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SEROLOGICAL HETEROGENEITY OF THE IgM COMPONENTS OF MIXED (MONOCLONAL IgM–POLYCLONAL IgG) CRYOGLOBULINS

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SUMMARY

Mixed IgG–IgM cryoglobulins were isolated from the sera of seven patients with macroglobulinaemia or cryoglobulinaemia. The IgM components of all seven cryoproteins were monoclonal, containing κ light chains only, whereas the IgG components were polyclonal, containing both κ and λ light chains. Despite their apparent immunological homogeneity, the IgM components showed a wide range of antiglobulin activity. The data indicate that serological specificity may vary from one mixed cryoglobulin to another and that the monoclonal IgM components of different mixed cryoglobulins represent a heterologous group of antiglobulins.

INTRODUCTION

Complex (mixed) cryoglobulins contain both IgG and IgM immunoglobulins, in contrast to simple cryoglobulins which are composed exclusively of IgG, IgA, IgM or Bence Jones proteins (Lerner & Watson, 1947; Mackay *et al.*, 1956; LoSpalluto *et al.*, 1962; Christian, Hatfield & Chase, 1963; Liss, Fudenberg & Kritzman, 1967). Previous studies of mixed cryoglobulins devoid of complement components (hereafter termed MCG) have shown that neither the isolated IgG nor the isolated IgM component is insoluble at reduced temperatures; the presence of both is required for cryoprecipitation (LoSpalluto *et al.*, 1962; Balázs, Fröhlich & Csáti, 1963). The IgM component of MCG is capable of interacting with normal IgG from any source (LoSpalluto *et al.*, 1962; Balázs *et al.*, 1963; Peetoom & van Loghem-Langereis, 1965; Curtain, Baumgarten & Pye, 1965; Meltzer *et al.*, 1966). The IgM fractions have, therefore, been considered to be incomplete cryoglobulins requiring IgG molecules for full expression. Antibody activity specific for IgG has been demonstrated in the IgM fractions of several mixed cryoglobulins, leading to the postulate that in at least

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some instances the cryoproteins may represent antigen–antibody complexes (Peetoom & van Loghem-Langereis, 1965; Meltzer *et al.*, 1966). Moreover, immunological characterization of a few MCG has shown that the IgM component of the cryoproteins studied contained only κ light chains, whereas the IgG component contained both κ and λ light chains, suggesting the interaction of a monoclonal* IgM with a random population of IgG molecules. The possibility that the IgM antiglobulins possess a selective specificity for certain IgG molecules, however, has not been excluded (Balázs & Fröhlich, 1966; Metzger, 1967).

IgM antiglobulins of a number of different antigenic specificities apparently exist in human sera, and indeed may co-exist in the same serum. Some of the IgM antiglobulins react only with native human IgG, whereas others react, in addition, with aggregated human IgG or rabbit IgG or both (Williams & Kunkel, 1963). The present investigation was undertaken to determine whether the IgM components of mixed cryoglobulins vary in antigenic specificity. For this purpose, we studied the serological interactions in three test systems of the IgM components isolated from seven different cryoglobulinaemic sera.

MATERIALS AND METHODS

Cryoglobulins were isolated from the sera of three patients with Waldenström's macroglobulinaemia (Br., La. and Pa.), two patients with Peetoom–Meltzer cryoglobulinaemia (purpura, joint pain, low serum complement level and positive immune adherence reaction) (Mc. and Wh.), one patient with essential cryoglobulinaemia and rheumatoid arthritis (Da.) and one with essential cryoglobulinaemia (Ri.).

For isolation of cryoglobulins, blood was drawn with a warmed syringe and allowed to clot at 37°C. The serum was collected, chilled to 2–4°C for at least 24 hr, and the supernatant was removed. The precipitate was dissolved in warm buffered saline solution, pH 8·0, at 37°C, then re-precipitated at 2–4°C. This procedure was repeated at least three times. After each precipitation the cryoprotein was washed three times with chilled saline solution. After the final washing the precipitate was suspended in acetate buffer, pH 4·0.

