Composition of immune complexes and their relation to plasma fibronectin in chronic myeloproliferative disorders

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SUMMARY High concentrations of circulating immune complexes were detected by polyethylene glycol precipitation in 11 of 20 patients with myelofibrosis secondary to chronic myeloproliferative disease. Circulating immune complexes showed a positive correlation with plasma IgG concentrations both in patients and controls. Covariance analysis of the two groups showed significantly increased polyethylene glycol precipitable IgG in patients when adjusted for plasma IgG concentrations, indicating that the patients had significantly increased concentrations of complexed IgG. The immune complexes contained IgG, C3, and fibronectin and were inversely correlated with plasma fibronectin concentrations, suggesting that this major non-specific opsonin is important for the normal clearance of immune complexes. Therapeutic plasmapheresis efficiently removed circulating complexes and produced an increase in plasma fibronectin. This suggests that plasmapheresis may be useful for controlling immune complex mediated complications of these disorders.

Increased concentrations of circulating immune complexes have been shown in primary myelofibrosis by several different methods^{1–4} but their pathophysiological importance remains unclear.^{1,2} Plasma fibronectin is an opsonin which regulates macrophage phagocytosis of micro-organisms and perhaps immune complexes.⁵ As plasma fibronectin is reduced in some patients with chronic myeloproliferative disorders⁶ we examined whether this is related to the high concentrations of circulating immune complexes. We also undertook a detailed study of the composition of immune complexes in these patients and examined the response to therapeutic plasmapheresis.

Patients and methods

Twenty patients with chronic myeloproliferative disorders and bone marrow fibrosis on trephine biopsy of the iliac crest were studied. There were five cases of primary myelofibrosis, 14 of polycythaemia vera, and one of essential thrombocythaemia. Sixteen healthy volunteers from the hospital staff served as control subjects.

Serum and plasma anticoagulated with edetic acid

were separated from venous blood samples within two hours of collection. Fibronectin, fibrinogen, and the third component of complement (C3) were determined by rocket immunoelectrophoresis. All other proteins were measured by radial immunodiffusion using specific antisera (Binding Site Ltd, Birmingham).

Immune complexes were precipitated from serum and plasma using 6000 molecular weight polyethylene glycol^{7,8} at a final concentration of 2%. Proteins were precipitated at 4°C for 18 hours, washed in 2% polyethylene glycol for 20 minutes, and resuspended in physiological saline. Immune complexes were expressed as mg/l of precipitated IgG. All samples were analysed in duplicate and the mean value determined. The mean (SD) percentage difference in 20 samples analysed in duplicate was 14(7). The plasma fibronectin precipitated by polyethylene glycol and the non-precipitable fibronectin remaining in the supernatant were also determined. For some experiments, fibronectin was purified by affinity chromatography.⁹ Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed under non-reducing conditions in 6.5% polyacrylamide gels.

The Mann-Whitney U test, correlation by "least squares fit," and covariance analysis were used to analyse the data.

Table 1 Details of 20 patients with primary myelofibrosis (PMF), polycythaemia rubra vera (PRV), and essential thrombocythaemia (ET)

Diagnosis	Age (years)	Duration of disease (months)	Haemoglobin (g/dl)	Neutrophil count ($\times 10^9/l$)	Platelet count ($\times 10^9/l$)	Spleen (cm)	Polyethylene glycol IgG (mg/l)	Chemotherapy in previous three months
PMF	80	1	8.5	11.0	175	7	968	No
PMF	66	1	10	1.5	200	10	786	No
PMF	49	28	12	2.0	200	12	512	No
PMF	61	37	10.5	3.0	150	14	492	No
PMF	51	35	15	4.5	400	1	412	No
PRV	70	74	14	17.0	500	4	800	No
PRV	48	48	14	10.0	200	11	760	Yes
PRV	64	37	16	5.0	200	0	672	No
PRV	66	20	14	9.0	1000	0	648	Yes
PRV	69	30	15	4.5	200	0	648	Yes
PRV	61	60	14	30.0	200	0	626	No
PRV	63	20	14	5.0	600	3	573	No
PRV	40	43	11	5.5	400	0	492	No
PRV	60	126	15	4.0	300	0	440	No
PRV	65	68	14	7.0	450	0	376	No
PRV	67	71	12	2.0	100	2	368	No
PRV	63	3	12	4.0	500	0	348	Yes
PRV	55	51	14	7.5	400	3	296	No
PRV	75	16	14	3.5	200	0	261	No
ET	75	13	14	3.0	300	0	672	No

Two patients each underwent three therapeutic exchanges of plasma on alternate days on a Hemometrics 30 cell processor to determine the effect of removing immune complexes on plasma fibronectin concentrations. A total of 4 litres of plasma from each patient was exchanged with 4.5% albumin (Blood Products Laboratory, Elstree, England).

