What proteins are present in polyethylene glycol precipitates from rheumatic sera?

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SUMMARY The proteins present in 4% polyethylene glycol (PEG) precipitates of 10 normal sera and 60 samples from patients with rheumatic diseases were studied. A variety of immunochemical methods were used, including estimation of the percentages of total serum proteins precipitated by PEG, gel filtration analyses of the precipitates, and affinity chromatography with protein A and anti-immunoglobulin columns. Substantial amounts of protein were precipitated from normal sera. Many non-immunoglobulin proteins were precipitated from patients' sera, including fibronectin, haptoglobin, albumin, transferrin, and α_1 -antitrypsin. Affinity chromatography with anti-immunoglobulin columns bound non-immunoglobulin proteins from PEG precipitates, but the protein A affinity column did not do so. The view that circulating antibody-antigen complexes alone are precipitated by 4% PEG is too simplistic; many non-immunoglobulin proteins are involved. They may either bind to immune complexes or be coprecipitated owing to non-specific protein aggregation.

Polyethylene glycol (PEG) precipitation is the basis for several techniques for the detection and measurement of circulating immune complexes.¹⁻¹¹ Doubts have been expressed about the specificity of PEG precipitation for this purpose,⁵ and there is still controversy about several points⁶⁻⁸—namely, the proportions in which the main classes of immunoglobulins occur in PEG precipitates; which other plasma proteins accompany the immunoglobulins in significant amounts; and what role (if any) these other proteins may have in complex formation or elimination. We looked at a series of 4% PEG precipitates from patients with rheumatic disorders and healthy controls using a variety of immunochemical techniques to re-examine these questions.

Patients and methods

PATIENTS

Table 1 gives details of patients and controls. Detailed analysis by gel filtration chromatography and affinity chromatography with protein A was carried out in one control and five selected cases: with rheumatoid arthritis and Felty's syndrome in

two instances, rheumatoid arthritis and vasculitis, systemic lupus erythematosus, and systemic vasculitis (as previously classified in ref. 12). Affinity chro-

Table 1 Patients and controls

Group	Total number	Men	Women	Age range (years)
Controls	10	6	4	16-47
Patients	60	19	41	12-76
Rheumatoid arthritis (all cases)	14	6	8	—
Rheumatoid arthritis vasculitis/Felty's syndrome	5	3	2	—
Systemic lupus erythematosus	14	0	14	—
Scleroderma	12	6	6	—
Primary Sjögren's syndrome	6	0	6	—
Immune vasculitis*	7	2	5	—
Other connective tissue diseases†	9	3	6	—

*Comprised two cases of systemic lupus erythematosus vasculitis; two with polyarteritis nodosa; one with Churg-Strauss vasculitis; two with Schönlein-Henoch purpura.

†Comprised five cases of Raynaud's syndrome; two with mixed connective tissue disease; one with viral arthritis; one with Reiter's disease.

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matography using antibodies to IgG, IgM, and IgA was carried out in those six cases plus two others with primary Raynaud's disease and viral polyarthritis.

SERUM SAMPLES

Serum was analysed on the day it was obtained. Freshly removed blood was clotted at 37°C for one hour on the morning of the study and centrifuged at the same temperature. When gel filtration or affinity chromatography were undertaken the serum was immediately loaded onto the columns. PEG precipitation (see below) was carried out on the same day. The precipitates were dissolved at 37°C overnight and analysed the following day.

POLYETHYLENE GLYCOL PRECIPITATION

The method used has been described in detail previously.¹³ In brief, PEG 6000 (BDH Chemicals) in borate buffer pH 8.3 at a concentration of 8% (w/v) was mixed with an equal volume of serum to give a final PEG concentration of 4%. The mixture was allowed to stand at room temperature (18–20°C) for 30 minutes and then centrifuged at 10 000 g for 30 minutes. The supernatant was discarded and the precipitate resuspended and washed three times in 4% PEG in borate buffer pH 8.3. The washed pellet was redissolved in 0.3 M phosphate buffered saline pH 7.4 overnight at 37°C and adjusted to the initial serum volume.

Gel filtration Sephacryl S400 superfine (Pharmacia) was used for gel filtration chromatography in Wright Amicon columns of bed volume 684 ml. The void volume (242 ml) was determined with Blue

Dextran 2000. The sample volume of serum or resuspended PEG precipitate applied to the columns was 3 ml. The optical density of the eluate was measured at 280 nm. Fractions (6 ml) were collected and those eluted between 240 and 600 ml stored at –20°C until analysed.

