Structural Studies of the Immunoglobulins. II. Antigenic and Chemical Properties of γA Myeloma Globulins*

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ABSTRACT: The polypeptide chain structure of γA myeloma globulins of antigenic types I and II has been studied by physical and chemical techniques. After reductive dissociation and alkylation, two kinds of chains were separated by gel filtration, heavy and light.

The light chains differ structurally for the two antigenic types but are virtually identical with the Bence-Jones protein of the same patient, both in amino acid composition and in tryptic peptide maps. Many of the peptides of the light chains of antigenic type I are identifiable with peptides of known sequence that occur in Bence-Jones proteins of the same antigenic type and in normal γG-globulins. However, part of the light chain appears to be unique for each specimen. The heavy chains have antigenic determinants and peptide maps characteristic for γA-globulins, but the number and structure of these chains is not well defined.

Of the three classes of human serum immunoglobulins,[1] least is known about the structure of the γA-globulins, partly because of the difficulty of purifying these proteins from normal human serum. γA myeloma globulins, on the other hand, can be obtained in relatively pure form and may serve as useful models for structural studies of normal γA-globulin (Ballieux, 1963), just as the γG myeloma globulins have facilitated structural analysis of normal γG-globulin (Putnam, 1962). In the latter case the γG-globulin molecule has been shown to contain two kinds of polypeptide chains: γ chains which are the heavy chains characteristic of the γG-globulin class and light chains which are common moieties throughout the immunoglobulin family (Edelman and Poulik, 1961; Fleischman *et al.*, 1963; Fahey, 1963). Likewise, in the case of γG-globulin, limited enzymatic hydrolysis with papain has produced biologically active fragments (Fc and Fab fragments) which retain many of the properties of the γG-globulin molecule (Porter, 1959; Hsiao and Putnam, 1961; Franklin, 1960).

In the case of the γA-globulins, however, papain cleavage results in poorly defined products that do not correspond to the Fc and Fab fragments of γG-globulin (Heremans, 1960; Deutsch, 1963). Reductive dissociation of γA myeloma globulins has yielded fractions separable by gel filtration that are analogous to the heavy and light chains of γG-globulin (Cohen, 1963; Carbonara and Heremans, 1963; Fahey, 1963). The present study was designed to define the polypeptide chain structure of γA myeloma globulins by partial enzymatic cleavage, by reductive dissociation of polypeptide chains, and by comparison of the γA myeloma globulin and Bence-Jones protein of individual patients. In this study, the two antigenic types of γA myeloma globulins (type I and type II) are shown to possess a common structural moiety that is representative of the α chain and differs from the γ chain. The γA myeloma globulins also possess light chains which define the antigenic type (I or II) and which are virtually identical with the Bence-Jones protein excreted by the patient.

Experimental Section

Proteins. γA myeloma globulins were prepared by ammonium sulfate precipitation, column or batch chromatography using DEAE-cellulose or carboxymethyl-cellulose, and in the case of 10–14 S γA-globulins gel filtration on Sephadex G-200. Bence-Jones proteins were prepared by ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration (Bernier and Putnam, 1964). The proteins are designated by abbreviations referring to the patient's name (*e.g.*, Ha, Ln, and Mo).

Antisera. The following rabbit antisera were employed in this study: antiserum to Ha γA myeloma globulin, antiserum to Ln γA myeloma globulin, antisera to Bence-Jones proteins of type I (Ag) and type II (Bo), antiserum to the Franklin protein (Cr) which possesses antigenic determinants of γG-globulin heavy chain only (Franklin *et al.*, 1964), and antiserum

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[1] The nomenclature used was proposed at the WHO Meeting on Nomenclature of Human Immunoglobulins on May 29–30, 1964, at Prague. Synonyms may be found in the draft memorandum (*Bull. Wld. Hlth. Org.* (1964), *30*, 447).

to reduced and alkylated heavy chains of γA myeloma globulin Ln and γA myeloma globulin Ha.

Analytical Techniques. Two-dimensional mapping of tryptic peptides was performed at pH 3.7 as described by Putnam and Easley (1965). The maps were stained with ninhydrin, Pauly, Sakaguchi, tyrosine, and chloroplatinic acid reagents both singly and in combination, as described by Easley (1965). Proteins were prepared for tryptic digestion by performic acid oxidation or reduction–alkylation. Amino acid analysis was performed in duplicate on the Beckman Model 120 automatic amino acid analyzer. Ultracentrifugation was done in the Spinco Model E at 59,780 rpm, and Tiselius electrophoresis was performed in a Spinco Model H apparatus. Thermosolubilities were tested in capillary tubes by a previously described method (Bernier and Putnam, 1964). Antigenic analysis was performed by double diffusion in agar and by immunoelectrophoresis (Migita and Putnam, 1963). Starch urea gel electrophoresis was performed in the discontinuous formate buffer system based on the method of Smithies (1962).

Reduction–Alkylation. Proteins were reduced and alkylated by the method of Fleischman *et al.* (1963) and by a modification of the method of Jaquet *et al.* (1964). In the latter method two sequential reduction–alkylations were performed. Gel filtration was performed on 250 mg of reduced–alkylated protein by passage through G-200 Sephadex, 0.5 M sodium phosphate, pH 6.8, 100 × 3.8 cm. The Sephadex was not equilibrated with detergent. The only detergent present was that which could not be removed by dialysis of the reduced–alkylated protein. Fractions obtained by gel filtration were pressure dialyzed, and the detergent was removed by batch chromatography using Dowex 1 (acetate).

TABLE I: Physical Properties and Antigenic Classification of γA Myeloma Globulins (γA) and Bence-Jones Proteins (BJ).