The protein content of the cryoglobulins was measured quantitatively by a modified Folin technique (Lowry *et al.*, 1951) and analysed qualitatively by immunoelectrophoresis (Scheidegger, 1955) and gel double diffusion, using rabbit antisera to whole human serum and monospecific antisera to various human serum proteins [Cohn Fraction II (IgG), pooled myeloma IgA, pooled macroglobulinaemia IgM and isolated κ and λ light chains].

The protein components of each cryoglobulin were separated by gel filtration chromatography on Sephadex G-200 columns equilibrated with 0·2 M-sodium acetate buffer, pH 4·0, or by ion exchange chromatography on DEAE-cellulose (Selectocel, Carl Schleicher and Schuell Co., Keene, New Hampshire) at 37°C, using 0·02 M-Tris–HCl buffer, pH 8·0, as starting buffer and linear gradient elution with 0·5 M-NaCl in the reservoir. The purity of the fractions was tested by immunoelectrophoresis and ultracentrifugal analysis. Ultracentrifugation was carried out in a Spinco model E analytical ultracentrifuge at 20° and 37°C by standard methods.

The antiglobulin activities of the whole sera, the isolated protein components and the cryoglobulin-free supernatants were determined in three test systems; positive and negative control sera were included in all experiments.

* The term monoclonal in this text means an immunoglobulin with one antigenic type of heavy chain and one antigenic type of light chain.

(1) Latex agglutination slide tests were performed with commercial reagents (Sylvana Co., Milburn, New Jersey). Agglutination was scored after 1 min, and the results were expressed as the highest dilution capable of agglutinating the latex particles.

(2) Agglutination tests were performed with human group O Rh-positive red blood cells sensitized for 1 hr at 37°C with a 1:20 dilution of the diagnostic anti-CD serum Ripley (Waller & Vaughan, 1956) and tested for adequacy of coating with rabbit antiglobulin reagents. One drop of a 2% suspension of the sensitized cells was added to one drop of serially diluted (two-fold) test material. The slides were agitated for 10 min at room temperature and read visually for agglutination. The highest dilution producing visible agglutination was taken as the end point; only those whole sera with titres of 1:20 or greater were considered abnormal.

(3) Agglutination tests with cells sensitized with rabbit IgG were done by a modified Rose–Waaler technique (Seligmann *et al.*, 1967). Normal control sera in dilutions greater than 1:32 failed to agglutinate the sensitized cells.

The specificity of antibodies reactive with red cells sensitized with human IgG and rabbit IgG was further tested by inhibition of agglutination. For these tests, four agglutinating units of IgM component were incubated with native or aggregated human IgG or with rabbit IgG (2·5 mg/ml) before the addition of the sensitized cells. The mixture of agglutinator, inhibitor and coated cells was incubated and agglutination was then scored as before.

Tests for anti-antibody activity (Milgrom, Dubiski & Woźniczko, 1956) were carried out in similar fashion with native and heat-aggregated human IgG in concentrations of 6 mg/ml.

RESULTS

The seven mixed cryoglobulins and their isolated IgG and IgM components had similar antigenic properties. In all instances Sephadex G-200 filtration of the isolated cryoproteins yielded two component peaks, which eluted in positions expected of IgM and IgG proteins (Fig. 1). On immunoelectrophoresis, the intact cryoproteins contained both IgM and IgG (Fig. 2). Immunoelectrophoresis of the isolated peak material showed that peak I consisted solely of IgM and peak II solely of IgG (Fig. 3). The IgM component of each of the cryoglobulins contained κ light chains only, whereas the IgG component of each contained both κ and λ light chains (Fig. 4). Both the IgM and IgG components of the seven cryoglobulins were soluble at 4°C in neutral buffers.

Ultracentrifugal analyses of the isolated intact cryoglobulins at 37°C (Fig. 5) showed two major peaks with sedimentation values of about 7S and 19S; small amounts of undissociated complexes were also present. The ultracentrifugal patterns of the whole sera at 20°C revealed, in addition to the major 19S peak, complexes with sedimentation rates greater than 19S. The ultracentrifugal pattern obtained with serum Wh., which contained a series of rapidly sedimenting polymers, is shown in Fig. 6.