AFFINITY CHROMATOGRAPHY

Two techniques, based on the methods of Johnstone and Thorpe were used.¹⁴ Firstly, protein A-Sepharose CL-4B (Pharmacia), as a preformed column, was used to bind IgG. The running buffer was 0.2 mM phosphate pH 7.0 and the eluting buffer 0.1 M glycine-HCl pH 3.0. Secondly, specific anti-IgG, -IgA, and -IgM columns, containing the respective antisera covalently bound to Sepharose 4B activated by cyanogen bromide, were used for these individual immunoglobulins. Three columns were set up in series (one for each immunoglobulin) with a running buffer of 0.5 M sodium chloride containing 20 mM sodium dihydrogen orthophosphate pH 7.3. Proteins were eluted with 3 M potassium thiocyanate in 0.5 M ammonium hydroxide.

Samples were passed through a Sepharose 4B column to remove proteins bound non-specifically, and then 0.5 ml of serum or redissolved PEG precipitate, thus pretreated, was loaded onto the affinity columns. After elution the material detected in the main peaks was pooled and dialysed against phosphate buffered saline for 48 hours at 4°C with three changes of buffered saline. The samples were gently concentrated back to their starting volumes using Sartorius collodion bags and a vacuum of 67 kPa. The samples were stored at –20°C until their

Table 2 Amounts of immunoglobulin and fibronectin in polyethylene glycol precipitates in different diagnostic groups. Mean percentage serum concentration (95% confidence intervals) are shown

Group	Total number	IgG	IgA	IgM	Fibronectin
Controls	10	2.1 (1.9 to 2.9)	3.1 (2.1 to 4.1)	15 (11 to 19)	1.5 (1.0 to 2.0)
All patients	60	4.3 (4.1 to 4.5)	8.0 (6.0 to 10.0)	36 (30 to 42)	4.3 (2.2 to 6.4)
RA* (all cases)	14	4.7 (1.7 to 7.7)	10.2 (4.5 to 15.9)	36 (19 to 53)	3.6 (1.9 to 5.3)
RA vasculitis/Felty's syndrome	5	9.4 (4.6 to 14.2)	18.6 (9.4 to 27.8)	66 (50 to 82)	8.1 (3.9 to 12.3)
Immune vasculitis	7	2.0 (0.6 to 3.4)	3.2 (0 to 6.4)	36 (17 to 55)	1.7 (0 to 3.4)
SLE*	14	3.1 (2.0 to 4.2)	5.6 (2.8 to 8.4)	35 (28 to 42)	2.4 (1.3 to 3.5)
Scleroderma	12	3.3 (2.5 to 4.2)	4.9 (3.3 to 6.5)	37 (20 to 54)	2.6 (1.4 to 3.8)
Primary Sjögren's syndrome	6	3.4 (1.2 to 5.6)	8.9 (5.5 to 12.3)	33 (24 to 42)	3.7 (1.4 to 6.0)

*RA=rheumatoid arthritis; SLE=systemic lupus erythematosus.

protein concentrations were estimated immunochemically.

IMMUNOCHEMICAL ESTIMATION

IgG, IgA, IgM, haptoglobin, complement C3, α_1 -antitrypsin, transferrin, and albumin were determined by radial immunodiffusion. Rocket immunoelectrophoresis was used to estimate fibronectin. The monospecific antisera necessary for these estimations were obtained from Dakopatts Ltd. A standard of suitably diluted pooled human serum was used in determining each protein. The standard serum was previously calibrated with a highly purified sample of the appropriate protein.

The concentration of each protein in serum or PEG precipitate was expressed as a percentage of its level in the standard serum (as g/l or mg/l). The concentration of PEG precipitates was also expressed as a percentage of the same protein in the serum

from which it was derived. Duplicate and triplicate estimations showed coefficients of variation of less than 6%.

Results

Small amounts of protein were precipitated from control sera by 4% PEG. About twice as much protein was precipitated from the patients' sera (Table 2). There were similar increases in the amounts of immunoglobulins and fibronectin in the precipitates. Subdivision of the patients into different diagnostic categories showed that most PEG precipitate was present in the five cases of RA vasculitis with or without Felty's syndrome. The least PEG precipitate was present in the seven patients with other forms of immune vasculitis.