Results

The clinical features, haematological variables, and concentrations of immune complexes (polyethylene glycol IgG) in 20 patients are shown in table 1. Concentrations of immune complexes did not increase with age or duration of disease and were constant in stable patients on no treatment: 24 measurements made in seven patients over nine months showed a variation of 10.5 (7)% (mean $\pm$ SD). Three patients with primary myelofibrosis, however, showed two to threefold increases in concentrations of immune complexes during the terminal phase of their disease. Immune complex values did not correlate with spleen size or peripheral blood counts or with myelosuppressive chemotherapy over the preceding three months.

The normal range for plasma immune complex concentrations in 16 control subjects (mean $\pm$ 2SD) was 103 to 507 mg/l of polyethylene glycol precipitable IgG. Values higher than this were found in three of five patients with primary myelofibrosis, seven of 14 with polycythaemia rubra vera, and one with essential thrombocythaemia. Immune complexes and plasma IgG concentrations showed a significant positive correlation both in patients ($r = 0.77$, $p < 0.001$) and controls ($r = 0.68$, $p < 0.01$). Plasma IgG concentrations were 13.1 (4.1) g/l and 11.9 (2.5) g/l, respectively. Polyethylene glycol precipitable IgG expressed as a percentage of the plasma IgG concentration was, however, significantly greater in patients compared with that in normal subjects (4.3% v 2.6%, $p < 0.001$). Covariance analysis of controls and

Fig 1 Lines of regression from covariance analysis of polyethylene glycol (PEG) IgG and plasma IgG in 20 patients —: $PEG\ IgG(mg/l) = 34.4 \times plasma\ IgG(g/l) + 107.2$; and 16 controls ---: $PEG\ IgG(mg/l) = 34.4 \times plasma\ IgG(g/l) - 80.4$. The difference between the two groups, adjusted for the covariate, was highly significant ($t = 4.75$, $p < 0.001$).

Table 2 Percentage precipitation of plasma proteins by 2 polyethylene glycol

	Patients	Controls	p Value
Fibronectin	79.0	67.0	<0.02
IgG	4.3	2.6	<0.001
C3	2.4	0.7	<0.01
IgM	3.4	3.1	NS
Fibrinogen	1.9	2.0	NS
α_2 -macroglobulin	1.2	1.3	NS
Haptoglobin	0.9	0.6	NS
Albumin	1.2	0.9	NS

patients showed significantly increased polyethylene glycol precipitable IgG when adjusted for plasma IgG ($t = 4.75$, $p < 0.001$) (fig 1). These results show that when a correction was made for plasma IgG concentrations, all the patients had significantly increased immune complex concentrations.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of plasma polyethylene glycol precipitates showed up to nine protein bands. Table 2 compares the percentage of polyethylene glycol precipitation of the four opsonic proteins fibronectin IgG, C3, and IgM with the four non-opsonic proteins fibrinogen, α_2 macroglobulin, haptoglobin, and albumin. The opsonic proteins all showed high polyethylene glycol precipitability compared with non-opsonic proteins, and up to 79% of patients' plasma fibronectin was polyethylene glycol precipitated in these experiments. Polyethylene glycol precipitable fibronectin, IgG, and C3 were significantly higher in patients than control subjects and precipitable IgG and C3 showed a significant positive correlation ($r = 0.6$, $p < 0.005$).

The precipitation of the opsonic proteins fibronectin, IgG, and C3 was significantly higher in plasma than in serum; 79% v 21% for fibronectin,

4.3% v 1.6% for IgG, and 2.4% v 0.4% for C3 ($p < 0.001$).

When plasma fibronectin was purified by sepharose-gelatin affinity chromatography its polyethylene glycol precipitability fell from 70% to 1%. These results and the detection of multiple opsonic proteins in the polyethylene glycol precipitates from plasma suggest that fibronectin is polyethylene glycol precipitated because of the formation of complexes with other plasma proteins.