Symbol	Class	Antigenic Type	$S_{20,w}$[a]	Mobility[b]
Ha	γA	II	7.0, 10.1, 12.6, 15.0	2.9
Ln	γA	I	6.6, 10.1, 12.6, 15.4	—
Mo	γA	I	6.6, 9.8, 12	2.4
Ha	BJ	II	3.5	0.88
Ln	BJ	I	3.3	—
Mo	BJ	I	3.1	3.4

[a] Expressed in Svedberg units. [b] Expressed in units of 10^{-5} cm^2 sec^{-1} v^{-1} at 0° in pH 8.6 Veronal buffer, 0.1 ionic strength.

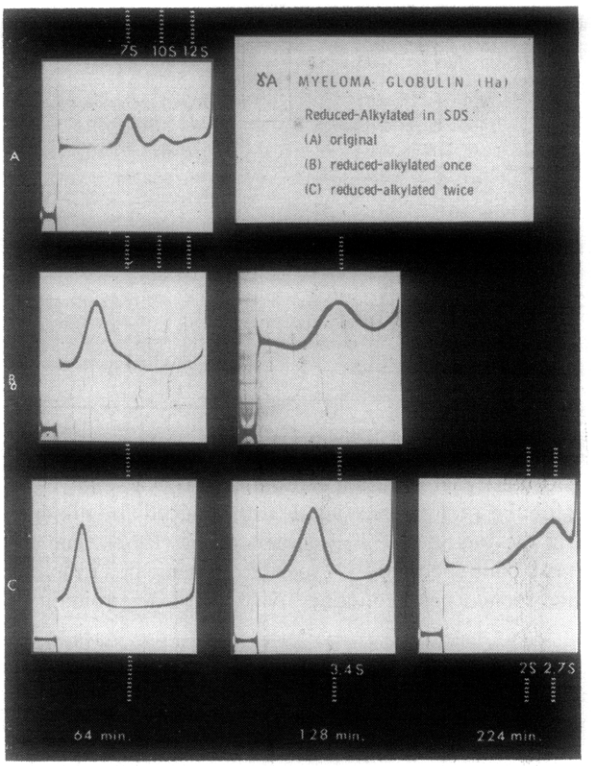

FIGURE 1: Ultracentrifugal pattern comparing (A) the original γA myeloma globulin (Ha) with (B) the preparation after reduction and alkylation in detergent (SDS), and (C) after a second reduction and alkylation in detergent. Note that the twice-reduced material shows only 2 S and 2.7 S components. The vertical dash line indicates the approximate sedimentation coefficient at the time indicated.

Papain Cleavage. Limited proteolytic cleavage of two γA myeloma globulins (Ha and Ln) was performed with crystalline papain (Hsiao and Putnam, 1961). The digest was fractionated by gel filtration with Sephadex G-200 with the aid of the Beckman Model 130 Spectrochrom. The eluted fractions were assayed for hexose content by the method of Dubois *et al.* (1956) and for reactivity with ninhydrin (Moore and Stein, 1954).

Results

The three γA myeloma globulins studied (Ln, Mo, and Ha) manifested polymer-type heterogeneity in the ultracentrifuge and in starch-gel electrophoresis, although by Tiselius electrophoresis they appeared rather homogeneous. Some of the physical properties of these proteins are summarized in Table I. Two preparations (Ln and Mo) contained large amounts of 10 S γA; these components were isolated by gel filtration using Sephadex G-200 equilibrated in 1 M NaCl, 0.1 M Tris–HCl, pH 9.0 buffer. In these two preparations, the γA myeloma globulin was isolated virtually free of con-

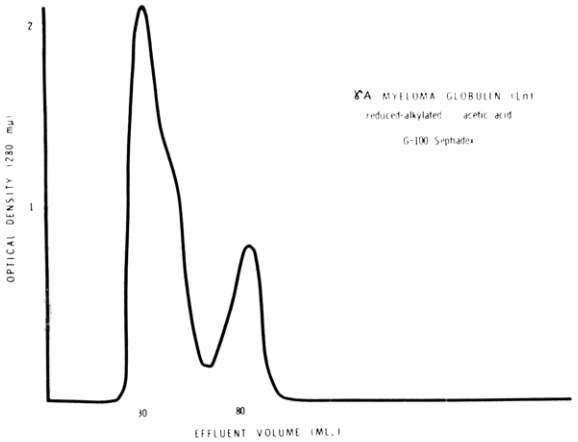

FIGURE 2: Gel-filtration elution pattern of a γA myeloma globulin of antigenic type I (Ln), reduced and alkylated by the method of Fleischman *et al.* (1963). The first peak contained only γA specific antigenic determinants; the second peak contained only type I determinants.

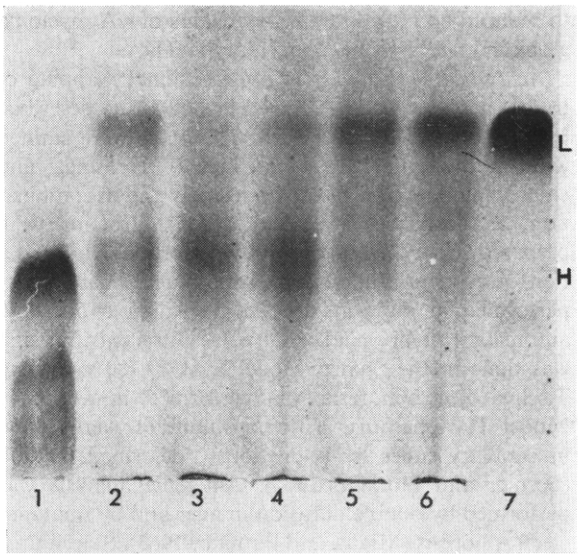

FIGURE 3: Starch urea gel electrophoretic pattern comparing proteins and separated chains from patient Ha. (1) Untreated γA myeloma globulin; (2) γA myeloma reduced and alkylated twice in detergent; (3) heavy chain; (4) intermediate fraction containing heavy and light chains; (5) light chain; (6) Bence-Jones protein, reduced and alkylated twice in detergent; and (7) untreated Bence-Jones protein. H and L represent the position of the heavy and light chains, respectively.

tamination with γG-globulin. The Ha γA myeloma globulin contained trace amounts of γG-globulin, detectable by immunoelectrophoresis. The three purified γA myeloma globulin preparations were studied by the methods of reductive dissociation, papain cleavage, and comparative peptide mapping.