More detailed analyses were undertaken in five patients and one control. The total amounts of

Fig. 1 Amounts of eight serum proteins in the polyethylene glycol precipitates from one control and five patients selected for additional studies. Results are expressed as the mean percentage total serum protein concentration (95% confidence intervals are shown). □=control case; ▨=mean results from five patients.

Table 3 Non-immunoglobulin proteins in polyethylene glycol precipitates analysed by column chromatography and immunoassay of eluant. The presence of complexed (C) or monomeric (M) protein is indicated by molecular size of eluant as present (+) or absent (0)

Protein	Molecular size	Patients				
		Normal	Felty's syndrome		RA* vasculitis	SLE* Systemic vasculitis
Complement	C	0	+	+	0	+
	M	+	+	+	0	+
Fibronectin	C	0	+	+	0	0
	M	0	0	+	0	0
α_1 -Antitrypsin	C	0	+	+	+	0
	M	0	+	+	0	0
Transferrin	C	0	+	+	+	0
	M	0	+	+	0	0
Albumin	C	0	+	+	+	+
	M	0	+	+	+	+
Haptoglobin	C	0	+	+	+	0
	M	0	0	+	0	0

*RA=rheumatoid arthritis; SLE=systemic lupus erythematosus.

immunoglobulins and fibronectin in their PEG precipitates were similar to those of the larger group (Fig. 1). These PEG precipitates also contained substantial amounts of C3 complement and measurable amounts of α_1 -antitrypsin, transferrin, and albumin. Haptoglobin (results not shown) was present at a similar level to that of transferrin.

Gel filtration chromatography of the PEG precipitates, immunoassay of the eluates, and similar analysis of their related whole serum samples showed that aggregates of immunoglobulins were often associated in the PEG precipitates with many non-immunoglobulin proteins. This was most obvious in the patients with Felty's syndrome. The PEG precipitate from the normal control showed that no non-immunoglobulin was detected in the PEG precipitate on gel filtration and immunoassay, with the exception of a small amount of monomeric C3. By contrast, patients' PEG precipitates contained large amounts of complexed (high and low molecular weight) and low molecular weight monomeric non-

immunoglobulin proteins. Table 3 summarises these results. There were considerable differences between patients, with less PEG precipitable non-immunoglobulin protein in patients with systemic lupus erythematosus and systemic vasculitis.

Affinity chromatography with protein A bound IgG in all patients' sera and PEG precipitates (Table 4). Small amounts of some other immunoglobulin and non-immunoglobulin proteins were bound from whole sera. Their associated PEG precipitates only bound IgA in two cases, and C3 in one case.

Affinity chromatography with anti-immunoglobulins showed a different pattern of binding (Table 5). Non-immunoglobulin proteins were bound from all patients' sera and in many instances from PEG precipitates.

Discussion

If 4% PEG preferentially precipitates only circulating

Table 4 *Relative amounts of different proteins eluted from protein A affinity columns in five patients' sera and polyethylene glycol precipitates*

Proteins eluted	Serum (concentration range mg/l)					PEG* precipitate (concentration range mg/l)				
	>1000	100-1000	10-100	1-10	ND*	>1000	100-1000	10-100	1-10	ND
IgG	5	0	0	0	0	0	1	4	0	0
IgA	0	4	1	0	0	0	0	2	0	3
IgM	2	3	0	0	0	0	0	0	0	5
Fibronectin	0	0	0	3	2	0	0	0	0	5
C3	0	0	0	4	1	0	0	0	1	4
α_1 -Antitrypsin	0	0	0	1	4	0	0	0	0	5
Transferrin	0	0	0	4	1	0	0	0	0	5
Albumin	0	0	4	0	1	0	0	0	0	5
Haptoglobin	0	0	3	1	1	0	0	0	0	5

*PEG=polyethylene glycol; ND=not detected.