The normal mean (SD) range for edetic acid anti-coagulated plasma fibronectin was 319–795 mg/l compared with a mean of 557 mg/l in controls; patients had significantly lower concentrations (mean 404 mg/l, $p < 0.001$). In patients plasma fibronectin concentrations showed a significant inverse correlation with those of immune complexes ($r = -0.63$, $p < 0.001$) (fig 2).

The plasma fibronectin not precipitated by 2% polyethylene glycol may be viewed as free fibronectin not incorporated into immune complexes. This was significantly lower in patients with high concentrations of immune complexes (mean 42 mg/l) compared either with controls (mean 190 mg/l) or with patients with low concentrations of immune complexes (mean 137 mg/l, $p < 0.001$).

Plasmapheresis reduced circulating concentrations of immune complexes from 640 mg/l to 204 mg/l in one patient and from 584 mg/l to 159 mg/l in the second. Non-precipitable fibronectin concentrations rose from 21 to 157 mg/l and 68 to 123 mg/l, respectively (normal > 100 mg/l). Total fibronectin rose from 258 to 433 mg/l in the first patient and remained at 290 mg/l in the second.

Discussion

Immune complexes are activators of the platelet release reaction,¹⁰ and the release of platelet derived growth factors by this mechanism may be an important stimulus to bone marrow fibroblasts in patients with myelofibrosis.² We observed high concentrations of immune complexes in 11 of 20 patients with myelofibrosis due to various chronic myeloproliferative disorders. When the polyethylene glycol precipitable IgG was adjusted for plasma IgG concentrations the result suggested that increased concentrations of circulating immune complexes were present in all 20 patients. These are important observations if the immune complex theory of myelofibrosis² is to remain tenable.

Plasma fibronectin concentrations are significantly reduced in patients with myeloproliferative disorders,⁶ but the relation between immune complexes and fibronectin concentrations has not previously been determined. Clearance of immune complexes by

Fig 2 Correlation between concentrations of circulating immune complexes and plasma fibronectin in 20 patients ($r = -0.63$, $p < 0.001$).

the fixed macrophage system may depend on effective opsonisation, and fibronectin is the major opsonin which regulates macrophage function.⁵ In the presence of effective concentrations of opsonic fibronectin the clearance of immune complexes could prevent complex concentrations from rising, but if immune complex formation were to exceed fibronectin production, an accumulation of complexes might ensue. The negative correlation between immune complex concentrations and plasma fibronectin, and the restoration of total and non-precipitable plasma fibronectin concentrations following removal of immune complexes by plasmapheresis support this hypothesis. Plasma exchange was performed with 4.5% albumin which contains no fibronectin, and so the recovery of plasma fibronectin concentrations cannot be attributed to therapeutic replacement.

All patients were free from infection at the time of study and only one had biochemical evidence of liver dysfunction, but this patient did not have raised concentrations of immune complex, and plasma fibronectin was normal. Fibronectin depletion and high concentrations of immune complexes were therefore not a result of either infection or impaired liver function.

Using a fixed concentration of polyethylene glycol, protein precipitation by polyethylene glycol is proportionate to the protein concentration and to the molecular weight of the protein.⁸ If, therefore, protein precipitation is expressed as a percentage of the initial protein concentration increased polyethylene glycol precipitation is determined by the molecular weight of the protein and its formation of high molecular weight complexes with other proteins.⁷ With 2% polyethylene glycol, we found that the percentage precipitation of non-opsonic proteins was minimal in both patients and controls. The increased precipitation of IgM was in keeping with its high molecular weight; the pronounced precipitation of the low molecular weight opsonic proteins fibronectin, IgG, and C3 was significantly higher in patients, and was most likely explained by the formation of high molecular weight complexes with other proteins.

The high precipitation of fibronectin in the presence of other plasma proteins compared with the isolated protein is also strong evidence in favour of complex formation. Fibronectin will form cryoprecipitable complexes with fibrinogen at -20°C but not in the 4°C to 22°C temperature range that we used. The minimal precipitation of fibrinogen and the much higher precipitation of fibronectin are strong evidence against this mechanism of polyethylene glycol precipitation of fibronectin. Furthermore, precipitation of

fibronectin from serum that contained complexed IgG and C3 but no fibrinogen was also high.

As fibronectin is bound to fibrin during clot formation¹¹ the lower concentrations of immune complexes which we found in serum compared with those in plasma may be explained by incorporation of opsonised immune complexes into the fibrin clot.

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