Reductive Dissociation of Polypeptide Chains. Figure 1 shows the change in ultracentrifugal pattern on progressive reduction by the detergent method of γA myeloma globulin Ha. Although fair separation of heavy and light chains of detergent-treated proteins was achieved on Sephadex G-200, the separation of these chains was generally more complete using the method of Fleischman *et al.* (1963) which involves reductive dissociation in aqueous buffers and separation of the heavy and light chains on Sephadex G-100 equilibrated in acetic acid (Figure 2).

By various physical and chemical criteria the reduced–alkylated light chains of the γA myeloma globulin appeared similar to or identical with the Bence-Jones protein from the same patient. The reduced–alkylated light chain of the γA myeloma globulin Ha (prepared by the detergent method) was similar to the Bence-Jones protein Ha in electrophoretic mobility in starch urea gel, while the heavy chain had a markedly slower mobility (Figure 3). In thermal coagulability the Ha light chain and the Bence-Jones protein were virtually identical; both proteins were precipitated by heating at 54° and underwent thermal dissolution at 110°.

Antigenic Identity of the Light Chains and Bence-Jones Proteins. Immunodiffusion analysis confirmed the identity of the light chains of a γA myeloma globulin and the Bence-Jones protein of the same patient, which was suggested by their similarity in chemical and physical properties. In both cases tested (Ha and Ln), the light chain and the Bence-Jones protein were antigenically identical when treated with antiserum to the original γA myeloma globulin (Figures 4A and 4B). In Figure 4A the intact γA myeloma globulin spurs with the precipitin lines of both proteins, reflecting additional antigenic determinants due to the heavy chain. In Figure 4B the nonidentity of the heavy and light chains of the γA myeloma globulin is illustrated. In this case, the original γA myeloma globulin spurs with the heavy chain reflecting the presence of light chain determinants in the intact globulin.

Papain Digestion. Under conditions which produce the structural and biologically active fragments of γG-globulin, papain cleavage of the γA myeloma globulin Ha resulted in a partial loss of antigenic determinants of the heavy chain, whereas the light chain determinants remained intact. This is indicated in Figure 4C by the fact that the intact γA-globulin spurs with the product of papain digestion while the latter spurs with the Bence-Jones protein.

When the products of papain digestion were characterized by gel filtration of the undialyzed digest on the Spectrochrom apparatus using Sephadex G-200, the major protein fraction eluted had an $s_{20,w}$ of 3.5 S in the ultracentrifuge; it was completely precipitable with trichloroacetic acid, contained little carbohydrate, and had low reactivity with ninhydrin. This fraction had a multibanded pattern when subjected to starch gel electrophoresis. The bands were uniformly spaced as if representing a series of components differing regularly in electrical charge.

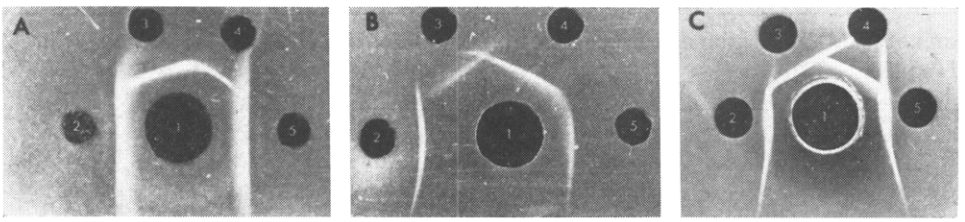

FIGURE 4: Ouchterlony double diffusion in agar. A: (1) Anti-Ha γA-globulin; (2) and (5) untreated Ha γA-globulin; (3) Ha light chain; and (4) Ha Bence-Jones protein. B: (1) Anti-Ln γA-globulin; (2) untreated Ln γA-globulin; (3) Ln heavy chain; (4) Ln light chain; and (5) Ln Bence-Jones protein. C: (1) Anti-Ha γA-globulin; (2) and (5) untreated Ha γA globulin; (3) papain digest 3.5 S fraction; and (4) Ha Bence-Jones protein.

The intermediate fraction eluted was very rich in carbohydrate, poor in trichloroacetate-precipitable material, and had a low optical density at 280 mμ. This fraction contained most of the glycopeptide portion of the γA myeloma globulin.[2]

The last fraction consisted entirely of dialyzable peptides strongly positive with ninhydrin and not precipitable by trichloroacetic acid. No protein was detectable by starch gel electrophoresis or ultracentrifugation. Presumably, papain treatment resulted in proteolytic destruction of a portion of the heavy chain (that portion analogous to the Fc fragment of γG-globulin); this was accompanied by release of most of the glycopeptide, while leaving the remainder of the molecule largely unchanged. This conclusion was supported by peptide map studies.

Amino Acid Composition. Amino acid analysis was completed for all three γA myeloma globulins, the corresponding Bence-Jones proteins, the papain digests of the Ha and Ln globulins, and the heavy and light chains of the Ha globulin. In the latter case, and also for the Ha Bence-Jones protein, the reduced–alkylated preparation was analyzed. Table II summarizes data for the three γA-globulins in comparison to the results of Heimburger *et al.* (1964) for normal human γA-globulin and our own data for normal human γG-globulin. Table III presents the amino acid composition of the Ha γA myeloma globulin in relation to its heavy and light chains, the autologous Bence-Jones protein, and the product of papain digestion of the globulin. The amino acid composition of the Ha and Mo Bence-Jones proteins has been discussed in a comparative study of a number of specimens of antigenic types I and II (Putnam and Easley, 1965).

