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Original Research Article

Detection of high molecular weight IgG fibronectin complexes in various types of Acute Myeloid Leukemia - A study in third world country

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Abstract

To detect presence of high molecular weight complexes of IgG and fibronectin, plasma of patients with Acute Myeloid Leukemia was examined by polyethylene glycol (PEG) precipitation, analytical ultracentrifugation, and immunoaffinity chromatography. Ultracentrifugation identified abnormal circulating high molecular weight IgG in all patients. This was precipitated by PEG and was shown by exclusion chromatography to contain IgG in a high molecular weight form. Examination of plasma by immunoaffinity chromatography supported evidence for complex formation between IgG and fibronectin and further showed that abnormal high molecular weight IgG complexes are a prominent feature of Acute Myeloid Leukemia and implicate IgG fibronectin complex formation.

Key words

Acute Myeloid Leukemia, Immune complexes, Fibronectin.

Introduction

It is established that serum of patients with primary myelofibrosis yields positive results in a variety of assays of immune complexes [1, 2, 3, 4]. Positivity for rheumatoid factor and anti-nuclear antibody (ANA) is well documented in

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Myeloproliferative disorders (MPD) and Acute Myeloid Leukemia (AML). Though the composition of any high molecular weight antigen-antibody complexes is yet to be fully defined, these findings have been attributed to the presence of high concentrations of circulating immune complexes.

Increased concentrations of polyethylene glycol precipitable IgG (PEG IgG) are suggestive of circulating high molecular weight forms of IgG in patients with primary myelofibrosis, and their demonstration in Myeloproliferative disorders indicates a common underlying pathophysiology [5]. A negative correlation between plasma concentrations of PEG IgG and fibronectin, and the high PEG precipitability of native plasma fibronectin compared with purified protein, suggests that some fibronectin may exist as a high molecular weight complex with IgG in the plasma of these patients [5]. The purpose of this study was to establish the presence of high molecular weight complexes in the plasma of patients with Acute Myeloid Leukemia and to determine if these consist of complexed IgG and fibronectin.

Material and methods

Five patients with Acute Myeloid Leukemia of different French-American-British (FAB) subtypes as per **Table - 1** and five healthy controls were investigated. The five patients were selected from a larger group of patients when they attended Oncology OPD (Out Patient Department) of Medical College, Kolkata on the basis that none had received chemotherapy or transfusion of blood products in the three months preceding the study. Plasma of such patients was evaluated in School of Tropical Medicine, Kolkata from January, 2012 to May, 2012.

Polyethylene glycol (PEG) precipitation

Proteins were precipitated from plasma anti-coagulated with Ethylenediaminetetraacetic acid (EDTA) by 2% PEG (molecular weight 6000) at 4°C. The precipitate was washed in 2% PEG, resuspended in saline, and the precipitated IgG quantitated by radial immunodiffusion. Immunoprecipitation of PEG precipitated IgG was determined from a plot of immuno-precipitated reference standard IgG concentrations. Thus values should be regarded as mg equivalents because enhanced immunoprecipitation of macromolecules or complexed proteins leads to underestimation when concentrations are calculated from a standard plot derived from immunoprecipitation of low molecular weight native proteins.

Table - 1: Demography and FAB subtype of study cases.

	FAB subtype	Age (Years)	Sex
Case 1	M 5a	26	Male
Case 2	M 5b	34	Female
Case 3	M 3	46	Male
Case 4	M 2	49	Male
Case 5	M 6	56	Female

Exclusion chromatography

A gel column of sepharose 4B was equilibrated with 50 mM TRIS-HC1 (pH7-5) and calibrated with proteins of known molecular weight. Protein elution was recorded by ultraviolet absorption at 280 nm. Samples were centrifuged at 1000 x g for 10 minutes, filtered through a 2 pm filter to remove any particular debris, and applied to the gel in filtered, deaerated TRIS-HC1 buffer. The eluted fractions were analyzed for individual proteins by radial immunodiffusion in gels containing appropriate antisera.

Analytical ultracentrifugation

Samples were centrifuged at 100 000 x g at 20°C in an MSE Centriscan 75 analytical

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ultracentrifuge. The analytical cells were scanned at four minute intervals against a phosphate buffered saline reference solution and sedimentation was observed by Schlieren optics. Sedimentation coefficients were calculated from a minimum of three sedimentation distances per sample.

