Immunochemical Characterisation of Cryoglobulins*

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SUMMARY **An immunochemical characterisation of four cryoproteins is presented. Two of them were typical "mixed" cryoglobulins with two protein peaks on gel filtration at low pH values, the first peak was monoclonal IgM kappa in one and monoclonal kappa IgA in the other. The other two cryoproteins were unusal. One of them showed only one protein peak on gel filtration; it was monoclonal IgG kappa. The other cryoprotein showed the first peak of aggregated gammaglobulins and the second peak of gamma heavy chains devoid of light chain activity. Functional analysis revealed rheumatoid factor activity in three of the cryoproteins and antinuclear antibody in one of them. It is concluded that all four of the cryoproteins were "mixed" in nature but that two of them were unusual in nature.**

ASIAN PACIFIC J ALLERG IMMUN 1984; 2 : 222-226.

While cryoglobulinaemia is occasionally seen as a primary or idiopathic disease, it is most often associated with monoclonal gammopathies, Raynaud's phenomenon and purpura.[1] Recently, it has become apparent that a small amount of cryoglobulin can also occur in other diseases, most often associated with collagen diseases, glomerulonephritides and generalised vasculitides.[2-5]

Cryoglobulins appear to fall within the two general categories. In one group, usually two immunoglobulin classes are present, one acting as the antibody directed against the other which acts as the antigen.[6] The other type of cryoglobulin includes those which usually have no antibody activity but exhibit increased inter-molecular attraction at low temperatures. A single homogenous immunoglobulin component is found in this category.[7,8]

In this report, we present the data on the analysis of four cryoproteins.

MATERIALS AND METHODS

In a routine screening for the cryoproteins in patients with clinical features of cryoproteinaemia, four cryoproteins were obtained in significant amounts enabling detailed analysis. For convenience they were labelled cryo-1, cryo-2, cryo-3 and cryo-4.

Isolation of cryoprotein

The cryoproteins were isolated from the serum obtained by allowing blood to clot at 37°C for 1-2 hours. Serum was kept at 4°C and observed for up to 10 days for the appearance of chalky precipitate or gellification. If cryoproteins were observed, the serum was centrifuged at 4,000 rpm at 4°C for 30 minutes and the cryoprecipitate was washed twice with cold 0.1 M phosphate buffered saline (PBS) at 4°C. In each case, the cryoprecipitate could not be solubilised in neutral or slightly alkaline buffer (pH 7.0 to 8.0 at room temperature). However, it could be solubilised in 0.1 M sodium acetate buffer, pH 4.0 at room temperature. The experiments with a native cryoprotein solution were, therefore, conducted under these conditions unless otherwise stated.

Separation of cryoprotein components

The reconstituted cryoglobulin was fractionated on Biogel A-1.5 M column (Biorad, Richmond, Cal., U.S.A.) (15x2.5 cm) prequilibrated with 0.1 M sodium acetate buffer, pH 4.0. Fractions were monitored spectrophotometrically at 280 nm, elution peaks were pooled, concentrated, dialysed against PBS and analysed immunochemically for

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This work was supported by grant No. HCS/DST/679/79 from the Department of Science and Technology, Government of India.

precipitation with isolated IgG and for rheumatoid factor and anti-nuclear antibody activity.

Immunological characterisation

Immunoelectrophoresis was carried out using 1.5% Noble agar (Difco, Detroit, Mich, U.S.A.) in 0.05 M veronal buffer (pH 8.6) and a field strength of approximately 2 mA per slide. Light chain activity was screened by Ouchterlony's double diffusion in 1.5% agarose in veronal buffer as above. Heavy chain specific rabbit anti-human IgG, IgA and IgM and human anti-kappa and anti-lambda light chain antisera were obtained from Cappel Lab, West Chester, Pens, U.S.A. Anti-whole human serum was prepared in our laboratory.

Anti-nuclear antibody (ANAB) was screened in the whole serum and isolated cryo-components by standard immunofluorescence technique.[9] Rheumatoid factor was detected by passive agglutination of latex particles sensitised with human IgG.[10] The test was carried out both at 37°C and 4°C with whole serum and isolated cryo-components.

The effect of cross-mixing of cryo-components with normal immunolobulin was studied by mixing equal amounts (20 μl each) of the isolated fractions with IgG purified on the DEAE cellulose column (2x 20cm) pre-equilibrated in PBS and eluted by increasing the salt concentration (0.005 x 0.2 M). The resulting precipitate was assessed visually and tested for its cryo-precipitability by warming to 37°C. The protein content of cryoprotein was estimated quantitatively by using Folin phenol reagent.[11] Immune complexes were detected by the C_1q radiolabelled binding assay test.[12] Polyacrylamide gel electrophoresis (PAGE) was conducted using 7.5% gel in 0.1 M tris-glycine buffer.[13] The gels were stained with 0.25% Coomasie Brilliant Blue in methanol: acetic acid: water [5:1:5 (v/v)] and destained with 7% acetic acid.