The γA myeloma globulins differ individually in their amino acid content. The most striking differences are in lysine, histidine, and isoleucine. The methionine content of the Ha γA myeloma globulin is diminished in accord with the lack of methionine in the Ha Bence-Jones protein. However, all three of the γA myeloma globulins are much more similar to normal γA-globulin than to normal γG-globulin.

TABLE II: Amino Acid Composition of Myeloma γA-Globulins and of Normal γA- and γG-Globulins.

Amino Acid	Normal[a]		γA Myeloma		
	γG	γA	Mo	Ln	Ha
	(g amino acid residues/100 g protein)[b]				
Lysine[c]	7.03	5.29	6.44	4.93	5.44
Histidine	2.26	2.18	1.75	2.36	2.53
Arginine[c]	3.99	5.15	5.04	5.55	5.40
Aspartic acid	7.80	6.96	8.38	8.31	7.87
Threonine[c]	7.54	8.65	8.55	8.44	8.99
Serine	9.71	8.97	8.86	8.81	9.54
Glutamic acid	10.96	11.90	11.78	11.47	11.00
Proline[c]	6.67	7.08	7.13	6.96	8.04
Glycine	3.39	3.64	3.49	3.72	4.25
Alanine[c]	3.24	4.38	4.31	4.21	4.86
Half-cystine[c]	1.92	2.37	2.51	2.66	2.44
Valine[c]	7.98	6.79	6.90	7.10	6.85
Methionine	0.79	0.90	0.92	0.97	0.66
Isoleucine	2.23	1.97	2.36	2.55	1.44
Leucine[c]	7.39	8.78	8.98	9.71	9.45
Tyrosine[c]	6.08	4.94	4.88	5.12	4.35
Phenylalanine	4.28	4.14	4.68	4.19	4.23
Tryptophan	3.12	3.73	Not determined		

[a] Data of Heimburger *et al.* (1964) for γA. [b] Expressed as the percentage of the total recovered amino acids. Tryptophan was not determined for the myeloma globulins but was assumed to be 3% for calculation purposes. Correction factors were applied for amino acids subject to partial destruction during acid hydrolysis. [c] Amino acids which are either uniformly higher or uniformly lower in normal and myeloma γA-globulins compared to normal γG-globulins.

The glucosamine content of the γA-globulins determined with the amino acid analyzer ranged from 1 to 2%, but these values are uncertain because up to 50% of the amino sugar is destroyed during 24-hr

[2] The structure of the carbohydrate prosthetic group of γA myeloma globulins is being investigated by Dr. John R. Clamp of the Department of Medicine, University of Bristol, Bristol, England. The glycopeptide fraction isolated in this experiment contains hexose, aminohexose, fucose, and *N*-acetylneuraminic acid in the approximate molar ratio of 8:6:1.2.

TABLE III: Amino Acid Composition of a Type II γA Myeloma Globulin (Ha), the Autologous Bence-Jones Protein, and Related Products.

Amino Acid	γA-Globulin	Papain Digest	Bence-Jones Protein	Reduced Alkylated Bence-Jones Protein	Light Chain (acetic acid)	Light Chain (SDS)	Heavy Chain (SDS)
	(g amino acid residues/100 g protein)[a]						
Lysine	5.44	5.37	6.27	6.28	6.22	5.81	5.27
Histidine	2.53	2.05	2.37	2.18	2.27	2.36	2.67
Arginine	5.40	5.60	5.42	5.26	5.32	5.76	5.55
Aspartic acid	7.87	7.92	6.77	6.86	7.01	7.43	8.26
Threonine	8.99	8.47	8.32	8.49	8.48	8.51	8.38
Serine	9.54	9.80	11.06	11.45	10.83	9.77	8.13
Glutamic acid	11.00	10.49	11.52	11.57	11.41	11.12	11.24
Proline	8.04	8.31	6.77	7.23	7.22	7.63	8.93
Glycine	4.25	4.31	4.04	4.00	4.17	4.31	4.49
Alanine	4.86	4.57	5.80	5.69	5.69	5.24	4.95
Valine	6.85	7.51	7.22	7.07	7.36	6.91	6.36
Methionine	0.66	0.46	0	0.03	0.12	0.26	1.00
Isoleucine	1.44	1.72	1.96	1.96	1.87	1.75	1.90
Leucine	9.45	8.07	7.57	7.66	7.58	8.92	10.02
Tyrosine	4.35	5.33	6.99	6.79	(6.10)[b]	5.10	2.96
Phenylalanine	4.23	4.48	2.79	2.66	(3.50)[b]	4.19	4.71
CM-cysteine	0	0	0	2.17	0.44	1.94	1.14
Half-cystine	2.44	2.64	2.14	0	1.43	trace	1.06

[a] Expressed as the percentage of the total recovered amino acids. Tryptophan was not determined but was assumed to be 3% for calculation purposes. Correction factors were not applied for partial destruction of amino acids during hydrolysis. [b] Parentheses signify poor resolution of phenylalanine and tyrosine.

acid hydrolysis (Clamp and Putnam, 1964).[3] Glucosamine was absent in the Bence-Jones proteins and the light chains.

The lack of methionine in the Ha Bence-Jones protein was the most striking difference to emerge on comparison of it with the Ha γA-globulin (Table III). Pronounced differences were noted in the content of serine, leucine, tyrosine, and phenylalanine, and smaller differences in other amino acids such as lysine, aspartic acid, proline, and alanine. By contrast, the nearly identical amino acid content of the Ha Bence-Jones protein and the light chain of the Ha γA-globulin indicates their close structural relationship. Generally, the composition of the light chain prepared by the acetic acid method more closely resembles that of the Bence-Jones protein than does that of the light chain prepared by the SDS method. This is consistent with the known, greater contamination of the latter with heavy chain. The amino acid contents of the heavy and light chains are complementary with respect to the composition of the intact γA-globulin. The complementarity is best illustrated by the difference in methionine content of the two chains. It was also confirmed by the presence of glucosamine in the heavy chains and its virtual absence in the light chain.