In tests against three different IgG antigens, the whole serum from each of the seven patients showed a wide spectrum of antiglobulin activity (Table 1). The isolated IgG components of the cryoglobulins were devoid of antiglobulin activity in all three systems. In contrast, the isolated IgM components had high titres of serological activity against aggregated human IgG and human anti-Rh antibody, although the results with rabbit IgG were variable. All the IgM components were monoclonal and all reacted with human IgG; therefore, to facilitate comparison of the data, the antiglobulin titres were expressed as the

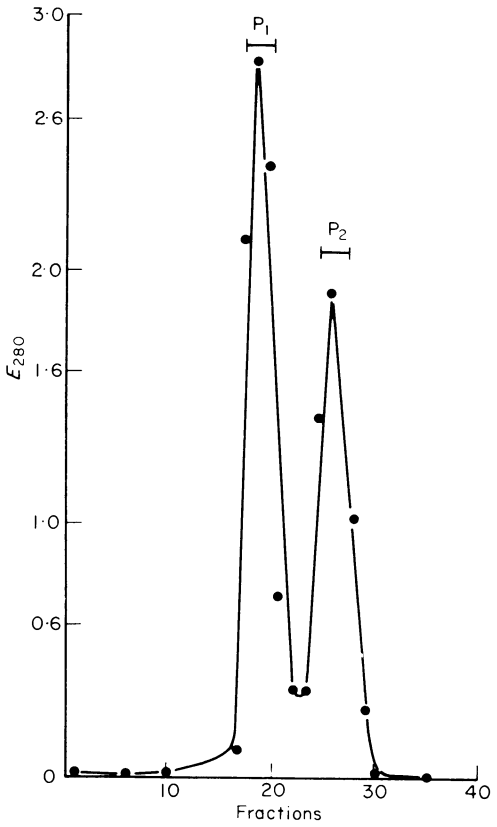

FIG. 1. Elution pattern (Sephadex G-200) of a representative isolated mixed cryoglobulin. Two peaks were obtained as measured by optical density at E_{280}. Sodium acetate, 0·2 M, pH 4·0.

TABLE 1. Antiglobulin activities of whole serum of seven patients with macroglobulinaemia or cryoglobulinaemia*

Patient	Diagnosis	Latex test (aggregated human IgG)	Sensitized cells	
			Human IgG (anti-CD Ripley)	Rabbit IgG
Mc.	Cryoglobulinaemia	160	160	1024
Wh.	Cryoglobulinaemia	2560	160	64
Br.	Macroglobulinaemia	4096	4096	64
La.	Macroglobulinaemia	20	80	32
Da.	Essential cryoglobulinaemia and rheumatoid arthritis	320	256	2048
Pa.	Macroglobulinaemia	1280	1280	4096
Ri.	Essential cryoglobulinaemia	80	80	64
Normal serum		<20	<20	<32

* Expressed as the reciprocal of the highest dilution showing agglutination.

reciprocal of titre of 1 mg of 'purified' antibody IgM. On this basis, as shown in Table 2, the antiglobulin titres of the IgM fractions ranged from 10 to 180 in the latex test and from 10 to 360 in tests against human IgG compared with a range of 0 to 140 in tests against rabbit

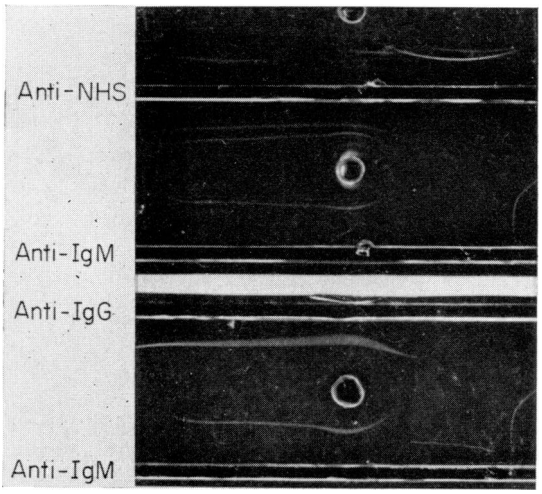

FIG. 2. Immunoelectrophoretic pattern of a representative isolated mixed cryoglobulin, demonstrating the IgM and IgG components. Normal human serum in top well and mixed cryoglobulin in second and third wells; antisera to normal human serum (NHS), IgM and IgG as indicated.