Affinity chromatography

Affinity gels were prepared according to the method of Axen, et al. [6]. Cyanogen bromide activated sepharose 4B was coupled to substrate and uncoupled protein removed by washing in carbonate buffer. Any remaining active groups were blocked with molar ethanolamine (pH90). The gel was then repeatedly washed in alternating carbonate buffer and 01 M acetate buffer and stored at 4°C in 1 % azide. Before use gels were packed into polypropylene columns and equilibrated with 50 mM TRIS-HC1. Loading and elution of gels was performed at a constant flow rate and protein elution monitored by ultraviolet absorption at 280 nm. Individual proteins were identified and quantitated by rocket immunoelectrophoresis using precipitating antibodies.

Affinity gels were prepared with gelatin, arginine, and sheep anti-human fibronectin. A fibronectin coupled sepharose gel was made from fresh human plasma fibronectin, prepared with the gelatin and arginine gels according to the method of Vuento and Vaheri [7].

Results

Analysis of PEG precipitates by exclusion chromatography

Fresh PEG precipitates were fractionated by exclusion chromatography and the IgG content of each fraction was measured to determine the molecular weight profile of PEG precipitated IgG. From case 4 is compared with fractionation of an untreated solution of polyvalent IgG. Compared with fractionation of the solution of IgG, which showed a single peak of molecular weight 150 kD, the PEG precipitated IgG from case 4 contained a broad high molecular weight band up to 1000 kD. Similar results were found on studying the PEG precipitates of the other patients, and to a lesser extent in two of the normal controls, though less material was precipitated.

Fractionation of a PEG precipitate of the IgG solution (BPL) showed minimal PEG precipitation of IgG (01%) with no high molecular weight component.

Analytical ultracentrifugation of fresh untreated plasma

Ultracentrifugation of untreated fresh plasma from the five patients showed multiple peaks of high molecular weight material ranging from 8S to 21S (sedimentation coefficient in plasma). Ultracentrifugation of plasma from five normal controls did not show any high molecular weight components in four subjects and only a single 9S peak in one.

The plasma of case 3, which contained three high molecular weight peaks of 14S, 15S, and > 15S was PEG precipitated and the precipitate and the supernate analyzed by ultracentrifugation. The PEG precipitate contained three high molecular weight peaks (15S, 18S, and 33S, sedimentation coefficients in saline), while the supernatant plasma contained no high molecular weight material. Sedimentation coefficients in plasma and saline are not comparable as the higher viscosity of plasma results in a reduced sedimentation velocity and hence a lower sedimentation coefficient as per **Table - 2**.

Analysis of PEG precipitates and fresh untreated plasma by affinity chromatography

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A fibronectin coupled sepharose gel was prepared and used to affinity purify a polyvalent sheep anti-human fibronectin antiserum. The resultant purified antibody was itself coupled to activated sepharose for use as a fibronectin immunoaffinity gel. About half of the purified fibronectin was retained by this gel and was eluted with 0-5 molar glycine (pH 2-5). The gel was saturable with fibronectin and therefore the percentage of fibronectin retained decreased when the initial load was in excess. IgG loaded on to the gel appeared in the breakthrough with no elution of IgG during subsequent treatment of the gel with glycine. Thus the gel was specific for fibronectin and had no affinity for IgG as per **Table - 3**.

PEG precipitate from two patients was passed through an uncoupled sepharose gel column to exclude non-specific adsorption of IgG by sepharose, and the eluate was loaded on to the anti-fibronectin antibody immunoaffinity gel. Elution of the column with 0-5 molar glycine yielded demonstrable amounts of IgG in addition to fibronectin. The column also retained small quantities of IgG from the PEG precipitate of a normal control subject. To determine if the column retained any IgG from untreated plasma, fresh plasma samples from a patient and a control were applied to the anti--fibronectin antibody column. IgG was retained from both plasmas but in greater amounts from the patient plasma as per **Table - 2**.

The plasma of case 5 was examined before and after plasmapheresis. The disappearance of a 20S component from the plasma was associated with a reduction in PEG IgG from 477 mg/l to 338 mg/l as per **Table - 2**.