The half-cystine content of the Ha Bence-Jones protein corresponds to five residues/22,000 g. This would permit two intrachain disulfide bonds and one disulfide bond between the heavy and light chains, as postulated by Milstein (1964, 1965) for type I and type II immunoglobulins. However, the number of disulfide bonds is still uncertain since the half-cystine content of the light chain is equivalent to only four residues. Although all of the half-cystines of the Bence-Jones protein were carboxymethylated on reduction–alkylation in 8 M urea with mercaptoethanol and monoiodoacetic acid, only one CM-cysteine/22,000 g was found in the light chain prepared by the acetic acid method. Presumably this was derived from the interchain disulfide bond, the location of which is considered in the Discussion. In the light chain prepared by the SDS method, the reduction was apparently complete. In the heavy chain CM-cysteine and half-cystine were present in almost equal amounts. The quantitative recovery for half-cystine is not complete because the data have not been corrected for partial destruction of CM-cysteine during acid hydrolysis.

Amino Terminal Groups. No NH_2-terminal residue

[3] The carbohydrate content of a type I γA-globulin (Ln) and of a type II γA-globulin (Ha) is compared by Clamp and Putnam (1965). The 2 proteins had equivalent amounts of hexose and hexosamine, but the Ha globulin had a higher content of fucose and sialic acid.

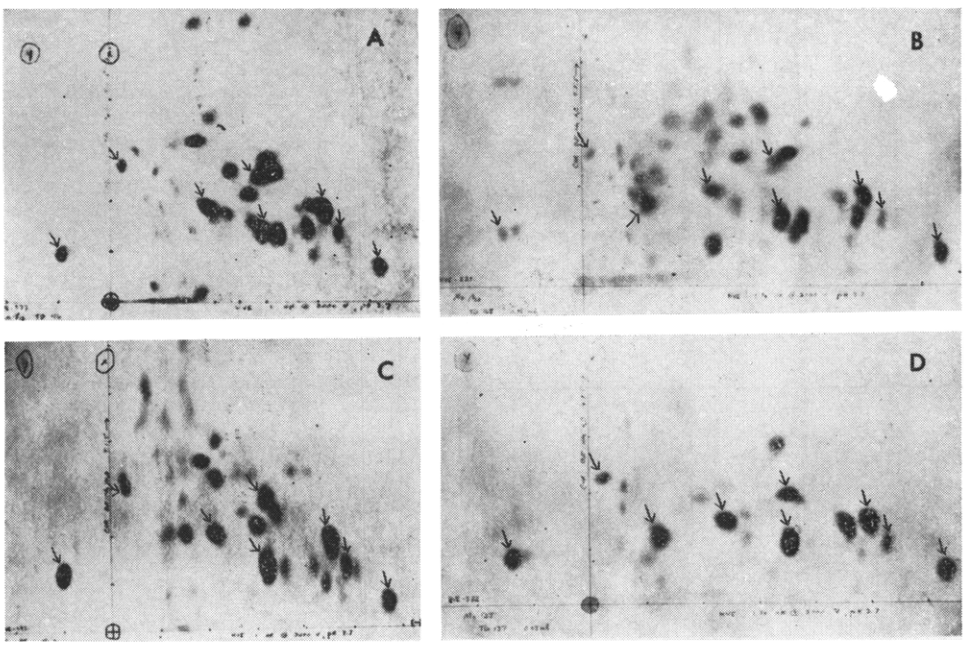

FIGURE 5: Peptide maps of tryptic digests of performic acid oxidized γA myeloma globulins and Bence-Jones proteins of antigenic type I. (A) γA myeloma globulin Ln; (B) γA myeloma globulin Mo; (C) Bence-Jones protein Ln; and (D) Bence-Jones protein Mo. Chromatography is in the *vertical* direction; electrophoresis is in the *horizontal* direction with the cathode to the *right*. The *circled y* marks the position of the indicator phenol red. The arrows in each map denote certain peptides referred to in the text. Reading from left to right in B and D, these are B_1 (the carboxylterminal peptide), B_2, B_3 (the amino-terminal peptide), B_6, B_9 (upper), and B_{11} (lower), B_{13} (free arginine), B_{14} (free lysine), and B_{15} (histidyl lysine). Exactly the same peptides are indicated by the arrows in A and C, except that the arrow is omitted for peptide B_3 which appears to be absent in the Ln protein.

was detectable by the dinitrophenyl method in the Ha Bence-Jones protein even after reaction in 8 M urea. This accords with the frequent failure to detect a free NH_2-terminal residue in type II Bence-Jones proteins (Putnam and Miyake, 1957). The Ln γA myeloma globulin had 1.0 mole of NH_2-terminal aspartic acid/160,000 g; its light chain had 0.3 mole of NH_2-terminal aspartic acid/22,000 g, but no significant amino terminal groups were detected on the heavy chain. The Mo Bence-Jones protein had 0.83 mole of NH_2-terminal aspartic acid/22,000 g, whereas the Mo γA-globulin had 1.3 moles of aspartic acid and 1.7 moles of glutamic acid as NH_2-terminal groups for each 160,000 g. The latter data suggest that the amino terminal group of type I light chains is generally aspartic acid, as is the case for type I Bence-Jones proteins (Putnam and Easley, 1965).