IgG. The IgM components of four of the seven cryoproteins lacked detectable activity against rabbit IgG, although the four corresponding whole sera showed significant serological activity against the antigen. The cryoglobulin-free supernatant from all seven sera retained activity against rabbit IgG (Table 3).

TABLE 2. Antiglobulin activities of IgM components of mixed cryoglobulins*

Patient	Latex test (aggregated human IgG)	Sensitized cells	
		Human IgG (anti-CD Ripley)	Rabbit IgG
Mc.	25	50	0
Wh.	30	15	0
Br.	20	227	0
La.	10	100	0
Da.	50	360	20
Pa.	35	10	30
Ri.	180	360	140

* Expressed as reciprocal of titre per milligram of 'purified' IgM. Initial concentration of each IgM was 1 mg/ml.

In similar control tests on two simple IgM and two simple IgG cryoglobulins, no antiglobulin activity could be detected.

To assess the specificity of the antiglobulins reactive with human IgG and with rabbit

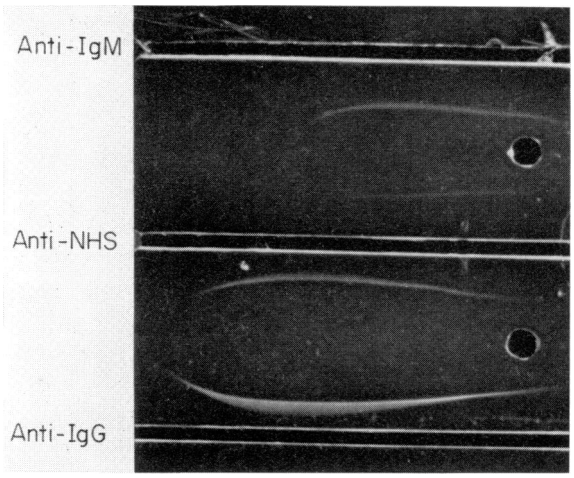

FIG. 3. Immunoelectrophoretic patterns of the peaks obtained by gel filtration of the isolated mixed cryoglobulin (Fig. 1). Peak I (upper) consists entirely of IgM, and peak II (lower) entirely of IgG.

IgG, attempts were made to inhibit the agglutinating activity of the IgM components of the cryoglobulins of two sera. One of the isolated IgM components (serum Pa.) had significant activity against cells sensitized with human IgG and with rabbit IgG; both agglutination

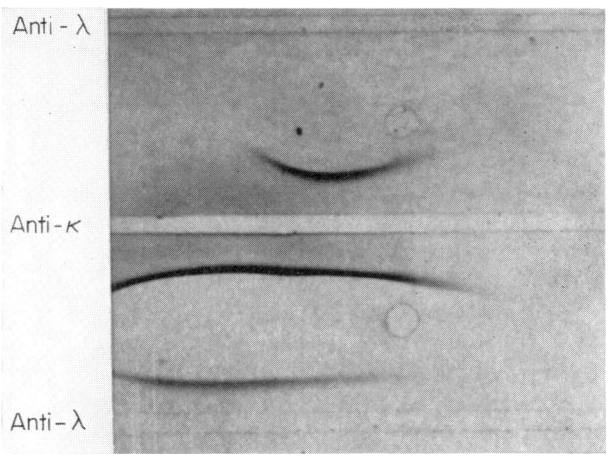

FIG. 4. Immunoelectrophoretic patterns of the isolated peaks (Fig. 3), showing light chain typing. The IgM component, peak I, (upper) has only κ determinants; the IgG component, peak II, (lower) contains both κ and λ determinants.

reactions were inhibited by native human IgG and by rabbit IgG. In contrast, the other IgM component (serum La.) had activity only against the human IgG coat; this activity was inhibited by human IgG but not by rabbit IgG (Table 4).

Anti-antibody activity was demonstrable in three of the seven isolated IgM components

TABLE 3. Antiglobulin activities of cryoglobulin-free supernatant sera

Patient	Latex test* (aggregated human IgG)	Sensitized cells†	
		Human IgG (anti-CD Ripley)	Rabbit IgG
Mc.	+	+	+
Wh.	+	+	+
Br.	+	+	+
La.	+	+	+
Da.	+	+	+
Pa.	+	+	+
Ri.	+	+	+

* Titres greater than 1:20.
† Titres greater than 1:32.

