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Immunological and structural properties of human monoclonal IgG cryoglobulins

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SUMMARY

Structural and immunological properties were determined for sixteen IgG and one Bence–Jones, human monoclonal cryoglobulins. The heavy chain subclass percentages were 47% IgG_1, 14% IgG_2 and 29% IgG_3, and were different from previously reported distributions of myeloma proteins. In addition, 69% (eleven out of fifteen) of the cryoglobulins and 100% (seven out of seven) of the IgG_1 cryos contained type lambda light chains. Electrofocussing of the cryoproteins by analytical liquid gradient column showed the isoelectric points to be included in the range of pH 6·3–8·9. The pI of six light chains and five out of six heavy chains were at acidic and slightly basic pH, respectively. The pI of the intact cryoglobulins were thus close to those of their constituent heavy chains. Six out of seven of the heavy chains were subjected to automated Edman degradation and were classified as containing vH-i or vH-ii variable region subgroups on the basis of their blocked amino termini. One type lambda light chain was unusual in that it contained an amino terminal sequence initially described in an amyloid fibril protein and is the first instance in which light chains with this sequence have been isolated from IgG. The data support the notion that the cryoglobulins are IgGs with unique structural and immunological properties which separate them from non-cryoprecipitable IgGs.

INTRODUCTION

Self-associative monoclonal IgG and IgA cryoglobulins without defined antibody activity have been shown to comprise the smallest percentage of the various types of human immuno-cryoglobulins described by Meltzer & Franklin (1966) and later classified by Brouet *et al.* (1974). In a few instances, IgG cryoproteins have been studied as a group of proteins with a unique physicochemical property and shown to have certain unusual characteristics which separate them from other IgGs. As an example, the monoclonal IgG cryoglobulins were shown by Virella & Hobbs (1971) to have different distributions of their gamma heavy chain subclass determinants when compared to those in normal sera and a random population of monoclonal IgG myeloma proteins. In their study, ten out of fourteen (71%) of the IgG cryoproteins were of the IgG_2 or IgG_3 heavy chain subclass, a distinctly unusual percentage. However, the kappa/lambda ratio obtained for this same group of proteins was 1·9/1 which was the same as that found for 139 non-cryoprecipitable IgG myeloma proteins.

In addition to unusual distributions for the gamma chain subclass markers, which are in effect heavy chain constant region determinants, Wang *et al.*, (1974) provided some evidence that the IgG cryos may also contain unusual distributions for their heavy and light chain variable region subgroups. Although only five monoclonal IgGs were investigated, these all contained the vH-i heavy chain variable region subgroup and thus had pyrrolidone carboxylic acid (PCA) as an amino-terminal amino acid residue. Two

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of these IgGs had unblocked type lambda (vλ-iii or vλ-iv) and three type kappa (vκ-i or vκ-iii) light chain variable region subgroups.

Thus, the preliminary evidence suggests that the monoclonal IgG cryoglobulins may have different immunochemical and serological properties from other groups of IgGs. In the present study, fifteen monoclonal IgG cryoglobulins and one type lambda Bence–Jones cryoprecipitable dimer were investigated in order to determine these characteristics for one group of cryoproteins and thus confirm previous notions of their unique properties. The IgG cryoglobulins were obtained from patients diagnosed as having either primary idiopathic cryoglobulinaemia or multiple myeloma.

MATERIALS AND METHODS

Isolation of IgG cryoglobulins. The cryoglobulins were precipitated from serum or plasma collected at 37°C after being incubated at 4°C for up to 7 days. The cryoprecipitate was collected by centrifugation, washed in ice-chilled 0·1 M borate buffered 0·154 M NaCl pH 7·8, redissolved by warming to 37°C, and then reprecipitated by further incubation at 4°C for 5 days. This procedure was repeated four or five times until the cryoglobulin was immunoelectrophoretically pure. Occasionally, if contaminating proteins remained, the washed and reprecipitated cryoglobulin was suspended and dissolved in 0·015 M phosphate buffer pH 7·4 and passed through a DEAE-cellulose column at 37°C equilibrated with the same buffer. The fall-through peak was collected and tested for the presence of contaminating serum proteins by immunoelectrophoresis with a polyvalent horse anti-whole human serum antiserum.