Comparative Peptide Maps. In a series of experiments with use of multiple staining techniques, the tryptic peptide maps of the three γA myeloma globulins and the corresponding Bence-Jones proteins were compared. In several instances the papain digestion products of the myeloma globulin were examined, and also the reduced–alkylated light chain. The comparison was extended to reference samples of other type I and II Bence-Jones proteins, normal γG-globulin, the 3.5 S papain fragments of normal γG-globulin, and the naturally occurring protein related to the Fc fragment of the heavy chain of γG-globulin (Franklin, 1964). Both performic acid oxidized and reduced–alkylated proteins were compared. For better comparison peptide maps were prepared simultaneously for the digests of the individual proteins and a mixture of the digests of the pair under study. The results are illustrated in Figures 5 and 6 which depict representative peptide maps of the individual proteins.

Comparison of Type I γA Myeloma Globulins and the Autologous Bence-Jones Proteins. Every tryptic peptide present in the map of the Mo Bence-Jones protein is present in the Mo γA myeloma globulin, and all but one or two of the peptides of the Ln Bence-Jones protein have a counterpart in the map of the Ln γA myeloma globulin (Figure 5). However, the globulins have a series of additional peptides presumably representing the heavy chain. In accord with Putnam and Easley (1965), the peptide maps of the two Bence-Jones proteins of type I differ entirely from the type II Bence-Jones protein illustrated in Figure 6. Furthermore, although the type I Bence-Jones proteins have many peptides in common, such as those denoted by the arrows, the maps of the two proteins differ in some respects. For example, peptide B_3 (identified in the figure legend) is present in the Mo proteins but missing in the Ln proteins.

Tryptic Peptide Maps of a Type II γA Myeloma Globulin, Its Heavy and Light Chains, and the Autologous

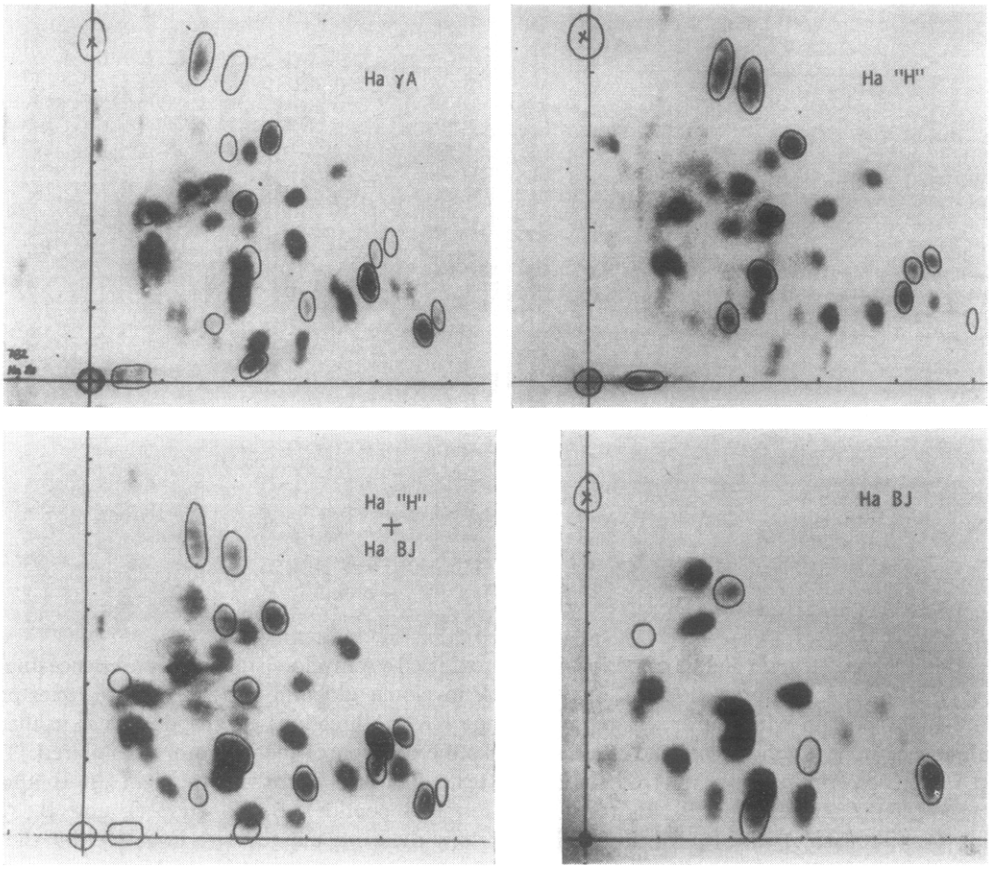

FIGURE 6: Peptide maps of tryptic digests of reduced-alkylated γA myeloma globulin Ha (upper left), the heavy chain prepared by the detergent method (upper right), the reduced-alkylated Bence-Jones protein (lower right), and a mixture of the heavy chain and the Bence-Jones protein (lower left). Peptides that give a positive Sakaguchi reaction for arginine are circled.

Bence-Jones Protein. Almost every tryptic peptide in the reduced–alkylated heavy chain prepared from γA myeloma globulin Ha is likewise found in the map of the reduced–alkylated protein from which it was derived (Figure 6). The intact globulin, of course, has additional peptides resulting from the light chain. In like manner, the majority of the peptides of the light chain have a counterpart in the map of the reduced–alkylated γA-globulin (not illustrated). Although some differences appeared between the light chain and the reduced–alkylated Bence-Jones protein, the maps of both the light chain and the Bence-Jones protein were quite similar and quite unlike that of the heavy chain. Indeed, the map of a mixture of the peptides of the heavy chain and the Bence-Jones protein (lower left, Figure 6) closely approximates the map of the intact γA-globulin. Thus, of the 16 arginine-positive peptides (circled spots) in the intact γA-globulin, all appear in the mixture of the heavy chain and Bence-Jones protein; of these, eleven are present in the heavy chain and the remaining five in the Bence-Jones protein.