(Table 5). The ability of these three cryoproteins to agglutinate cells sensitized with anti-CD Ripley was readily inhibited by heat-aggregated human IgG, but not by native human IgG. Additional evidence of anti-antibody activity was obtained with direct precipitation techniques (Warner, MacKenzie & Fudenberg, 1968). The ability of the remaining four IgM components to agglutinate such cells was inhibited by both native and heat-aggregated IgG.

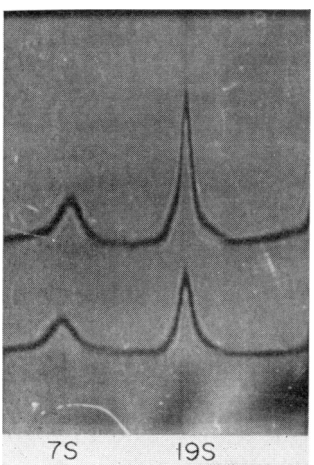

FIG. 5. Analytical ultracentrifugal pattern of the whole cryoglobulin of serum We. (upper) and Mc. (lower) at 37°C after 16 min at 59,780 rev/min. A 19S and 7S component are present.

DISCUSSION

The IgG components isolated from the seven mixed cryoglobulins contained both κ and λ chains, as has been reported previously of the IgG fractions of all MCG so far studied. The IgM components, however, were monoclonal and contained only κ light chains. IgM fractions consisting solely of light chains of κ type have been described in only several

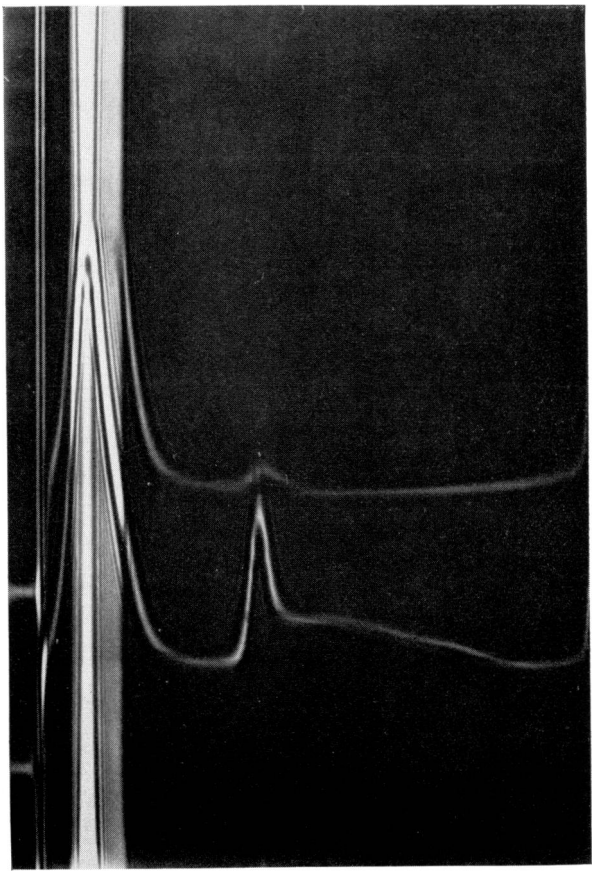

FIG. 6. Analytical ultracentrifugal pattern of normal human serum (upper) and whole serum from patient Wh. (lower) at 20°C after 16 min at 59,780 rev/min. Serum Wh., in addition to the major 19S peak, shows a series of high molecular weight polymers.

instances (Balázs & Fröhlich, 1966; Metzger, 1967). Other reports, however, have suggested that the IgM fractions of most MCG contain both κ and λ chains (Peetoom & van Loghem-Langereis, 1965; Meltzer *et al.*, 1966). The cryoglobulins studied by these investigators were obtained from sera with diffuse immunoglobulin elevations, and the lack of a definite homogeneous peak may have led to the conclusion that no monoclonal protein was present. In the present study the sera of four of the seven patients showed diffuse hypergammaglobulinaemia by electrophoresis; nevertheless, the isolated IgM components contained only

κ molecules. Although the possibility that the IgM fractions of some MCG are polyclonal cannot be excluded, such antigenic heterogeneity must be unusual.