In one instance, a Bence–Jones light chain dimer of the same light chain type co-precipitated with the IgG cryoglobulin. The IgG and light chain were then separated on a Sephadex G-200 column after concentration of the fall-through peak obtained by DEAE-cellulose chromatography. Another protein (Web) showed a minor contaminant of H_2L which co-precipitated with the intact molecule, i.e. H_2L_2. Since this was found in the last half of the DEAE fall-through peak, only the first third of the peak was utilized in these studies for this protein. The H_2L migrated with intact IgG-Web on immunoelectrophoresis but was noted as an anodal shoulder on the isoelectric focussing profile, a sharp band which migrated ahead of H_2L_2 on polyacrylamide gel electrophoresis using buffers which contained sodium dodecyl sulfate (SDS), and was subsequently converted to bands which migrated with heavy and light chains after it was totally reduced and alkylated and when analysed by the same technique (Abraham *et al.*, in preparation).

Isoelectric focussing. The intact IgG cryoglobulins were radiolabelled with ^{125}I by the iodine monochloride method (Bale *et al.*, 1966) and subjected to electrofocussing in liquid 40–10% sucrose gradients 1·0 M in urea as described previously (Trieshmann, Abraham & Santucci, 1975; Abraham, 1978). The peak tubes of the isofocussing profiles were pooled and 10 mg of unlabelled IgG cryoglobulin were added to each as a carrier. Cryoglobulins were reduced in 0·2 M Tris-HCl pH 8·6, 6·0–8·0 M in urea at a 0·02 M concentration of dithiothreitol (DTT), and alkylated with iodoacetamide in a 50% molar excess over DTT. Heavy and light chains were obtained by Sephadex G-100 chromatography in columns equilibrated with 1·0 M acetic acid, 6·0 M in urea. These were dialysed against 0·1 M acetic acid 6·0 M in urea and then a large volume of 4·0 M urea prior to electrofocussing.

Primary amino acid sequencing. Some of the totally reduced and carboxy-methylated light chains were also used for automated primary amino acid sequence analysis. These were exhaustively dialysed against 0·05 M or 0·1 M acetic acid and lyophilized prior to sequencing. For amino terminal analysis 1·5–5·0 mg of heavy or light chain were utilized and sequence analysis was performed with a 0·5 M Quadrol buffer, single cleavage programme (Beckman Instruments). The procedures for conversion of the thiazolinone amino acids into the stable phenylthiohydantoin (PTH) derivatives and the methods of identification for the PTH amino acids as performed in this laboratory have been described previously in detail (Johnston, Abraham & Welch, 1975; Abraham *et al.*, 1978). Automated sequencing was performed for six sequenator cycles and if no amino acids were identified the run was terminated, the protein removed from the reaction cup and presumed to contain an amino terminal PCA. When amino termini were unblocked and PTH amino acids identified, sequencing was continued until the variable region subgroup was able to be assigned, i.e. for at least twelve residues. No protein which had an unblocked amino terminus differed from a prototype sequence of the same variable region subgroup within the first twelve residues. Prototype sequences and classification of variable region subgroups are as described by Kabat, Wu & Bilofsky (1978).

RESULTS

Each cryoglobulin was tested for gamma chain subclass with rabbit or primate antisera specifically absorbed for the human subclass determinants. Some antisera were provided by Dr J. P. Leddy of the University of Rochester School of Medicine. The subclass of IgG-Web was confirmed by Dr S. Litwin, Department of Human Genetics, Cornell University Medical Center, New York. The subclass of each protein was determined by at least two and in some instances three methods. These were the radio-

precipitin inhibition assay, immunodiffusion analysis in agar-gel with known reference proteins, and a recently described micro-haemagglutination inhibition assay (Johnston & Abraham, 1979).

TABLE 1.

Protein	Heavy chain subclass	Light chain type	pI
Bak	γ-1	λ	7·18
Bow	γ-1	λ	6·47
Kin	γ-1	λ	6·33
Log	γ-1	λ	6·38
Mit	γ-1	λ	7·30
Pea	γ-1	λ	6·82
Pie	γ-1	λ	8·85
Dub	?	κ	7·98
Web	γ-2	κ	7·40
Per	γ-2	κ	6·70
Cuy	γ-3	κ	7·05
Gon	γ-3	λ	6·98
Kep	γ-3	κ	7·13
Clo	γ-3	λ	8·21
Kel	γ-4	λ	7·00
Bl	—	λ	7·90

Table 1 lists the heavy chain subclasses, light chain types and isoelectric points (pI) for these proteins. The heavy chain subclass for IgG-Dub could not be determined. Of the proteins whose heavy chain subclasses were able to be classified, seven out of fourteen (50%) were IgG_1, two out of fourteen (14·3%) IgG_2 and four out of fourteen (28·6%) IgG_3. The percentages for the sum of the IgG_2 and IgG_3 proteins (43%) were increased and the IgG_1 decreased when compared to the subclass frequencies previously reported for IgG myeloma proteins by Virella & Hobbs (1971). An unusual and previously unreported finding for the cryoglobulins in the present study was that eleven out of sixteen (69%) and seven out of seven (100%) of the IgG_1 proteins had type lambda light chains.