Peptides of the Heavy Chains. By use of the multiple staining technique for study of the whole γA-globulins and the autologous Bence-Jones proteins and by study of the separated heavy and light chains as illustrated in Figure 6, the peptides characteristic of the heavy chains of the three γA-globulins were identified. Ten peptides were found in common among all three γA-globulins regardless of antigenic type. These are represented by the solid bars in Figure 7. One of these was the glycopeptide which runs as a streak (or double spot) from the origin toward the cathode in electrophoresis but fails to move in chromatography.[4] However, each of the γA-globulins had seven to nine additional spots characteristic of its heavy chain but which did not overlap with peptides of the other two γA-globulins (depicted schematically in Figure 7). These peptides, which had no counterpart in the autologous Bence-Jones protein or in the light chains, seemed to represent a part of the heavy chain that is variable in structure, yet is characteristic for a given specimen. In this respect there is an analogy to the structure of the light chains (Bence-Jones proteins)

[4] The presence of a second glycopeptide spot to the right of the origin in some of the peptide maps probably results from partial loss of *N*-acetylneuraminic acid from the naturally occurring glycopeptide (Clamp and Putnam, 1964).

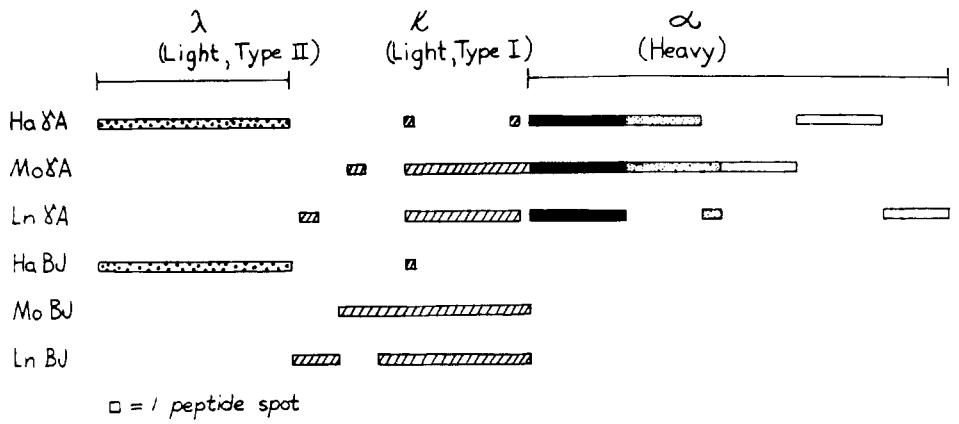

FIGURE 7: Schematic drawing showing peptide overlaps among three γA-globulins and the autologous Bence-Jones proteins. One small square represents one peptide spot, regardless of the size of the peptide. The coarsely stippled bars represent λ-chains. κ-chain peptides are shown by diagonally shaded bar segments. Solid bars represent the "constant" portion of the α chain. The finely stippled segments represent α-chain peptides shared by two of the three globulins. The α-peptides peculiar to an individual globulin are depicted by unfilled bars.

which consist of a portion common to all proteins of the same antigenic type and a portion prone to change in the individual specimen (Putnam and Easley, 1965). An alternative explanation is that there may be two kinds of heavy chains in γA-globulins, one kind common to all γA-globulins and another which may differ with the individual specimen.

Although not illustrated, it was established that the peptides of the heavy chains of γA-globulins differ greatly from those of the 3.5 S fragment of the heavy chain of γG-globulin (the Fc fragment) and also from the naturally occurring protein resembling this fragment (the Franklin protein specimen Cr). Surprisingly few overlaps were seen in composite maps, and even some of these could be differentiated by specific staining on the individual maps. The peptide maps of the γA heavy chains also differed markedly from those for γM heavy chains. Very few peptides, if any, appear to be in common among the three kinds of heavy chains.

Discussion

Largely from studies of antigenic determinants and electrophoretic mobility it is now widely assumed that immunoglobulins contain two major groups of polypeptide chains designated "light" and "heavy". The light chains are common to the three major classes of immunoglobulins (γG, γA, and γM); the heavy chains determine the distinctive properties of each class. However, as was pointed out in the proposal for a new international nomenclature for human immunoglobulins,[1] "the possibility that the "heavy chain" is in fact several chains must be considered." Likewise, largely from similarity in antigenic determinants and electrophoretic mobility, it is generally assumed that Bence-Jones proteins are equivalent to the light chains of the myeloma globulin (or macroglobulin) of the same patient. Substantial biochemical evidence for the latter conclusion has hitherto been adduced only for γG-globulins (Edelman and Gally, 1962; Putnam, 1962; Cohen, 1963). In the present work the conclusion that Bence-Jones proteins are the light chains of γA-globulins is established for three well-characterized pairs through a combination of comparative peptide mapping, amino acid analysis, and NH_2-terminal group determinations, as well as by immunochemical study and electrophoretic mobility. On the other hand, although the heavy chains have antigenic determinants and peptide maps characteristic for γA-globulins, the number and structure of these chains remains uncertain.[5]

The comparative immunochemical and structural study of many individual myeloma globulins and related proteins is needed, for the classification of human immunoglobulins on which the new nomenclature is based rests mainly on antigenic study of a veritable host of myeloma proteins, each showing discrete differences. There are two recent examples of how study of one or two individual proteins may alter this tentative classification. First, since this nomenclature[1] was proposed in May 1964, the study of heavy chain fragments excreted by two individuals has led to the recognition of the Cr and Zu subtypes of γG heavy chains (as well as other new subtypes) (Ballieux *et al.*, 1964) and has aroused suspicion that the heavy chain is composed of two distinct pieces. Second, the discovery by Rowe and Fahey in 1965 of a unique myeloma protein from a single patient has led to recognition of a fourth class of

[5] Preliminary molecular weight estimation of the heavy chains of γA myeloma globulins by the sedimentation equilibrium method has yielded values of 35,000 for the Ha γA heavy chain and 39,000 for the Ln γA heavy chain (K. E. Neet, personal communication). The molecular weight of the glycopeptide suggests the presence of three oligosaccharide units in the intact Ln γA myeloma globulin (Clamp and Putnam, 1965).

immunoglobulins (γD) not considered in the nomenclature proposal. The problem, of course, is to achieve judgment in the selection of proteins for study and in the integration of the results so that the systematic pattern emerges, knowledge of which is necessary for the understanding of the structure and biosynthesis of antibodies.