Despite their immunological homogeneity, the IgM components isolated from the cryoglobulins of our seven patients differed considerably from one patient to another in sero-

TABLE 4. Inhibition of antiglobulin activity of IgM cryoprotein by human and rabbit IgG*

Patient	Inhibitor	Sensitized cells	
		Human IgG (anti-CD Ripley)	Rabbit IgG
Pa.	Human IgG	−	−
	Rabbit IgG	−	−
	Saline	+	+
La.	Human IgG	−	−
	Rabbit IgG	+	−
	Saline	+	−

* Agglutinating system: 4 agglutinating units of IgM cryoprotein, sensitized red cells and inhibitor (2·5 mg/ml). + = Agglutination; − = inhibition.

logical specificity. The antiglobulin activities of the IgM fractions were determined in three test systems differing in the antigenic site of IgG available for interaction. The latex test detects antiglobulins directed toward antigenic determinants present on aggregated human IgG (Singer *et al.*, 1960). Antiglobulins directed against antigens of native human IgG and

TABLE 5. Inhibition of antiglobulin activity of IgM cryoprotein by heat-aggregated and native human IgG*

Patient	Inhibitor		Saline control
	Aggregated human IgG†	Native human IgG	
Mc.	−	+	+
Wh.	−	+	+
Br.	−	+	+
La.	−	−	+
Da.	−	−	+
Pa.	−	−	+
Ri.	−	−	+

* Agglutinating system: 4 agglutinating units of IgM cryoprotein, sensitized red cells (anti-CD Ripley) and inhibitor (6 mg/ml). + = Agglutination; − = inhibition.

† Solution of 10 mg/ml heated at 63°C for 15 min.

determinants made available by antigen–antibody combination react with anti-CD (Ripley) sensitized red cells. Antiglobulins reactive with rabbit IgG presumably are directed toward antigens similar to the internal or 'buried' determinants of human IgG which are not usually available for interaction (Milgrom & Witebsky, 1960). The variation in the results obtained in tests on the seven IgM fractions (Table 2) indicated non-identity of specificity. This heterogeneity was especially striking with rabbit IgG. Only one of the IgM components (Ri.) showed significant activity against rabbit IgG. To our knowledge only one other instance in which rabbit IgG was as effective as human IgG in restoring the cryoprecipitability of an incomplete IgM cryoprotein has been reported (Metzger, 1967). The IgM fractions of two other cryoproteins (Da. and Pa.) showed slight activity against rabbit IgG, but the titres were much lower than those of the native sera. The minimal reactivity of the two IgM components, as shown by inhibition studies, resulted from cross-reacting antibodies. The IgM components isolated from the remaining four cryoproteins were devoid of activity against rabbit IgG, although such activity was present in the corresponding whole serum. Thus, the results obtained with the three test systems indicate that the IgM components of mixed cryoglobulins react preferentially with the exposed antigens of human IgG and have only limited reactivity with the buried determinants of human IgG.

The serological heterogeneity of the monoclonal (IgM K) antiglobulins is of considerable interest for several reasons. Demonstrable antibody activity is rare in the monoclonal immunoglobulins encountered in human diseases. One exception is another antibody active only at low temperatures, namely, the cold agglutinin of chronic cold agglutinin haemolytic anaemia, which is also almost invariably IgM (Fudenberg & Kunkel, 1957) and almost invariably consists of type κ light chains (Franklin & Fudenberg, 1964; Harboe *et al.*, 1965). In contrast to the IgM K antiglobulins, these cold agglutinins appear to have restricted specificity and interact only with the I antigen on the red cell surface (Wiener *et al.*, 1956). Whether limited specificity is a property of other monoclonal antibodies has not been established. The results of the present study, however, indicate that immunological homogeneity does not necessarily imply limited antigenic specificity.

At present, little is known about the mechanism whereby the interaction of the IgM fraction with IgG at reduced temperatures produces cryoprecipitability. The frequent finding of antiglobulin activity in the IgM component of MCG suggests that antigen–antibody interactions may be involved. Whether cold alters the IgM component, making it reactive with native IgG, or whether reduced temperatures alter the IgG molecule, thereby exposing reactive sites, remains to be determined.

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