The results of the electrofocussing experiments show that the pI of these proteins are included within a relatively narrow pH range and with only one exception (Pie) are near neutrality (i.e. pH 7·0), a characteristic not previously determined for IgG cryoglobulins. All of the proteins except Dub produced single spikes and were very restricted in their charge heterogeneity. Fig. 1 shows isofocussing profiles for IgGs Bow, Pea and Per which demonstrate the monoclonal nature of these proteins by this technique.

The heavy and light chains of seven proteins were also isoelectric focussed. These results are shown in Table 2. With the exceptions of Pea whose heavy chain pI was at 4·85 and Web whose light chain pI was at pH 7·60, the tendency was for heavy chains to have pI which were nearly neutral or slightly basic in pH, and for light chains pI at acidic pH.

Fig. 2 shows the charge restriction for the intact IgG and the heavy and light chains for proteins Bak, Gon and Pie. These profiles are typical of those obtained previously for heavy and light chains isolated from monoclonal proteins and analysed in these laboratories (Trieshmann *et al.*, 1975).

Heavy and light chains from ten of the IgG cryoglobulins and the Bence–Jones protein Bul were subjected to automated amino acid sequence analysis in order to assign a variable region subgroup. These results are shown in Table 3a. When a type lambda light or heavy chain was blocked to automated Edman degradation, it was presumed to have pyrrolidone-carboxylic acid (PCA) for an amino terminal amino acid residue. Since only the vH-i and vH-ii heavy chain variable region subgroups contain PCA as an amino terminal residue blocked proteins were assigned to either of these two subgroups. The

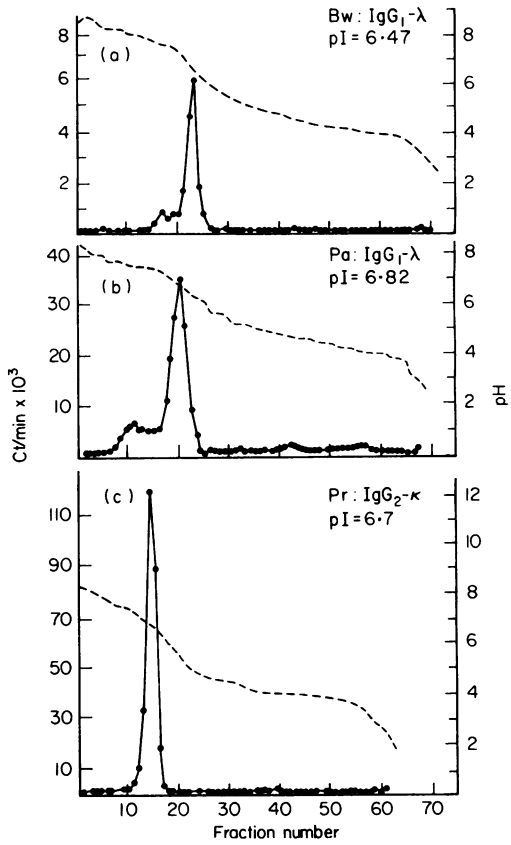

FIG. 1. Isoelectric focussing profiles of IgG_1 (a) Bow (Bw), (b) Pea (Pa) and (c) $IgG_{,2}$ Per (Pr). Each protein was radiolabelled with ^{125}I and isofocussed in a liquid sucrose gradient 110 ml analytical column containing 1·0 M urea. The effective pH range was pH 4–8·5. Gradients were fractionated as indicated and the entire fraction counted.

TABLE 2.

		pH of isoelectric point for:	
	Protein	Heavy chain	Light chain
IgG_1	Kin	7·00	5·90
	Pea	4·85	4·80
	Log	7·65	5·30
	Pie	7·28	6·50
	Bak	8·90	5·70
IgG_2	Gon	7·26	6·20
IgG_3	Web	7·20	7·60

