial fluid form of the molecule. The altered pattern of immunoreactivity of serum fibronectin on two dimensional immunoelectrophoresis can be explained by both partial degradation and by complexing with other proteins. Such complexing could be similar to the IgA/ α_1 antitrypsin complex seen in rheumatoid arthritic sera and synovial fluids.^{29 30} Enzymically formed fibronectin fragments possess altered biological activity compared with the active molecule.^{31 32} It has also been shown that some fragments may have specific effects not present in the intact molecule with a significant stimulating action on fibroblasts. The implication is that the binding of native or fragmented fibronectin to IC may influence their biological role or their removal from sites of immune related inflammation and tissue damage. Bykowska *et al* have shown that proteolysis of plasma fibronectin influences its apparent concentration in two immunoassays based on electroimmunoassay and immunoturbidimetric techniques.³³ Our own results, however, suggest that in PEG precipitates the levels of fibronectin measured immunochemically are similar in two different assays—ELISA and rocket immunoelectrophoresis. This suggests that the levels of fibronectin in PEG

precipitates found in our assay systems do not result from an artefact produced by partial degradation.

The *in vitro* formation of IC shows the relative amount of fibronectin precipitated by PEG is similar in the presence and absence of serum. Only trace amounts of serum proteins will have been added with the antiserum to IgM, including a very small quantity of complement proteins. These results therefore suggest that fibronectin can bind directly to IC and that this binding is not mediated by C1q. Conversely, it cannot be argued from our results that C1q binding does not account for a part of fibronectin adherence to IC; indeed there is considerable evidence supporting such a suggestion.^{3 13 34-36} Nevertheless, the analyses of paired serum and synovial fluid PEG precipitates provide further evidence that fibronectin adherence does not depend on the amount of C1q in IC. The relative amounts of fibronectin and C1q in IC are dependent upon the site at which IC are formed.

Although these results point to a role for fibronectin in IC formation, this will only be of biological significance if it can alter their handling *in vivo*. The demonstration of specific macrophage receptors for fibronectin³⁷ suggested a potential role. The work of Saba and Jaffe showed that fibronectin is an opsonic protein,¹¹ and more recent studies by Simpson and Boughton have shown its opsonic role is equal to that of IgG and complement in certain circumstances.¹² Using the clearance of a charged colloid (dextran sulphate) as a model, Walton *et al* showed that high molecular weight dextran sulphate forms complexes containing fibronectin in rat plasma *in vivo*³⁸; its parenteral administration lowers plasma fibronectin acutely in a dose related fashion. The plasma changes are accompanied by deposition of dextran sulphate (shown histochemically as metachromatic material) and of fibronectin (shown by specific immunofluorescence) in an identical distribution within reticuloendothelial cells of rat liver and spleen. If one transposes IC for 'charged colloid' it is apparent that fibronectin has the potential to modify the clearance of IC and control some features of immunologically mediated disease. There is already evidence that fibronectin can bind to certain forms of IgG,³⁹ to DNA,⁴⁰ and possibly to C3.⁴¹ It also has a major role in binding to bacteria.^{42 43} It is thus probable that fibronectin is an important functional constituent of IC in rheumatic diseases. Our current results support this, though the exact pathogenic significance of the interaction of fibronectin with IC needs further definition.

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