Table 5 *Relative amounts of different proteins eluted from anti-immunoglobulin affinity columns in seven patients' sera and polyethylene glycol precipitates*

Proteins eluted	Serum (concentration range mg/l)					PEG* precipitate (concentration range mg/l)				
	>1000	100-1000	10-100	1-10	ND*	>1000	100-1000	10-100	1-10	ND
IgG	7	0	0	0	0	1	5	1	0	0
IgA	4	3	0	0	0	0	2	5	0	0
IgM	6	1	0	0	0	3	0	4	0	0
Fibronectin	0	1	5	1	0	0	0	1	2	4
C3	0	0	6	1	0	0	0	1	2	4
α_1 -Antitrypsin	0	0	6	1	0	0	0	1	3	3
Transferrin	0	0	1	6	0	0	0	1	1	5
Albumin	0	0	7	0	0	0	0	5	2	0
Haptoglobin	0	0	6	1	0	0	0	3	2	2

*PEG=polyethylene glycol; ND=not detected.

immune complexes, as some authors suggest,⁷ little or no protein should be brought down from normal sera and predominantly IgG or IgG and IgM complexes from patients' sera. Our results suggest this is not the case. There were substantial amounts of PEG precipitable proteins in normal samples, the amounts of immunoglobulins G, M, and A and fibronectin precipitated in patients' samples showed similar increases, and non-immunoglobulin proteins were often precipitated. There is disagreement about the composition of precipitates produced by 4% PEG. Chia *et al* claim it is a valid measure of circulating immune complexes in pathological sera.⁹ Other studies have not found a close correlation between serum and PEG precipitable immunoglobulins.^{10 11} It seems too simplistic to consider that 4% PEG solely precipitates antibody-antigen complexes; many other proteins are involved.

The optimal concentration of PEG for these assays is the subject of debate. Although many workers use 4% PEG, other groups prefer lower concentrations, such as 2% or 2.5% PEG. In a large initial study we evaluated the effectiveness in this assay of four different concentrations of PEG from 1.6 to 4.0%.¹⁵ We found that lower concentrations of PEG are considerably less sensitive, but it has been argued that they are more specific. Our own view is that 4% PEG has the advantage of sensitivity in determining the constituents of putative complexes. In vitro studies with one non-immunoglobulin protein found in PEG precipitates, fibronectin, have shown that the purified protein alone is not precipitated by 4% PEG.¹⁶

Non-immunoglobulin proteins could be present in PEG precipitates owing to 'trapping' or non-specific binding to the columns, but the evidence suggests this is not the case. The washing procedure we used was extensive. The proteins were present in association with immunoglobulins in high molecular weight fractions of sera and PEG precipitates. They were eluted from the anti-immunoglobulin column after affinity chromatographic analysis of PEG precipitates, but not from the protein A column. This last observation makes it very difficult to argue for a non-specific binding effect. Several studies have shown that fibronectin is associated with circulating immune complexes,^{13 15-17} and it is widely accepted that complement components can bind to these complexes. The present results suggest many other plasma proteins bind to circulating immune complexes, albeit at low concentrations. This has been reported by others,^{18 19} though it has not been fully evaluated. How could non-immunoglobulins become associated with circulating immune complexes? One possibility is that these proteins are altered by free radical²⁰ or enzyme damage and then become non-

specifically adherent. Alternatively, many of the apparent circulating immune complexes in rheumatic diseases may represent heterogeneous macromolecular complexes which do not have an obvious antigen within them. Such complexes would be expected to show rheumatoid factor activity in rheumatoid arthritis but not in other diseases.

There were clear differences between the type of proteins eluted from the protein A affinity column and the anti-immunoglobulin affinity columns. It was only with the latter columns that non-immunoglobulin proteins were bound from PEG precipitates. The implication behind this finding is that non-immunoglobulin proteins are associated with certain immunoglobulins, but not the IgG which binds to protein A. If sera contains circulating immune complexes it may be that several different types of complex are present, only some of which bind small amounts of other plasma proteins.

There were interesting differences between subgroups of patients (Table 2). Rheumatoid vasculitis differed quite markedly from the other cases of 'immune' vasculitis. Only patients with rheumatoid vasculitis had large amounts of PEG precipitable immunoglobulins. Clearly different pathological mechanisms must exist in these two groups, and it is unlikely that circulating immune complexes are the basis of the vascular damage in patients with conditions like polyarthritis nodosa.

Our principal objective was to reconsider exactly what 4% PEG precipitates contain. Many non-immunoglobulin serum proteins are precipitated by it, and other methods like gel filtration and affinity chromatography suggest that these proteins are closely associated with immunoglobulins. Either circulating immune complexes bind many different proteins or non-specific protein aggregation is common. In both cases the simplistic views that PEG specifically precipitates circulating immune complexes is incorrect.