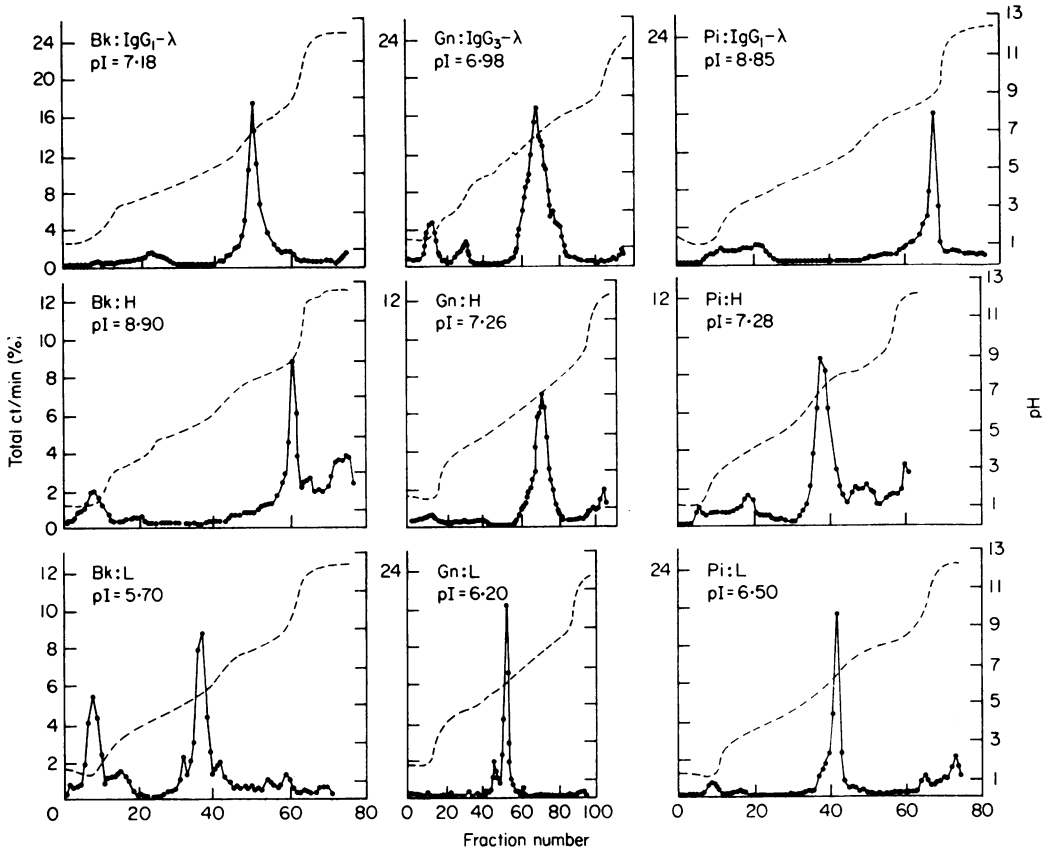

FIG. 2. Isoelectric focussing profiles for IgG-1 Bak (Bk), Pie (Pi) and IgG-3 Gon (Gn) and their purified heavy and light chains. Conditions for the experiments are as described in Fig. 1 and the Materials and Methods section.

TABLE 3a.

Protein	Heavy chain	Light chain
Bak	Blocked	Blocked
Bow	Blocked	Blocked
Kin	vH-iii	λ*
Log	Blocked	vλ-iii
Pea	Blocked	Blocked
Pie	Blocked	Blocked
Mit	vH-iii	n.d.
Dub	Blocked	vκ-iii
Gon	Blocked	Blocked
Web	Blocked	vκ-i
Bul	—	Blocked

TABLE 3b.

	1	2	3	4	5	6	7	8	9	10	11
KIN	Asn	Phe	Met	Leu	Leu	Gln	Pro	His	Ser	Val	Ser
AR	—	—	—	—	Thr	—	—	—	—	—	—
YAM	—	—	—	—	Thr	—	—	—	—	—	—

N-terminal amino acid sequence of KIN λ chain compared to the two known λ chains with the same N-terminal variable region sequences, AR (Sletten *et al.*, 1974) and YAM (Wang *et al.*, 1976).

percentage of proteins with PCA represents the sum of these two heavy chain variable region subgroups. Eighty per cent of the heavy chains were blocked and thus were either vH-i or vH-ii. Two heavy chains, Kin and Mit, showed vH-iii sequences for the first twelve residues which were identical to prototype heavy chains (Kabat *et al.*, 1978). Six out of eight (75%) of the type lambda light chains were blocked, an expected percentage. The unblocked lambda light chains of IgG-Kin contained an amino terminal amino acid sequence similar to that reported by Sletten, Husby & Natvig (1974) for an amyloid fibril protein isolated from the spleen and liver of a patient with primary systemic amyloidosis. Wang *et al.*, (1976) also noted this amino terminal sequence in intact type lambda light chains isolated from a monoclonal IgA which demonstrated thermal precipitable properties of both cryo- and pyro-globulins. The amino terminal sequences of the first eleven residues of these proteins are compared in Table 3b. The amino terminal amino acid sequences of the type kappa light chains were unremarkable.