Discussion

In patients with Acute Myeloid Leukemia, high concentrations of plasma PEG precipitable IgG are suggestive of complexed high molecular weight IgG, possibly in the form of immune complexed IgG. Examination of plasma PEG precipitates by exclusion chromatography confirmed the presence of IgG with a high molecular weight profile. This finding may have been artefactual, however, as PEG may induce aggregation of IgG [8, 9]. In view of this, fresh untreated plasma was examined by analytical ultracentrifugation and this confirmed that patients with Acute Myeloid Leukemia have abnormal circulating high molecular weight material in the absence of any artefacts due to PEG precipitation. Furthermore, PEG precipitated the high molecular weight components, leaving a PEG plasma supernate free of such material.

High concentrations of PEG precipitable fibronectin and IgG may be due to complex formation between these two proteins [5]. Fibronectin is capable of binding to a variety of plasma proteins [10], and complex formation between fibronectin and aggregated IgG has been suggested by others [11, 12, 13]. Further evidence for complex formation between fibronectin and IgG was obtained in our study by examining plasma and PEG precipitates by immunoaffinity chromatography. An anti-fibronectin gel retained IgG from patients' plasma and PEG precipitates, and to a lesser degree from normal plasma and PEG precipitates. As the gel had no affinity for IgG its retention is highly suggestive of complex formation with the retained fibronectin. Rheumatoid factor activity has been reported in some patients with primary myelofibrosis [12] and the IgG present in the eluates might theoretically have been IgG which bound specifically with the sepharose coupled sheep antibody. No rheumatoid factor activity, however, was identifiable in the plasmas examined, though small amounts of IgG rheumatoid factor and anti-idiotypic antibody

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can't be excluded.

Immune complexes must reach a critical size to be removed by the reticuloendothelial system. When antibody affinity is low or there is antigen excess, the antigen-antibody network is small and elimination is slow [14]. The size of the complexes, and hence their clearance, may be increased by opsonisation by other proteins—for example, complement or rheumatoid factor [15]. Opsonisation of immune complexes by fibronectin may be a further means of increasing the size of immune complexes. Another possibility is an auxiliary clearance mechanism for immune complexes via macrophage fibronectin receptors [16].

Conclusion

In conclusion, abnormal circulating high molecular weight IgG is present in the plasma of patients with Acute Myeloid Leukemia. The results of PEG precipitation and analytical ultracentrifugation suggest that this material is IgG in a high molecular weight complex form. Examination of plasma by immunoaffinity chromatography supports our previous evidence for complex formation between IgG and fibronectin. Further studies are necessary to confirm complex formation between fibronectin and IgG and to evaluate the importance of this phenomenon not only in Acute Myeloid Leukemia but also in other disorders characterized by immune complex formation.

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Table – 2: Sedimentation coefficients and number of identifiable individual sedimentation peaks from examination of normal and patient plasmas and PEG precipitates by analytical ultracentrifugation.

Subject	Number of peaks	Sedimentation coefficients	PEG IgG (mg/l)
Controls			
1	0		86
2	0		115
3	1	9S	140
4	0		207
5	0		232
Patients			
1	2	14S 21S	759
2	3	13S 15S 20S	547
3	3	14S 15S > 15S	600
4	4	8S 9S 10S 17S	636
5	3	9S 11S 20S	477
5	2	9S 11S	338
(following plasmapheresis)			
Case 3			
Plasma	3	14S 15S >15S	600
PEG	3	15S 18S 33S	600
PEG	0	—	0

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Table – 3: Affinity chromatography of fibronectin and IgG from plasma and PEG precipitates (using an anti-fibronectin immunoaffinity gel). The percentage of IgG retained by the gel was calculated from the amount eluted divided by the amount loaded onto the gel. The gel retained purified fibronectin but not IgG.

Sample	Protein	Load	Recovery in glycine	° of Retained
Purified fibronectin	Fibronectin	390	189	
	IgG	0	0	0
IgG solution	Fibronectin	0	0	
	IgG	2370	0	0
Case 1 PEG	Fibronectin	1500	131	
precipitate	IgG	1440	105	70
Case 5 PEG	Fibronectin	699	319	
precipitate	IgG	623	75	120
Control PEG	Fibronectin	300	149	
precipitate	IgG	147	5	3-5
Case 5	Fibronectin	222	70	
plasma	IgG	20,000	259	1-3
Control	Fibronectin	670	241	
plasma	IgG	11,000	34	0-3