RESULTS

The gel filtration analysis(Fig. 1) of the four cryoproteins revealed two protein peaks in cryo-1, cryo-2 and cryo-4 but only one peak in cryo-3. Immunochemically, the first protein peak in cryo-1 and cryo-2 was the kappa type monoclonal IgM and IgA, respectively; the second peak was polyclonal IgG in both cases (Fig. 3). The elution profile of cryo-4 was rather peculiar (Fig. 2). A small peak in void volume was probably the undissociated cryoprotein complex which preceded the second peak. The second peak was devoid of any light chains but showed a gamma heavy chain and a trace of alpha heavy chain which were demonstrable by double diffusion in agar with specific antisera (Fig. 3). The first peak showed the presence of immune complexes and was equivalent to 4.97 mg of aggregated human gamma globulin as detected by C_1q binding assay. Figure 4 shows a characteristic cryoglobulin electrophoresed on PAGE. The cryoprecipitate (Fig. 4b) contains approximately five to six as yet unidentified bands including a few other components of normal serum basides immunoglobulins. The analysis of rheumatoid factor activity in the whole serum and in different isolated peaks of cryoproteins is given in Table 1. The whole

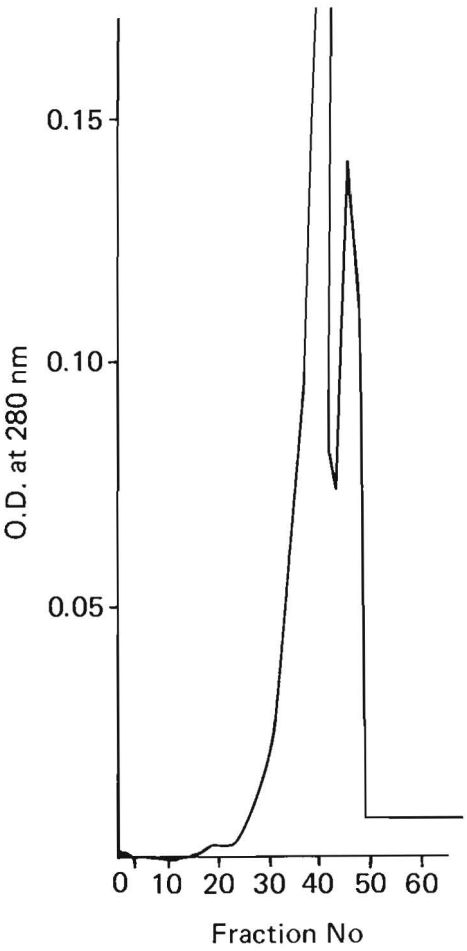

Fig. 1 Elution pattern (Biogel A-1.5 M) of a reprasentative isolated cryoglobulin. Elution buffer was sodium acetate (0.1 M), pH 4.1 with a fraction volume of 3 ml.

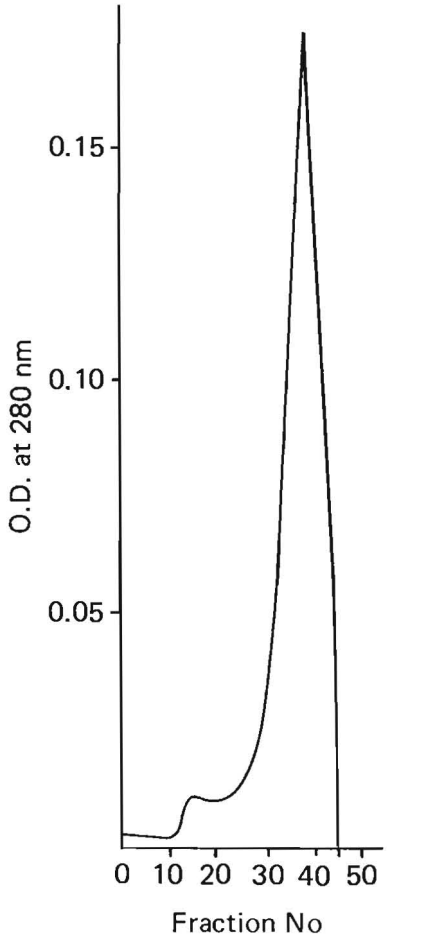

Fig. 2 Elution profile of cryoprecipitate of cryo-4. (Note small peak in void volume).