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References

- Harrington J C, Fenton J W, Pert J H. Polymer-induced precipitation of antigen-antibody complexes: 'precipitation reactions'. *Immunochemistry* 1971; **8**: 413-21.
- Creighton W D, Lambert P H, Miescher P A. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. *J Immunol* 1973; **111**: 1219-27.
- Barnett E V, Chia D. Quantitation of immunoglobulin G in serum and immune complexes isolated in 4% polyethylene glycol. *Ann Rheum Dis* 1977; **36**(suppl 1): 26.
- Digeon M, Lauer M, Riza J, Bach J F. Detection of circulating

- immune complexes in human sera by simplified assays with polyethylene glycol. *J Immunol Methods* 1977; **16**: 165–83.
- 5 Hyun J. Specific proteins precipitated by polyethylene glycol during assays for circulating C precipitable immune complexes. *Am J Clin Pathol* 1983; **80**: 777–8.
- 6 Soltis R D, Hasz D E. The effect of serum immunoglobulin concentration on immune complex detection by polyethylene glycol. *J Immunol Methods* 1983; **57**: 275–82.
- 7 Ritzman S E, Daniels J C. Immune complexes: characteristics, clinical correlations and interpretative approaches in the clinical laboratory. *Clin Chem* 1982; **28**: 1259–71.
- 8 Koethe S M, Melton H E, Jendrzeczek Z. A simple light-scattering method for detecting soluble immune-complexes in human serum. *Am J Clin Pathol* 1980; **74**: 804–8.
- 9 Chia D, Barnett E V, Yamagata J, Knutson D, Restivo C, Furst D. Quantitation and characterization of soluble immune complexes precipitated from sera by polyethylene glycol (PEG). *Clin Exp Immunol* 1979; **37**: 399–407.
- 10 Doan R, Ollier-Hartman M P, Schwarzenberg L, Hartmann L. Study of circulating immune complexes in three haematologic diseases: acute myeloid leukaemia, acute lymphatic leukaemia and haematosa. *Cancer Immunol Immunother* 1980; **8**: 161–6.
- 11 Rayner A A, Steele G, Rodrick M L, *et al*. Application of the PEG turbidity assay to detection of circulating immune complexes in cancer patients. *Am J Surg* 1981; **141**: 460–4.
- 12 Scott D G I, Bacon P A, Tribe C R. Systemic rheumatic vasculitis: a clinical and laboratory study of 50 cases. *Medicine (Baltimore)* 1981; **60**: 288–97.
- 13 Coppock J, Scott D L, Carter S, Moreland T, Robinson M W. Fibronectin in PEG precipitates: evidence for a role in immune complexes. *Rheumatol Int* 1986; **6**: 79–83.
- 14 Johnstone A, Thorpe R. *Immunochimistry in practice*. Oxford: Blackwell Scientific, 1982: 298.
- 15 Herbert K E, Coppock J S, Griffiths A M, Williams A, Robinson M W, Scott D L. Fibronectin and immune complexes in rheumatic diseases. *Ann Rheum Dis* 1987; **46**: 734–40.
- 16 Carter S D, Scott D L, Elson C J. Fibronectin associated with immune complexes from sera and synovial fluids in rheumatoid arthritis. *Br J Rheumatol* 1986; **25**: 353–8.
- 17 Cosio J, Bakaletz A. Binding of human fibronectin to antigen-antibody complexes. *J Lab Clin Med* 1986; **107**: 453–8.
- 18 Zhen Q, Walther S, Sodomann C. Small albumin-immunoglobulin complexes in patients with liver disease. *Immunology* 1983; **48**: 809–16.
- 19 Melsom R D, Smith P R, Maini R N. Demonstration of an unidentified 48 kD polypeptide in circulating immune complexes in rheumatoid arthritis. *Ann Rheum Dis* 1987; **46**: 104–9.
- 20 Lunec J, Bailsford S, Hewitt S D, Morris C J, Blake D R. Free radicals: Are they possible mediators of IgG denaturation and immune complex formation in rheumatoid arthritis? *International Journal of Immunotherapy* 1986; **11**: 6442–6.