By analogy to the elucidation of the structure and genetic control of the abnormal hemoglobins, the most fruitful approach for structural study of the immunoglobulins will be comparative peptide mapping of their light and heavy chains coupled with sequence analysis of a few proteins and of selected peptides from others. The present objective of our laboratory is to perform complete amino acid sequence analysis on at least one Bence-Jones protein of each antigenic type and to relate the tryptic peptides so determined to the peptides carefully defined by position and multiple staining in the light chains of γG and γA myeloma proteins, γM Wäldenström macroglobulins, and normal pooled samples of γG, γA, and γM. Although at least two other laboratories have engaged in sequence analysis since our first reports on structural relationships among normal γ-globulin, myeloma globulins, and Bence-Jones proteins, none to our knowledge is combining these techniques for the necessary comparative study.

To this end, we have thus far completed about three-fourths (148 residues) of the sequence of one Bence-Jones protein of type I, including the consecutive sequence of the last 118 residues (Titani *et al.*, 1965). Hilschmann and Craig (1965) have published a partial sequence for 96 widely distributed residues for another protein of type I (Roy) and fragments of the sequence for a third. Milstein (1965) has defined the COOH-terminal octapeptide sequences in type I and type II Bence-Jones proteins. Through comparison of these data, and through the relationship we have established from the peptide maps of the two proteins for which most sequence data is known, we have been able to deduce the probable sequence of many of the peptides of the two type I Bence-Jones proteins and γA light chains described herein. One of these light chains (Mo) not only has the amino-terminal octadecapeptide B_8 whose sequence we have reported (Titani and Putnam, 1965), it has all the tryptic peptides in the COOH-terminal half of Bence-Jones protein Ag for which the sequence is known (positions 106–212). Yet the Mo Bence-Jones protein differs from Ag in two soluble tryptic peptides. One of the Ag peptides (B_{16}) which is replaced in Mo is a tetrapeptide at positions 102–105 where four homologous interchanges have been observed in the three Bence-Jones proteins undergoing sequence analysis in the two laboratories. The other peptide of Ag (B_8) which is replaced in Mo immediately precedes B_{16} and is known to contain at least one nonhomologous interchange relative to the Roy Bence-Jones protein described by Hilschmann and Craig (1965). It should be necessary only to isolate the corresponding peptides in the Mo Bence-Jones protein and subject them to sequence analysis.

Evidently, through the detailed comparative peptide mapping of Bence-Jones proteins and light chains of the γG-, γA-, and γM-globulins, the myriad-seeming myeloma proteins may yet become subject to structural study in a meaningful way that will enable development of the theory for control of multiple amino acid replacements of light chains.

Unfortunately, the present prospect is less auspicious for heavy chains. The finding of immunological subtypes of γG heavy chains referred to above and the evidence herein from peptide maps of γA heavy chains continues to raise the suspicion that parts of the heavy chains may have individual differences for myeloma globulins, just as the light chains have. This possibility must be kept open and subject to continued investigation rather than be closed because a convenient classification and subunit structure has been proposed.

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Immunochemical Studies on the Tobacco Mosaic Virus Protein. II. The Specific Binding of a Tryptic Peptide of the Protein with Antibodies to the Whole Protein*

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ABSTRACT: Direct measurements are presented for the specific binding of a tobacco mosaic virus protein (TMVP) tryptic peptide having the amino acid sequence Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg to antibodies to TMVP. The specific binding of the acetyl-^{14}C tryptic peptide with anti-tobacco mosaic virus protein was shown by precipitation of the complex with ammonium sulfate, by gel filtration of the complex, and by equilibrium dialysis. No binding occurred with anti-tobacco mosaic virus protein which had been previously absorbed with tobacco mosaic virus protein.

Several protein fragments have been shown to possess immunological activity related to that of the whole protein from which they have been derived. This has been shown by methods which involve measuring the effect of the fragment on the reaction between the whole protein and the antibody or by demonstrating immunological cross reactivity between the whole protein and the fragment conjugated to an unrelated carrier.[1]

In studies on the immunological relationship of the tryptic peptides of tobacco mosaic virus protein (TMVP)[2] to the full antigen (Benjamini *et al.*, 1964), tryptic peptide 8[3] was found to specifically inhibit the fixation of complement by TMVP and anti-TMVP. This peptide has the amino acid sequence Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. Preliminary experiments directed toward the elucidation of the antigenic determinant(s) of peptide 8 revealed that acetylated peptide 8 was still fully inhibitory in the complement fixation system.

The data presented in this communication deal with the binding between ^{14}C-acetylated peptide 8 and anti-TMVP. These data offer direct evidence that peptide 8 specifically binds with antibodies to TMVP.

Materials and Methods

Tobacco Mosaic Virus Protein. TMVP was obtained

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[1] Tryptic peptide 12 of TMVP was conjugated to bovine serum albumin by Anderer (1963), who studied the immunological relationship between this peptide and the tobacco mosaic virus.

[2] Abbreviations used in this work: TMVP, tobacco mosaic virus protein; ^{14}C8, [2-^{14}C]acetyl peptide 8; AChE, acetylcholinesterase.

[3] Nomenclature according to that proposed by Tsugita *et al.*, 1960.