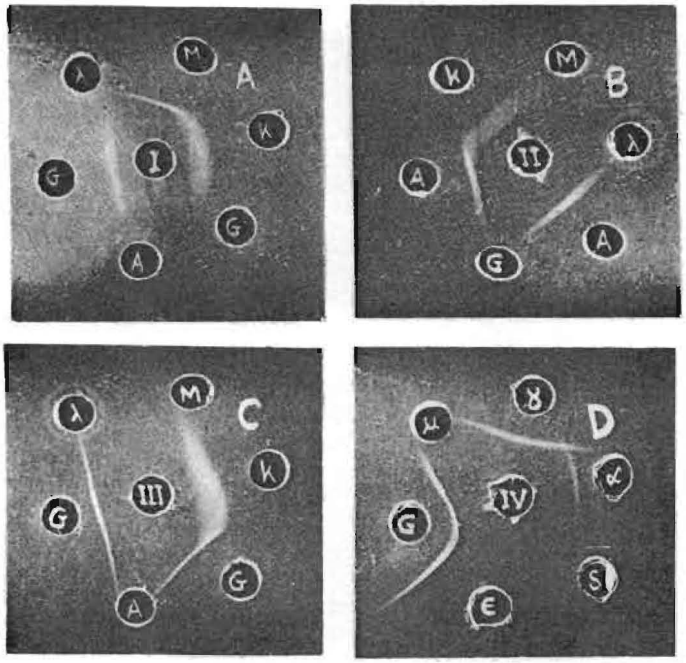

Fig. 3 Oucterlony double diffusion analysis for isolated first peak – centre well (A) cryo-1, (B) cryo-2, (C) cryo-3 and (D) cryo-4. Side well-G-anti IgG; M-anti-IgM; A-anti-IgA; kappa-anti-knppa light chain and lambda-anti-lambda light chain. Gamma, mu, alpha, delta and epsilon are heavy chain specific antisera to anti-IgG, anti-IgM, anti-IgA, anti-IgD and anti-IgE, respectively (cross reactivity in between anti-IgE and anti-whole-IgG was due to species specificity).

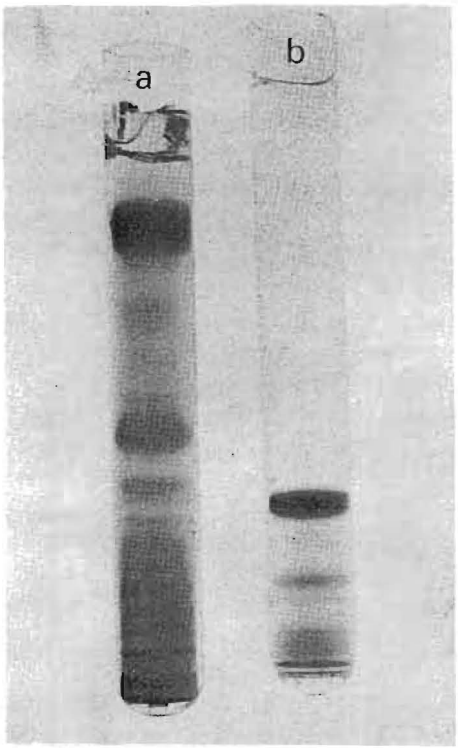

Fig. 4 Polyacrylamide gel electrophoretic pattern of (a) whole cryoglobulinaemic serum (case III) and (b) isolated cryoglobulin of the same serum.

Table 1 Immunochemical characterisation of isolated peaks

	Cryoprotein concentration (mg/ml)	Whole serum		1st peak					2nd peak		Characterisation	Classification
		RF	ANAB	Immunoglobulin	Light chain	ANAB	RF 37°C	RF 40°C	Immunoglobulin	Light chain		
Cryo-1	1.1	+	–	IgM	k	–	–	++	IgG	k	Monoclonal IgM, kappa type with polyclonal IgG	Type II
Cryo-2	0.55	+	–	IgA	k	–	–	+	IgG	k	Monoclonal IgA, kappa type with polyclonal IgG	Type II
Cryo-3	4.5	+	–	IgG	k	–	–	++	–	–	Monoclonal IgG, kappa type	Type II
Cryo-4	0.6	–	+	–	–	+	–	–	IgG	missing	Gamma heavy chain with trace of alpha heavy chain	Type II

serum of cryo-1, cryo-2 and cryo-3 showed rheumatoid factor activity while cryo-4 serum was negative. Rheumatoid factor activity was also demonstrable in the first peak of cryo-1 and 2, but only at 4°C. The single peak of cryo-3 also showed RF activity. No RF activity was demonstrable in any of the second peaks of any of the cryoproteins, neither at 4°C nor at 37°C. As the presence of RF activity has been shown to be a definite indicator of "mixed" nature of cryoproteins,[14] cryo-1, 2 and 3 could be labelled as "mixed" cryoglobulins.