DISCUSSION

The present report provides data and extends observations which suggest that monoclonal human IgG cryoglobulins have unusual distributions for serological and structural determinants used to classify the variable and constant regions of IgG heavy and light chains.

With regards to the heavy chains, an increase in the percentages of gamma-2 and gamma-3, and a decrease in gamma-1 heavy chains were noted. The IgG_2 and IgG_3 subclasses comprised 43% of the proteins in this series which is less than the 71% incidence noted for IgG cryoglobulins in the series reported by Virella & Hobbs (1971). Fifty per cent of the IgGs in the present series were IgG_1 and for the first time an IgG_4 cryoprotein was noted. These distributions should be constrasted with those obtained for nearly 600 non-cryoprecipitable myelomas reported in numerous series and tabulated by Virella & Hobbs (1971). Seventy-five per cent were IgG_1, 11% IgG_2, 10% IgG_3 and 4% IgG_4.

In order to obtain a more comprehensive representation of the subclass distributions for the IgG cryoglobulins, the data from the three previous reports in which subclass distributions were noted were pooled with those from the present study and summarized in Table 4. The combined frequency for IgG_2 and IgG_3 was 53%; that of IgG_1 42%.

TABLE 4.

	IgG_1	IgG_2	IgG_3	IgG_4	?	Total
Virella & Hobbs (1971)	4	6	4	—		14
Brouet *et al.* (1974)	6	4	1	—		11
Wang *et al.* (1974)	2	2	1	—		5
Present study	7	2	4	1	1	15
Totals	19	14	10	1	1	45
Percentage	42·3%	31·0%	22·2%	2·22%		

An unusual distribution for the variable region subgroups of the heavy chains of cryoproteins was suggested by Wang *et al.* (1974) who found no heavy chains of the vH-iii variable region subgroup in the five monoclonal IgG cryoglobulins investigated. Førre, Natvig & Kunkel (1976) have recently determined that vH-i and vH-ii together comprise 49% of the heavy chain variable region subgroups of any IgG population which contrasts with the 80% (eight out of ten) incidence obtained for the cryoglobulins in this report. Whether these heavy chains have unusual amino acid sequences in their variable or hypervariable regions is as yet not known.

Another unexpected finding was the unusually high percentage of type lambda light chains associated with this group of cryoproteins. Since 100% of the IgG_1 cryos contained type lamba light chains, the decrease in IgG_1 is selective for those which contain kappa chains. Further, IgG-Kin was unusual

among these since it contained a type lambda light chain with an animo terminal sequence similar to that noted by Wang *et al.* (1976) for an IgAλ which had both pyro- and cryo-precipitable properties. This variable region sequence was first reported by Sletten *et al.* (1974) for an amyloid fibril protein. These investigators suggested that this sequence may actually constitute a new lambda chain variable region subgroup. The subsequent isolation of intact lambda chains containing this sequence from IgA (Wang *et al.*, 1976) and from IgG-Kin supports this notion. It is possible that this peculiar variant of type lambda chains may show an increased incidence in the IgG cryoglobulins and contribute to the insolubility of these proteins at reduced temperatures.

In order to determine if a chemical property was shared by the cryo-IgGs, they were subjected to analytical isoelectric focussing. The isoelectric focussing profiles noted were typical of monoclonal proteins but were found in a more restricted pH range than expected especially since a heterogeneous pool of normal IgG, which contained all IgG subclasses, was previously shown to have isoelectric points which ranged from pH 4·5 to pH 9·5 (Abraham, Clark & Vaughan, 1972). Further no correlation could be made between the heavy chain subclass and the isoelectric point of the cryoglobulins since all were near neutrality. When the heavy and light chains of seven proteins were electrofocussed, the isoelectric points of the intact molecules were most nearly equivalent to the pI of the heavy chain. This is of some interest, since the isoelectric points of the light chains were at acidic pH and therefore did not significantly decrease the pI of the intact IgG cryoglobulins.

The present study has, in sum, confirmed that the monoclonal IgG cryoglobulins have some shared and unique structural and serological properties which are apparently not found for other non-cryoprecipitable monoclonal IgGs. It is possible that these properties may be related to the unusual ability of these proteins to cryoprecipitate.

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