The first three cryoglobulinaemic sera and isolated cryoproteins gave negative results in the indirect immunofluorescence test for antinuclear antibody. However, cryo-4 showed positive ANF which was also present in the first peak of fractioned cryoproteins. Considering that auto-antibody activity is usually seen in mixed cryoproteins, even cryo-4 would be considered as a "mixed" cryoprotein in spite of having a monoclonal component without rheumatoid factor activity.

Since three of the cryoglobulins consisted of a mixture of IgM, IgA or IgG globulin with polyclonal IgG and possessed RF activity in the para-protein component, attempts were made to determine whether IgG, IgM and IgA or both fractions were necessary for cryoprecipitability and RF activity. The isolated components did not exhibit cold insolubility separately. Recombination (20 μl each) again produced a cryoprecipitate. Recombination of monoclonal component with isolated IgG from human serum also produced a precipitate which was insoluble on heating to 37°C. The reason for this could be that isolated IgG may be partially denatured whereas the native state may be essential for the reversibility of the phenomenon.

DISCUSSION

Immunochemically, cryoproteins are classified into three categories.[14-17] Type I is monoclonal cryoglobulin made of immunoglobulins with only one class or subclass of heavy and/or light chains. Type II is mixed cryoglobulinaemia with components made of immunoglobulin belonging to two different classes, one of which is monoclonal. Type III is polyclonal mixed cryoglobulin which comprises a heterogeneous immunoglobulin molecule belonging usually to two or more different classes and sometimes is made of additional serum proteins.

In the present study, cryo-1 and 2 were the straightforward type II cryoglobulins, as they showed a monoclonal component (IgM in case I and IgA in case II) with polyclonal IgG. Previous reports also mention that in cryoglobulinaemia, the first component is usually either monoclonal IgM,[18] IgA[19] and IgG[20] or a polyclonal immunoglobulin.[2,21]

Cryo-3 apparently had only a monoclonal component. Thus, on gel filtration it appeared that this cryoprotein was a type I cryoprotein which is usually seen in cases of multiple myeloma. However, the clinical features and follow-up observations of the patient with cryo-3 were not consistent with the diagnosis of multiple myeloma. On the other hand, functional studies were compatible with essential mixed cryoglobulinaemia because the monoclonal component was showing antiglobulin (RF) activity (Table 1). It is possible that, in such cases, part of the IgG population behaves like an antigen while the other IgG molecules represent antibody.[22] Thus, cryo-3 may belong to a very rare category called essential mixed monoclonal cryoglobulin. There is a report of a patient who was initially described as having essential cryoglobulinaemia but after 15 years he developed multiple myeloma from a benign condition.[23]

Cryo-4 was unusual because, in spite of the low pH, complete separation of the different components of the cryoprotein was not effected. The reason for this is not clear. The second peak was devoid of light chains and showed the presence of more than one heavy chains thus indicating the mixed nature of the cryoprotein. However, it has been pointed out by Skravil and Barandun[24] that for the detection of light chain activity in the agar double diffusion test, a very precise balance of antigen and anti-serum is necessary. Therefore, the possibility of a technical difficulty in demonstrating the light chain in cryo-4 cannot be excluded. Whole serum of cryo-4 was positive for the DS-DNA antibody which could not be traced out in isolated components because of low concentration. Therefore, cryoprecipitation of cryo-4 might have been dependent either on non-covalent bonds or on some unique antigen present in the cryoglobulin itself.

Results of cross-mixing studies involving cryoproteins and normal immunoglobulin are suggestive of immunological interaction. Moreover, since the paraprotein alone is not cryoprecipitable and since it co-precipitates with IgG, it appears that the paraproteins act as autoantibodies to IgG thus representing an antigen-antibody complex formed at low temperature.[25] This is in concurrence with the suggestion of Wager and coworkers that autologous IgG may become auto-antigenic after reacting with a primary antigen or antigen-antibody complex.[26] The immune system of the host would then respond to these pathogenic complexes (primary antigen/IgG antibody) by producing a corresponding antibody. It is also possible that their prescence is coincidental and of no immunopathogenic significance.

The ambient temperature in our country generally is not very low; therefore, clinical symptoms of cryoproteinaemia usually do not appear. A careful screening for cryoproteinaemia in the suspected clinical conditions may be helpful

both for diagnosis and may also give a clue to the possible mode of treatment including plasmapheresis. Primary over-production of cryoglobulin may occur in cases of myeloma and other lymphoproliferative diseases whereas secondary cryoglobulinaemia develops with infections, auto-immune diseases and collagen vascular diseases. Besides these practical implications, the study of cryoglobulin is extremely interesting as it yields excellent information regarding the reactivities of different immunoglobulins.

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